D-aspartate exerts an opposing role upon age-dependent NMDAR-related synaptic plasticity and memory decay

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In the present study, we demonstrated that D-aspartate acts as an *in vitro* and *in vivo* neuromodulatory molecule upon hippocampal NMDAR transmission. Accordingly, we showed that this D-amino acid, widely expressed during embryonic phase, was able to strongly influence hippocampus-related functions at adulthood. Thus, while up-regulated levels of D-aspartate increased LTP and spatial memory in four-month old adult mice, the prolonged deregulation of this molecule in thirteen-month old animals induced a substantial acceleration of age-dependent decay of synaptic plasticity and cognitive functions. Moreover, we highlighted a role for D-aspartate in enhancing NMDAR-dependent synaptic plasticity through an inducible "turn-on/turn-off-like mechanism". Strikingly, we also showed that D-aspartate, when administered to aged mice, strongly rescued their physiological synaptic decay and attenuated their cognitive deterioration. In conclusion, our data suggest a tantalizing hypothesis for which this in-embryo-occurring D-amino acid, might disclose plasticity windows in the aging brain.

INTRODUCTION

Cognitive processes are believed to depend on changes in synaptic efficacy in certain key brain regions, including the hippocampus[1]. Among different forms of synaptic plasticity, hippocampal N-methyl D-aspartate receptors (NMDARs)-dependent Long-Term Potentiation (LTP) has been proposed as one of the most probable neuronal substrates underlying plastic changes associated with spatial learning and memory[1-4]. Accordingly, antagonists of NMDARs blocked the induction of LTP in hippocampal slices[5-7] and impaired reference memory in rats[8, 9]. Similarly, synaptic memory and cognition were influenced by genetically manipulated NMDARs. Indeed, hippocampus-related synaptic plasticity and learning were improved in mice over-expressing NR2B subunit of these receptors[10]. Intriguingly, up-regulation of this subunit has been also described beneficial for improving cognitive functions in aged mice[11]. On the other hand, extensive evidence suggests that the hippocampus is the main brain region sensitive and vulnerable to age-related loss of functional synapses and NMDAR-mediated responses[12]. Age-related deficits in reference memory have been explored across a wide variety of species, especially in rodents, where a decline in the hippocampal ability to produce plastic synaptic changes has been documented during aging[13-15]. In this respect, alterations of synaptic connectivity and plasticity are thought to be important neurobiological determinants in causing the memory deterioration observed in old animals[12, 16]. According to this view, aged rats exhibit reduced NMDAR binding sites, which correlate with their impairments in synaptic plasticity and cognition[17].

Although in the past free D-amino acids were considered to be involved only in the "bacterial world", recently, mounting evidence pointed out their neuromodulatory role in controlling neuronal functions in mammals. Specifically, the D-amino acid D-serine, by acting as an endogenous co-agonist at glicyne-binding-sites of NMDARs, has been shown to be able to rescue age-related impairment of NMDAR-mediated synaptic potentials in old mice and rats[18, 19]. Besides D-serine, today well characterized for its pharmacological properties and clinical implications in schizophrenia treatment[20], another D-amino acid, namely D-aspartate, occurs in the mammalian

brain. In contrast to the former, the central role of the latter remains, so far, an issue of controversy. However, neuro-anatomical and biochemical analyses indicated that in both humans and rodents, D-aspartate occurs at abundant levels in developing brain to decrease at low amounts during postnatal life[21, 22]. This time-dependent regulation of D-aspartate brain content has been linked to the concomitant postnatal expression of D-Aspartate Oxidase (DDO), the only enzyme known so far to be responsible for its degradation[23, 24]. Moreover, *in vitro* studies pointed out that this D-amino acid binds NMDARs[25]. However, its *in vivo* relevance in modulating glutamatergic neurotransmission remains unclear, since the affinity of these receptors for D-aspartate is 10 times lower than for glutamate. Based on these findings, in the present work, we challenged the hypothesis by which "forcing" higher D-aspartate levels in aged animals, this in-embryo-occurring molecule might disclose neuronal plasticity features aimed to reduce the physiological synaptic and cognitive deterioration appearing during brain aging.

RESULTS

D-aspartate triggered NMDAR-dependent and NMDAR-independent currents.

The pharmacological features of D-aspartate were studied on CA1 pyramidal neurons. NMDA induced a remarkable inward current (I_{NMDA}), which remained constant after repeated applications of the agonist. Perfusion with NVP, a selective NR2A blocker, and cis-PPDA, a NR2C-D antagonist, reversibly reduced I_{NMDA} to great extent **(Fig. 1a,b)**. In the same neurons, I_{NMDA} was largely blocked, in an irreversible manner, by Ro 25-6981, a selective NR2B antagonist **(Fig. 1 a,b)**. Similarly, also D-aspartate-mediated inward currents ($I_{D\text{-}Asp}$) were reduced by each NR2 selective antagonist **(Fig. 1 a,b)**. Interestingly, while I_{NMDA} was totally blocked by the simultaneous combination of all NR2 selective antagonists or by the non-competitive NMDAR antagonist, MK801 **(Fig. 1b)**, under the same conditions D-aspartate was still able to excite CA1 pyramidal neurons **(Fig. 1b)**. Next, we studied the calcium dynamics mediated by D-aspartate, performing voltage-clamp recordings associated with microfluorometry with the use of a pipette solution filled with the calcium-sensitive dye fura-2. As expected, puff applications of D-aspartate caused an inward current that was associated with a transient increase of $[Ca^{2+}]i$ **(Fig. 1c)**. This current was largely, but not totally, antagonized in a reversible manner by a high concentration of D-AP5 or irreversibly by MK801 **(Fig. 1c)**. Finally, a further set of experiments conducted in the continuous presence of MK801 showed that the residual $I_{D\text{-}Asp}$ remained constant even with the co-application of the sodium-dependent glutamate/aspartate transporter inhibitor DL-TBOA **(Fig. 1d)**. This current is rather inhibited by switching the normal ACSF solution to one not containing calcium ions **(Fig. 1d)**. Notably, chelation of intracellular Ca^{2+} with EGTA, blockade of all glutamate receptors with CNQX and MCPG, and the use of non-specific Voltage Gated Calcium Channel (VGCC) blocker cadmium chloride, all added to the superfusion medium, did not affect the remaining Excitatory Post Synaptic Currents (EPSC) **(Fig. 1d)**. Therefore, our data indicate that the action of D-aspartate on CA1 pyramidal neurons occurs via Ca^{2+} currents through, at least, two alternative routes based on NMDAR-dependent and NMDAR-independent pathways.

Lack of D-Aspartate Oxidase induced a dramatic acceleration of age-dependent decay of hippocampal synaptic transmission and plasticity.

We have recently demonstrated that higher D-aspartate levels, in the hippocampus of two-month old *Ddo*$^{-/-}$ mice, enhance the NMDAR-dependent form of LTP, while maintain synaptic transmission unaltered[26]. Based on this study, here we determined the age-related effect of *Ddo* gene ablation on synaptic transmission and plasticity in area CA1.

Firstly, we examined D-aspartate levels in the hippocampus of *Ddo*$^{-/-}$ animals and their control littermates at 4, 9 and 13 months of age. According to the pivotal role of DDO in controlling D-aspartate levels, HPLC analysis indicated a strong and persistent hippocampal up-regulation of this D-amino acid content throughout all ages tested **(Fig. 2a)**.

Next, we measured basal synaptic transmission on hippocampal slices from mutants and controls, at the same ages at which we evaluated D-aspartate concentrations. At 4 months of age, *Ddo*$^{+/+}$ and *Ddo*$^{-/-}$ mice showed similar trendlines **(Fig. 2b)**. Also at 9 months of age, both genotypes exhibited comparable fEPSP slopes and fiber volley amplitudes at all stimulus intensities tested **(Fig. 2b)**. In contrast, thirteen-month old *Ddo*$^{-/-}$ animals had significantly reduced maximum fEPSPs, relative to *Ddo*$^{+/+}$ controls **(Fig. 2b)**. Therefore, these results show that the age-dependent decline in fast glutamatergic transmission is exacerbated in absence of DDO enzyme. We then investigated paired-pulse facilitation (PPF), a measure of short-term plasticity. Interestingly, no differences in the paired-pulse ratio between *Ddo*$^{-/-}$ and *Ddo*$^{+/+}$ mice were observed in the three age-group tested **(Fig. 2c)**. Finally, we explored the effects of deregulated levels of D-aspartate on age-related LTP at CA1 synapses. At 4 months of age, we showed a significant increase in the magnitude of LTP in *Ddo*$^{-/-}$ animals, compared to control mice **(Fig. 2d)**. In contrast, LTP was impaired either in nine-month **(Fig. 2d)** and, even more severely, in thirteen-month old mutants, when compared to age-matched controls **(Fig. 2d)**. Interestingly, at 13 months of age, a physiological decay of LTP appeared also in

Ddo$^{+/+}$ mice that, in any case, never reached the dramatic impairment observed in mutant CA1 synapses **(Fig. 2e)**.

Ddo$^{-/-}$ mice displayed spatial memory enhancement or worsening in an age-dependent fashion.

As putative behavioural correlate of hippocampal NMDAR-dependent synaptic plasticity, we investigated ontogenetic changes in learning and memory abilities of *Ddo*$^{-/-}$ mice, at 4, 9 and 13 months of age, in a reference memory task of the Morris water maze paradigm.

During acquisition phase, we found similar escape latencies between four-month old *Ddo*$^{+/+}$ and *Ddo*$^{-/-}$ animals **(Fig. 3a)**. In order to evaluate their spatial memory formation, we performed two retention tests, after a three-day (short) or five-day (long) training period. Interestingly, in probe 1, knockout mice displayed a forward ability in remembering the correct location of the platform, compared to controls **(Fig. 3b)**. However, after a longer training exposure, both genotypes showed a comparable bias spatial search in the target quadrant **(Fig. 3c)**. Then, in order to analyze the ability of the mice to erase previous spatial information and planning a new navigation strategy, we moved the platform to the opposite position. Also in the reversal phase, both groups displayed similar learning abilities **(Fig. 3a)**. Moreover, the results in the retention tests confirmed in *Ddo*$^{-/-}$ mice an improved spatial memory after a short training exposure, compared to controls **(Fig. 3d)**. In fact, although both groups showed a search preference for the new goal quadrant, *Ddo*$^{-/-}$ mice significantly spent more time in this location, compared to controls. After additional training, both genotypes showed comparable preferential search for the target quadrant **(Fig. 3e).**

Also at 9 months of age, both genotypes evidenced comparable spatial learning aptitudes in both acquisition and reversal phases **(Fig. 3f)**. Importantly, in probe 1, *Ddo*$^{-/-}$ group did not confirm improved spatial memory, evidenced at 4 months of age **(Fig. 3g)**, since the correct position of the platform was acquired only after a longer training **(Fig. 3h)**. Moreover, in the reversal phase, spatial retention in the new goal quadrant occurred only in *Ddo*$^{+/+}$ animals after five-day training exposure **(Fig. 3i,j)**, thus highlighting in mutants the occurrence of mild memory deterioration at this age.

The worsening of cognitive aptitudes of *Ddo*$^{-/-}$ animals was further evidenced in thirteen-month old mice. While comparable spatial learning curves were observed for both genotypes during acquisition **(Fig. 3k)**, only *Ddo*$^{+/+}$ animals showed preserved spatial memory abilities **(Fig. 3l,m)**. In the reversal phase, a prominent overall impairment in spatial learning and memory was observed in mutants **(Fig. 3k,n,o)**.

Alteration of hippocampal synaptic markers in *Ddo*$^{-/-}$ mice.

Here, we analysed whether synaptic and cognitive decline were mirrored by morphological variations in the hippocampus of *Ddo*$^{-/-}$ mice. First, toluidine staining showed no distinguishing abnormalities in the pyramidal cell layer and dentate gyrus (DG) of the hippocampus from *Ddo*$^{+/+}$ and *Ddo*$^{-/-}$ mice, at any age. This was confirmed by the counting of Neu-N-IR profiles (data not shown). A diffuse age-dependent increase in GFAP-IR **(Fig. 4h**,**i**,**m**,**n**,**s)** and astrocyte hypertrophy **(Fig. 4g**,**l)**, were observed in the CA3 subfield of the hippocampus, regardless genotype. Nevertheless, in the same area, these changes were selectively associated with a reduced immunostaining for the presynaptic marker synaptophysin in thirteen-month old *Ddo*$^{-/-}$ mice **(Fig. 4s)** and, interestingly, were anticipated by a decreased staining for the postsynaptic marker MAP2 in four-month old mutants **(Fig. 4s)**. Synaptophysin significantly decreased also in knockout mice in CA1/2 area, at both 4 and 13 months of age **(Fig. 4o-s)**, and in DG of aged mice, compared to respective controls **(Fig. 4s)**. Similarly, a reduced pattern of staining for MAP2 was detected in CA1/2 field of elderly *Ddo*$^{-/-}$ mice, compared to age-matched *Ddo*$^{+/+}$ animals **(Fig. 4a**,**b**,**d**,**e**,**s)**. Remaining dendrites resulted irregular in shape and size **(Fig. 4c**,**f)**. Overall, morphological alterations found in the hippocampus of old mutants are consistent with the behavioural and electrophysiological decline observed during aging.

D-aspartate induced a transient and reversible modulatory effect on NMDAR-dependent LTP.

To study the in *vitro* and in *vivo* consequences associated to non-physiological increased levels of D-aspartate, without interfering with *Ddo* gene expression, we recently developed a pharmacological mouse model based on one-month oral administration of this D-amino acid[26]. Taking advantage of this model, we further examined the neurochemical and electrophysiological consequences of a more prolonged chronic administration. According to our previous report, we found that D-aspartate oral treatment overall resulted in a significant increase of its hippocampal content. However, despite the different time-exposure extent, we failed to detect gross differences in D-aspartate amount following moderate (3 months) and long-term (12 months) delivery periods **(Fig. 5a)**.

After a three-month administration, C57BL/6J treated mice showed a higher I-O trend, although not significant, compared to their untreated controls **(Fig. 5b)**. Similarly, chronic one-year administration of D-aspartate produced only a slight decrease in basal transmission. Indeed, differently to what observed in mutants, this effect did not reach statistical significance **(Fig. 5b)**, demonstrating that even prolonged oral administration of this molecule does not perturb basal synaptic responses. Also PPF was indistinguishable in all groups **(Fig. 5c)**. Finally, we examined the effects of higher D-aspartate levels on synaptic plasticity in area CA1. Notably, we showed that three-month D-aspartate treatment induced a robust enhancement in LTP magnitude **(Fig. 5d)**. Interestingly, this plastic augmentation appeared to be regulated through an inducible mechanism, since three-week withdrawal was able to reverse this potentiating effect that was, conversely, re-instated following one-month D-aspartate re-administration **(Fig. 5d)**. In contrast, hippocampal slices from twelve-month chronically treated mice were dramatically unresponsive to the conditioning train, compared to age-matched controls **(Fig. 5e)**. Moreover, despite what was found in $Ddo^{+/+}$ mice, no signs of age-dependent LTP decay was detected in thirteen-month old untreated C57BL/6J mice **(Fig. 5f)**. These results mirrored, to a large extent, those obtained in $Ddo^{-/-}$ mice, thus highlighting a common opposing time-dependent effect of chronic D-aspartate exposure on hippocampal LTP. To summarize, our data clearly point out that D-aspartate is able to modulate

NMDAR-dependent LTP at CA1 synapses and, in turn, provide strong evidence about its role in influencing age-dependent changes in synaptic plasticity.

Chronic D-aspartate oral administration in C57BL/6J mice improved spatial memory in adulthood without perturbing cognitive functions in elderly phases.

Spatial learning and memory abilities in D-aspartate treated C57BL/6J mice were analyzed after its three-month or twelve-month chronic oral administration, according to the same behavioural protocol adopted for mutant animals. During acquisition and reversal phases, three-month D-aspartate treatment did not produce any visible change in learning aptitudes of mice **(Fig. 6a)**. Importantly, after a short training, treated mice evidenced an improvement of spatial memory, as indicated by longer search in the goal area, compared to controls **(Fig. 6b)**. Nevertheless, such slight difference between groups disappeared after a two-day additional training **(Fig. 6c)**. Similarly, during reversal phase, treated animals showed enhanced spatial memory skills, as indicated by the longer time spent in the new goal quadrant in the probe 3, compared to non-treated mice **(Fig. 6d)**, while both groups were similarly able to remember the position of the new target quadrant after five-day training **(Fig. 6e)**.

Like the shorter treatment, also one-year D-aspartate administration did not modify spatial learning performances of C57BL/6J mice **(Fig. 6f)**. Nevertheless, this long-term treatment induced a lack of the previous memory enhancement seen in three-month D-aspartate-treated mice. Indeed, no differences were detected between D-aspartate-treated and untreated groups in each of the retention tests performed **(Fig. 6g-j)**.

D-aspartate rescued CA1 synaptic plasticity decline associated with aging.

We have previously shown that one-month oral administration of D-aspartate was able to strongly increase LTP magnitude in young animals[26]. Here, we investigated whether such short-term treatment might also be beneficial to counterbalance the age-related decay of hippocampus-

dependent functions. Since it is known that rodent females exhibit a faster rate of deterioration in reference memory[27, 28], we decided to utilize for this study twelve-month old C57BL/6J females. Such females, treated for 1 month with D-aspartate, exhibited a significant increase of this D-amino acid levels in their hippocampi, compared to untreated mice **(Fig. 7a)**. Electrophysiological experiments indicated no main differences in basal synaptic transmission and short-term plasticity, as measured by I–O curves **(Fig. 7b)** and PPF (data not shown), respectively. In contrast, we found a strong enhancement of LTP in treated mice, compared to both age-matched controls **(Fig. 7c,d)** and two-month old C57BL/6J females **(Fig. 7c,d)**. Moreover, according to gender differences relative to age-related hippocampal functions, C57BL/6J females showed a pronounced age-dependent LTP decay, not seen in males **(Fig. 7f)**.

Finally, we explored spatial cognitive abilities of D-aspartate-treated aged females. Surprisingly, we found that treated animals displayed a better spatial learning, compared to untreated mice **(Fig. 7e)**. In this regard, it is important to notice that the difference seen after the first training day exclusively derives from the second daily session since, in the first test session, mice exhibited comparable escape latencies (untreated: 84.25 ± 2.26 s; treated: 76.50 ± 4.21 s). However, according to a more relevant age-dependent mnemonic decay likely due to hormonal influences, even after a five-day training exposure, both treated and untreated mice did not remember the correct location of the platform **(Fig. 7f,g)**. During reversal phase, both groups showed a comparable learning profile **(Fig. 7e).** Nevertheless, a mild improvement was detected at days 4 and 5 in D-aspartate treated animals. Conversely, no differences were found in the retention tests **(Figure 7h,i).**

DISCUSSION

While a relevant role for D-aspartate in controlling hormonal activity is well established[29], its involvement in central functions, so far, remains largely unclear. Indeed, although pharmacological studies demonstrated that this D-amino acid binds NMDARs, its *in vivo* neuromodulatory action is still actively debated. In accordance to our previous studies performed on single cells, in CA1 pyramidal neurons[26], here we confirmed the existence of an inward current triggered by D-aspartate, not fully suppressed by NMDAR antagonists. For the first time, we demonstrated that D-aspartate-mediated excitatory currents are exclusively calcium-dependent, since they are abolished by perfusion in a calcium-free medium. We were unable, so far, to identify any putative target, since the chelation of intracellular calcium and the blockade of all glutamate receptors and VGCC never affected this persistent inward current. Therefore, besides demonstrating a direct action on NMDAR via interaction with each of NR2A-D subunits, we indicated that the physiological function of D-aspartate is selectively mediated through a calcium-dependent signalling. Hence, we propose that D-aspartate in the brain acts presumably through, at least, two different pathways based on NMDAR-dependent and NMDAR-independent signalling. On the other hand, the proposed "dual" mechanism of D-aspartate action is also supported by biochemical findings which pointed out a lack of functional NMDARs in organs, like testis and adrenal glands, where high levels of this D-amino acid were also detected[21, 30].

The pharmacological properties of D-aspartate to act as an endogenous NMDAR agonist are in favour of its ability in modulating, among others, synaptic plasticity occurring in the hippocampus at CA1 synapses. Accordingly, we have recently demonstrated that non-physiological increase of D-aspartate levels in the mouse, achieved either by generating animals with a targeted deletion of *Ddo* gene or by treating mice with D-aspartate for 1 month, enhanced this NMDAR-dependent form of LTP[26]. Based on this, we explored the consequences of deregulated levels of D-aspartate upon

hippocampus-related functions during brain aging. Remarkably, we showed that a constitutive deregulated high level of D-aspartate in mutant hippocampi (roughly 15-fold, at each age tested) is able to act as potent biphasic modulator of neuronal activity: potentiating synaptic memory in adult mice (4 months), whilst dramatically accelerating age-dependent basal transmission and LTP decay during maturity stages (9-13 months).

Notably, behavioral studies failed, so far, to reveal consistent learning and memory changes induced by D-aspartate in adult animals[26, 31]. Therefore, in the present study, we carried out a more "stringent" spatial cognitive demanding task. In these new setting conditions, a selective enhancement of hippocampus-mediated reference memory was found in adult *Ddo*$^{-/-}$ mice compared to their *Ddo*$^{+/+}$ littermates. Interestingly, these *in vivo* data were confirmed in three-month D-aspartate treated mice. Taken together, such findings are in accordance to those reported in ddY/DAO^{-} mutants[32] and NR2B transgenic mice[10] in which enhanced NMDAR-dependent synaptic plasticity mirrored a spatial memory improvement. Moreover, according to Bach *et al.*[33], our studies indicated a good correspondence between the biphasic age-dependent LTP changes and spatial memory abilities. In fact, while four-month old knockouts displayed enhanced spatial memory, in nine- and, even more prominently, thirteen-month old *Ddo*$^{-/-}$ animals this improvement completely disappeared and a significant worsening of cognitive abilities occurred, compared to their relative age-matched controls.

We also showed that, when D-aspartate was administered under an intermittent regimen, this D-amino acid triggered a transient and reversible enhancement of LTP at CA1 synapses through an inducible "turn-on/turn-off-like mechanism". Hence, these data further corroborated a role for D-aspartate in regulating NMDAR signalling and, in turn, provided an attractive possibility when considering its clinical potential.

Furthermore, similarly to aged *Ddo*$^{-/-}$ mice, long-term exposure of D-aspartate evocated a severe decay of hippocampal synaptic plasticity. However, differently to the oldest *Ddo*$^{-/-}$ mice here tested

(13 months), one-year D-aspartate chronic administration in C57BL/6J animals failed to produce deficits in basal transmission and reference memory.

Overall, our data strongly supported a neuromodulatory role for D-aspartate on glutamatergic transmission and, in turn, showed that, according to its properties as NMDAR agonist, non-physiological amounts of this molecule in the brain could unmask the opposite neuronal influences exerted by this receptor[34, 35]. In fact, while physiological stimulation of NMDARs has a crucial adaptive role in brain processes such as neuronal survival and synaptic plasticity, on the other hand, their abnormal activation leads to deregulation of calcium homeostasis, responsible for loss of synaptic memory and neurotoxicity[34, 36, 37]. Consistently, altered LTP has been widely documented in hippocampal slices from several transgenic mouse models of Alzheimer Disease (AD)[38-41] and in senescence-accelerated mouse strains[42]. In this line, we showed that higher D-aspartate levels render glutamatergic synapses more prone to plastic changes, in both four-month old genetic and pharmacological animal models, while the prolonged activation of NMDARs in 9-13 month old *Ddo*$^{-/-}$ mice and in long-term treated animals produced a strong accelerated rate of age-dependent synaptic plasticity deterioration.

Nevertheless, our data pointed out also a direct involvement of the specific genetic background on which genetic or pharmacological manipulation are studied[43,44]. In fact, C57BL/6J animals, characterized to display very high performances in hippocampus-dependent functions, represent a "difficult" mouse strain for easily detecting changes associated to aging[45]. Thus, even though D-aspartate induced very strong changes on hippocampal LTP between three- and twelve-month treated animals, surprisingly, only mild, if any, differences were found in their spatial memory aptitudes.

On the contrary, based on the observations that C57BL/6J aged females manifested a more prominent hippocampal cognitive[27] and synaptic memory decline **(Fig. 7c**,**d)**, compared to males, we decided to test whether this embryonic D-amino acid may produce a "youth" influence on neuronal plasticity in this more aging-sensitive gender. Remarkably, we showed that one-month

oral administration of D-aspartate to one-year old females conferred considerable stronger plastic properties, relative to those observed in age-matched controls. The entity of this amelioration was even more surprising comparing the level of LTP of these D-aspartate-treated mice to that observed in two-month old untreated females. Moreover, this D-amino acid also slightly improved cognitive abilities of old females. Taken together, these evidences further extended to aged brains the ability of this endogenous D-amino acid to modulate NMDAR-dependent functions.

In conclusion, our data demonstrated an *in vitro* and *in vivo* neuromodulatory role for D-asparate in modulating synaptic plasticity and cognition and, in turn, highlighted a crucial, yet unknown, implication of DDO in controlling the age-related physiological decline of hippocampus-dependent functions. In fact, while the lack of DDO in adult mice induced an enhancement of both LTP and spatial memory, at maturity stages it determined a strong acceleration of aging processes, which resembled the synaptic and behavioural features described in AD-like animal models[38-41]. In this line, also alterations in synaptic markers MAP2 and synaptophysin found in old mutant hippocampi, are consistent to those seen in APP-null mice[46]. Moreover, the in dissociable opposite age-dependent electrophysiological and behavioural phenotypes seen in *Ddo*$^{-/-}$ mice, let retain these animals as a peculiar model for studying *in vivo* the "Yin and Yang" consequences associated to an abnormal NMDARs signalling[34]. Nevertheless, the lack of gross cognitive alterations after a long-term D-aspartate chronic administration further supported a potential interest for this D-amino acid when considering the beneficial effects of this molecule in attenuating schizophrenia-like symptoms induced by amphetamine and MK801 challenges (FE and AU unpublished data).

METHODS

Animals

Mutant mice for *Ddo* gene were generated as previously described[23]. Four-, nine- and thirteen-month (± 3 weeks) old male wild type ($Ddo^{+/+}$) and knockout ($Ddo^{-/-}$) mice were used in this study and derived from mating of heterozygous ($Ddo^{+/-}$) mice, back-crossed to F5 generation to C57BL/6J strain[47]. Animals were genotyped by polymerase chain reaction according to Errico *et al.*[23]. C57BL/6J male mice were used to test the effects of chronic three- and twelve-month oral administration of D-aspartate in neurochemical, cognitive and electrophysiological studies. D-aspartate was delivered in drinking water at the concentration of 20 mM to 45 days old mice until they were used for experiments. Thirteen-month old C57BL/6J female mice were used to test the effects of chronic one-month D-aspartate oral administration, delivered between the 12th and the 13th month of their life.

Mice were housed in groups (n = 4-5) in standard cages (29 x 17.5 x 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 to 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 (n. 86/609/EC). All efforts were made to minimize the animal's suffering.

HPLC analysis

Mice were killed and the hippocampus dissected and stored at -80°C. The determination of D-aspartate was performed by HPLC technique, based on the diastereomeric separation of D-aspartate from the L-form and other L-amino acids, as previously described[48]. Data were analysed using ANOVA or two-tailed Student's *t* test and data are expressed as means ± standard error of the mean (SEM).

Morris water maze

Morris water maze was performed according to a modified version of the protocol described by Errico *et al.*[26]. The apparatus consisted of a circular pool (100 cm in diameter), surrounded by three-dimensional visual cues, containing opaque water at 21°C (± 1°C) with a platform (8 cm in diameter) submerged 1 cm beneath the water surface. Mice were gently handled 5 min per day for a week before the experiment. The acquisition phase consisted of 2 sessions per day (3 h interval between sessions) over a five-day period. Each session was composed of 4 trials with an inter-trial

interval of approximately 5 min. On day 6, the platform was moved to the opposite position and the reversal learning was monitored for 5 additional days. Overall, 4 probe tests were performed (2 tasks per each training phase) in which animals were allowed to swim for 60 s in the absence of the platform, in order to evaluate time-dependent memory retention of mice. During acquisition phase, the first test (probe 1) was performed before starting the 4^{th} day of training, while the second (probe 2) was conducted at day 6, before the first session of the reversal phase. During reversal phase, the first retention task (probe 3) was done before starting the 9^{th} day of training, while the second (probe 4) was conducted at the end of the reversal phase, at day 11. Each retention test was conducted about 18 h after the second session of the previous day. In both acquisition and reversal phases, the time to reach the target was measured. In the probe tests, the percentage of time spent in each quadrant was recorded. A computerized video tracking system (Videotrack, Viewpoint S.A., Champagne au Mont d'Or, France) was used for all mentioned parameters. In the acquisition phase, the measure of the escape latency was used as dependent variable and data were examined using two-way ANOVA (genotype or treatment x days) with repeated measures. Data obtained in the probe trials were analysed by one-way ANOVA followed by appropriate *post-hoc* comparison. A significance level of $p < 0.05$ was accepted as statistically significant in all the experiments performed. All measures are expressed as mean ± SEM. All statistical analyses were performed with StatView software (version 5.0.1.0; SAS Institute, Cary, NC).

Electrophysiology

Patch-clamp recordings and microfluorometric measurements.

Experiments aimed at investigating the effects of D-aspartate application were performed in whole-cell path-clamp recordings on CA1 pyramidal neurons obtained from juvenile (thirteen- to fifteen-day-old) Wistar rats. All recordings were obtained in voltage-clamp mode (at –60 mV holding potential), in the continuous presence of TTX (1 μM) and nifedipine (10 μM). Vibratome-cut parasagittal slices (300 μm) were prepared from hippocampi, incubated for one hour and then transferred to a recording chamber submerged in a continuously flowing artificial cerebrospinal fluid (30°C, 2-3 ml/min) gassed with 95% O_2- 5% CO_2. The composition of the control solution was (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 11 Glucose, 25 $NaHCO_3$. The recording chamber was mounted on the stage of an upright microscope (Axioscope FS, Carl Zeiss) equipped for infrared video microscope (Hamamatsu, Japan) and video microfluorometry (ImproVision, Coventry, UK). Borosilicate glass electrodes (3-4 MΩ) were filled with (in mM): K-Gluconate 135; KCl 10; $MgCl_2$ 2; $CaCl_2$ 0.045; EGTA 0.1; HEPES 10; ATP 2; GTP 0.3 (pH 7.3, with KOH). Data were acquired using pClamp and Axoscope software (Axon Instruments). For

microfluorometric measurements, neurons were filled with calcium-sensitive dye through the patch pipette as described previously[49]. The fluorescent calcium indicator (fura-2 pentapotassium salt, Molecular Probes) was excited via a 40x water immersion objective by illumination with light provided by a 75-W Xenon lamp. Excitation light was band-pass-filtered alternatively at 340 or 380 nm whereas emission light passed a barrier filter (500 nm) and was detected by a CCD camera (Photonic Science, Milham, UK). Time courses of fluorescence values, obtained at 340 and 380 nm, were calculated over the cell soma (region of interest, ROI) and corrected for background fluorescence (BK), measured from a region > 100 μm away from the soma. Changes in fluorescence corresponding to calcium concentrations [Ca^{2+}] are reported as ratio values and were calculated from the following equation: R=(F340ROI-F340BK) / (F380ROI-F380BK), where F340 and F380 are the specific fluorescence values emitted by ROI and background at 340 and 380 nm excitation wave-lenghts. Drugs were bath-applied by switching the solution to one containing known concentrations of drugs. D-aspartate and NMDA were also applied via a patch pipette that was positioned in close vicinity of the cell body and was connected to a pressure application system (Picospritzer, 20–30 psi, 0.5–1 s).

Extracellular recordings.

Ddo$^{-/-}$ mice at different ages and mice chronically treated with D-aspartate (3 and 12 months) were compared to their respective controls. Hippocampal slices (400 μm thick) were prepared from halothane-anesthetized mice as described above. A bipolar nichrome wire stimulating electrode was used to evoke field excitatory postsynaptic potentials (fEPSPs) at approximately 50% of the maximal fEPSP amplitude in the Schaffer collateral/commissural fiber pathway. Stimulation pulses were delivered at 0.02 Hz for a duration of 0.02 ms. Extracellular recordings of fEPSPs in the stratum radiatum of the CA1 region were made using glass microelectrodes filled with ACSF (5–10 MΩ). I–O, paired-pulse facilitation (PPF) and baseline-normalized LTP fEPSP slope data, were grouped according to age and genotype. LTP was induced by a HFS protocol (1 train, 100 Hz, 1 s) and the effect of conditioning train was expressed as the mean (±SEM) percentage of baseline EPSP slopes measured at 60 min after stimulation protocol. Significance of data were determined using the Student's *t* test and ANOVA. The *p*-values were always set at a significance level of less than 0.05.

Drugs

D-aspartate, D-(-)-2-amino-5-phosphonoeptanoic acid (AP5), 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), Tetrodotoxin (TTX), Nifedipine, N-Methyl-D-aspartic acid (NMDA) from

Sigma-Aldrich, Milan, Italy; (2S*,3R*)-1-(Phenanthren-2-carbonyl)piperazine-2,3-dicarboxylic acid (cis-PPDA), 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), (aR,bS)-a-(4-Hydroxyphenyl)-b-methyl-4-(phenylmethyl)-1 -piperidinepropanol maleate (Ro 25-6981) and MCPG, (RS)-a-Methyl-4-carboxyphenylglycine (MCPG) from Tocris Cookson Ltd, Bristol, UK. NVP-AAM077 was a generous gift from Novartis Pharma AG, Switzerland.

Histology

All animals were deeply anesthetized with Ketamine (Ketalar, Parke Davis, Italy) 10 mg/kg body weight, i.p. (+ diazepam. 2 mg/kg i.m.) and perfused through the ascending aorta with Tyrode-Ca^{2+} free, pH 6.9, followed by 4% paraformaldehyde in Sorensen phosphate buffer 0.1 M pH 7.0. During perfusion, animals were bathed in ice-cold water. The brains were then removed and immersed for 90 min in the same ice-cold fixative, before being rinsed for at least 48 h in 5% sucrose in 0.1M phosphate buffer. Brains were frozen in CO_2 and 14 μm thick coronal sections were then obtained from the dorsal hippocampus at rostro-caudal level –1.58 to –1.94 from the bregma. For immunofluorescence experiments, sections were first incubated in 0.1 M phosphate buffered saline (PBS) at room temperature for 10-30 min, followed by incubation at 4°C for 24h in a humid atmosphere with the primary antibodies diluted in PBS containing 0.3% Triton X-100, v/v (rabbit polyclonal anti-MAP-2, Santa Cruz Biotecnology, INC; rabbit polyclonal anti-GFAP, EuroDiagnostica; goat polyclonal anti-synaptophysin, Santa Cruz Biotecnology). After rinsing in PBS, the sections were incubated at 37°C for 30 min in a humid atmosphere with the secondary antisera Red™ X- and Cy™2- conjugated antisera (Jackson Immunoresearch West Grove PA). Sections were then rinsed in PBS (as above) and mounted in glycerol containing 1,4-phenylendiamine 0.1 g/l.

Images from tissues were taken by Olympus AX70-PROVIS microscope equipped with motorized z-stage control and F-VIEW II CCD Camera and processed using Cell^P software (Soft Imaging System, GmbH, Munster Germany). Three sections (bilaterally) from each hippocampus were taken for analysis of each antigen. The hippocampus was divided into three distinct fields, which were labeled as CA1/2, CA3 and DG. Quantification of the tissue staining was performed morphometrically by the same investigator who was blinded to the experimental protocol. Shading error correction was performed before measurements to correct for irregularities in illumination of the microscopic fields. Background levels were equalized and the detection threshold was tested and kept at the same level for all samples. MAP2 and GFAP immunostainings were estimated as fraction area, whereas synaptophysin staining was examined as optical density. For each animal and

histochemical staining, multiple areas were measured and the results were averaged. More details on the sampling areas and statistical analysis are included in the legend to the figure.

FIGURE LEGENDS

Figure 1. D-aspartate triggers NMDAR-dependent and NMDAR-independent inward currents on CA1 pyramidal neurons. **(a)** Plot of EPSC amplitude *vs* time showing the effect of pressure applied NMDA and D-aspartate on CA1 pyramidal neurons. NMDA-mediated inward current (I_{NMDA} 173.6 ± 47.2 pA, $p < 0.001$, Student's *t* test) was reversibly reduced to 66.47 ± 3.18% of control by NVP (0.4 μM) ($p < 0.001$) and to 67.40 ± 3.43% of control by cis-PPDA (10 μM) ($p < 0.001$). Ro 25-6981 (5 μM), inhibited I_{NMDA} amplitude to 66.99 ± 3.55% of control ($p < 0.05$) and this effect was irreversible at the wash. Similarly, NVP, cis-PPDA and Ro 25-6981 reduced the inward current triggered by D-aspartate ($I_{D\text{-}Asp}$ 260 ± 84.4 pA) to 66.68 ± 4.88% ($p < 0.001$), 65.79 ± 5.61% ($p < 0.001$) and 67.11 ± 4.62% ($p < 0.001$) of control, respectively. On top of each plot, traces are shown, acquired at the times indicated by the corresponding letters in the plots. Bars indicate the time duration of each compound. **(b)** Summary bar chart representing the pharmacological effects of different NMDAR antagonists on the inhibition of current (expressed as mean percentage of change ± SEM) triggered by pressure application of NMDA and D-aspartate on at least seven separate cells. Notably, I_{NMDA} amplitude is totally blocked by the simultaneous application of the three selective NR2 antagonists (98.98 ± 0.67% of control, $p > 0.1$) as well as MK801 (10 μM) (99.54 ± 5.18% of control, $p > 0.1$). In contrast, the $I_{D\text{-}Asp}$ amplitude is largely, but not totally, inhibited when blocking all NR2 subunits (80.65 ± 0.55% of control, $p < 0.05$) or by MK801 (82.43 ± 5.92% of control, $p < 0.05$). **(c)** Time course of $[Ca^{2+}]_i$ in response to a puff-application of D-aspartate (2 μM) in the presence of D-AP5 (450 μM) and MK801 (10 μM). On top, corresponding inward currents elicited by D-aspartate are shown. On the right, images are representative examples of a CA1 neuron patch clamped with a pipette containing fura-2 under the three different experimental conditions (scale bar, 20 μm). **(d)** Normalized pooled data (n = 9) of $I_{D\text{-}Asp}$ amplitude against time, in response to perfusion with MK801 (10 μM), DL-TBOA (100 μM), ACSF calcium free, CNQX (30 μM), MCPG (200 μM) and cadmium chloride (100 μM). On top, representative raw traces are shown, acquired at the times indicated by the corresponding letters in the plot. Bars indicate the time duration of each treatment.

Figure 2. Age-related synaptic deterioration in *Ddo*$^{-/-}$ mice **(a)** D-aspartate levels in the hippocampus of *Ddo*$^{+/+}$ and *Ddo*$^{-/-}$ mice were measured by HPLC at 4 (*Ddo*$^{+/+}$, n = 6; *Ddo*$^{-/-}$, n = 3), 9 (n = 3, per genotype) and 13 (n = 3, per genotype) months of age. At each age analysed, D-aspartate amount in mutants was significantly higher, compared to their wild type counterpart, although no differences were found within groups at different ages. *** = $p < 0.0001$, compared

with control groups (Student's *t* test). Values are expressed as mean ± SEM. Genotypes are as indicated. **(b)** Input/output (I/O) curves measured by plotting the fEPSP slopes and their corresponding presynaptic fiber volley amplitudes at increasing stimulus strengths are shown for $Ddo^{-/-}$ and $Ddo^{+/+}$ mice at 4, 9 and 13 months of age. At 4 months of age, the trendlines for $Ddo^{+/+}$ and $Ddo^{-/-}$ were: y = 2.30x, R^2 =0.96 and y = 2.58x, R^2 = 0.88, respectively ($p > 0.1$). I-O were similar also at 9 months of age (y = 1.41x, R^2 =0.91 for $Ddo^{+/+}$, y = 1.37x, R^2 = 0.88 for $Ddo^{-/-}$, $p > 0.1$). Conversely, at 13 months of age, basal transmission was impaired in $Ddo^{-/-}$, relative to $Ddo^{+/+}$ (y = 1.10x, R^2 =0.89 for $Ddo^{+/+}$, y = 0.68x, R^2 =0.81 for $Ddo^{-/-}$, $p < 0.05$). Each data point is an average of two recordings. **(c)** Paired-pulse facilitation is not altered between the two genotypes in the three age groups under observation ($p > 0.1$, for all comparisons). The facilitation ratio (slope of second EPSP/slope of first EPSP) was plotted as a function of interpulse interval, 20, 50, 100, 200 and 500 ms. For each group, the mean ± SEM is indicated. **(d)** Superimposed pooled data showing the normalized changes in field potential slope (± SEM) in $Ddo^{+/+}$ and $Ddo^{-/-}$ mice induced by HFS protocol. fEPSP slopes were recorded and expressed as the percentage of the pre-tetanus baseline. A stimulation intensity that evoked 50% of maximal fEPSP response was used. There was a significant increase ($p < 0.05$) in the magnitude of LTP in four-month old mutants (n = 8), compared to control mice (n = 6) (43.9 ± 10.3% above baseline in $Ddo^{+/+}$, and 61.5 ± 3.3% in $Ddo^{-/-}$). At 9 months of age, this tendency was inverted and LTP values were 39.7 ± 9.3% above baseline in control (n = 8), and 19.5 ± 2.5% in mutant slices (n = 7). Finally, fEPSP was potentiated to only 8.5 ± 12.5% in thirteen-month old $Ddo^{-/-}$ mice (n = 6), compared to 30.07 ± 10.4% in age-matched $Ddo^{+/+}$ animals (n = 7). At 9 and 13 months of age, the genotypic effect on LTP was always significant ($p < 0.001$). The arrow represents the HFS used to induce potentiation **(e)** Summary graph (mean ± SEM) showing genotype- and age-effects on the fEPSP slopes (% of baseline) quantified 50-60 min after HFS (100 Hz for 1 s).

Figure 3. Memory abilities of $Ddo^{-/-}$ mice improve at adulthood but worsen during aging. **(a)** Four-month old $Ddo^{+/+}$ (n = 14) and $Ddo^{-/-}$ (n = 14) mice were trained for 5 consecutive days in a submerged platform version of the Morris water maze (acquisition phase). Two-way ANOVA, with escape latency as repeated measure, revealed a significant days effect [$F_{(4, 104)}$ =24.723, $p < 0.0001$], non significant genotype effect [$F_{(1, 104)}$ = 0.211, p = 0.6500] and genotype x days interaction [$F_{(4, 104)}$ = 0.261, p = 0.9026]. Acquisition phase was followed by a five-day reversal phase, in which the submerged platform was moved to the opposite position of the pool (as indicated in the figure). Two-way ANOVA showed a significant days effect [$F_{(4, 104)}$=5.147, p = 0.0008] but no differences between genotypes [$F_{(1, 104)}$ = 0.717, p = 0.4047] and no interaction

between genotype and days [$F_{(4, 104)} = 0.898$, $p = 0.4681$]. **(b)** A 60 s transfer test was performed after 3 days of training (probe 1). *Ddo*$^{-/-}$ animals spent significantly more time in the goal quadrant ($p < 0.05$, compared to right and opposite, $p < 0.01$ compared to left; Fisher's *post-hoc* comparison) while *Ddo*$^{+/+}$ mice did not show any preference for the target quadrant. **(c)** A second probe test was carried out at the end of acquisition phase (probe 2). After a longer training, targeted spatial searching was comparable between wild type and knockout mice (*Ddo*$^{+/+}$: $p < 0.01$, compared to others; *Ddo*$^{-/-}$: $p < 0.05$, compared to opposite, $p < 0.01$, compared to right, $p < 0.0001$, compared to left). **(d)** After a three-day reversal phase (probe 3), both *Ddo*$^{+/+}$ and *Ddo*$^{-/-}$ animals displayed a preference for the new goal quadrant (*Ddo*$^{+/+}$: $p < 0.05$, compared to others; *Ddo*$^{-/-}$: $p < 0.01$, compared to old goal and right, $p < 0.0001$, compared to left) even if knockouts spent significantly more time in this area, compared to wild type mice ($p < 0.05$; Student's *t* test). **(e)** At the end of the reversal phase (probe 4), both *Ddo*$^{+/+}$ and *Ddo*$^{-/-}$ mice showed a comparable preference for the new goal area (*Ddo*$^{+/+}$: $p < 0.05$, compared to right and left, $p < 0.01$, compared to old goal; *Ddo*$^{-/-}$: $p < 0.05$, compared to right, $p < 0.01$, compared to left and old goal). **(f)** At 9 months of age, *Ddo*$^{+/+}$ (n = 10) and *Ddo*$^{-/-}$ (n = 10) mice showed similar learning abilities both during acquisition [two-way ANOVA with repeated measures: days effect, $F_{(4, 72)} = 35.729$, $p < 0.0001$; genotype effect, $F_{(1, 72)} = 0.135$, $p = 0.7179$; genotype x days interaction, $F_{(4, 72)} = 0.668$, $p = 0.6160$] and reversal phase [two-way ANOVA with repeated measures: days effect, $F_{(4, 72)} = 10.090$, $p < 0.0001$; genotype effect, $F_{(1, 72)} = 0.045$, $p = 0.8352$; genotype x days interaction, $F_{(4, 72)} = 0.270$, $p = 0.8963$]. **(g)** In the first retention test, wild type and mutant mice did not show preference for the goal quadrant while, **(h)** after a longer training, both groups spent a significantly longer time in the proper quadrant (*Ddo*$^{+/+}$: $p < 0.01$, compared to others; *Ddo*$^{-/-}$: $p < 0.05$, compared to right, $p < 0.01$, compared to left and opposite). Similarly, **(i)** in the first retention test of the reversal phase, *Ddo*$^{+/+}$ and *Ddo*$^{-/-}$ mice did not properly discriminate among quadrants. On the other hand, **(j)** after a five-day reversal training, only *Ddo*$^{+/+}$ animals remembered the new targeted location of the platform ($p < 0.01$, compared to right, $p < 0.0001$, compared to left and old goal). **(k)** At 13 months of age, both *Ddo*$^{+/+}$ (n = 11) and *Ddo*$^{-/-}$ (n = 9) mice showed similar acquisition. Two-way ANOVA revealed a significant days effect [$F_{(4, 72)} = 8.534$, $p < 0.0001$], non significant genotype effect [$F_{(1, 72)} = 4.641E\text{-}4$, $p = 0.9830$] and genotype x days interaction [$F_{(4, 72)} = 1.353$, $p = 0.2588$]. During reversal phase, differently to controls, *Ddo*$^{-/-}$ animals show altered spatial learning [two-way ANOVA: genotype x days interaction, $F_{(4, 72)} = 3.129$, $p = 0.0198$]. **(l)** In the transfer test, performed after 3 days of training, both *Ddo*$^{+/+}$ and *Ddo*$^{-/-}$ animals showed an unbiased spatial search. **(m)** In the probe 2, only *Ddo*$^{+/+}$ mice acquired the information about the correct spatial location of the platform ($p < 0.05$, compared to left, $p < 0.01$, compared to right and opposite). **(n)** After a three-day reversal training, mice from

both genotypes did not show searching preference for the new goal area. **(o)** Conversely, in the probe 4, wild type, but not knockout mice, remembered the new spatial location of the platform ($p < 0.05$, compared to right, $p < 0.01$, compared to left and old goal). Escape time, expressed in seconds, was used as dependent variable in the acquisition and reversal phases. Search quadrant, expressed as percentage of time, was used as dependent variable in the probe tests. The dashed lines in panels **b**-**e**, **g**-**j** and **l**-**o** indicate the chance level (25%) of search in the four quadrants. * = $p < 0.05$ in **b**, **d**, **j** and **m**; ** = $p < 0.01$ in **o**, between genotypes (Student's *t* test). All values are expressed as mean ± SEM. Genotypes are as indicated.

Figure 4. Hippocampal synaptic markers are reduced in *Ddo*$^{-/-}$ mice. Representative, confocal images of MAP2 (**a**-**f**, **o**-**r**, green), NeuN (**a**, **d**, red), GFAP (**g**-**n**) and synaptophysin- (**o**-**r**, red) immunostaining in the CA1/2 (**a**-**f**, **o**-**r**) and CA3 (**g**-**n**) hippocampal subfield in four- and thirteen-month old *Ddo*$^{+/+}$ and *Ddo*$^{-/-}$ mice. In old *Ddo*$^{-/-}$ mice, a severe reduction of the MAP2-positive dendritic tree of pyramidal neurons is observed, compared to wild type (*Ddo*$^{+/+}$: **a**, low power; **b**, high power; *Ddo*$^{-/-}$: **d**, low power; **e**, high power). Remaining dendrites are irregular in shape and size (**c**: *Ddo*$^{+/+}$; **f**: *Ddo*$^{-/-}$). No significant variations in cell density (NeuN-positive nuclei) were observed in the pyramidal layer of CA1/2, between genotypes (**a**: *Ddo*$^{+/+}$; **d**: *Ddo*$^{-/-}$). In both genotypes, GFAP-IR increases in old (**m**: *Ddo*$^{+/+}$, **n**: *Ddo*$^{-/-}$) compared to young mice (**h**: *Ddo*$^{+/+}$, **i**: *Ddo*$^{-/-}$), and high power confocal images of astrocytes indicate hypertrophy of these cells in old animals, regardless genotypes (**g**: young, **l**: old; *Ddo*$^{+/+}$). Synaptophysin, associated to synaptic vesicles, depicts apical dendrites and soma of pyramidal neurons in CA1/2 hippocampal field of old mice (**o**, **p**: *Ddo*$^{+/+}$; **q**, **r**: *Ddo*$^{-/-}$).

(s) The semiquantitative evaluation of the different immunostainings is shown in the column graphs, where black bars refer to *Ddo*$^{+/+}$ and white bars to *Ddo*$^{-/-}$ mice. The percentage area for MAP2 and GFAP, and the optical density synaptophysin for young (4 months) and old (13 months) mice are illustrated. Data are expressed as mean ± SEM of 4 animals/group. The value in each animal derives from 6 measures on adjacent sections. The sampled areas were 15,000 μm^2 for MAP2 and GFAP; 60,000 μm^2 for synaptophysin. One-way ANOVA and Tukey *post-hoc* test were used for statistical analyses. Genotype differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$) and age differences are indicated by hashes ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$).

Abbreviations: *o*: stratum oriens; *p*: stratum pyramidale; *r*: stratum radiatum; *lm*: stratum lacunosum-moleculare; *m*: stratum molecular. Bars: **a**, **d**, 100 μm; **b**, **e**, 50 μm; **c**, **f**, 10 μm; **h**, **i**, **m**, **n,** 100 μm; **g**, **l,** 10 μm; **o**, **q**, 20 μm; **p**, **r**, 10 μm.

Figure 5. Oral administration of D-aspartate modulates hippocampal synaptic functions at CA1 synapses. **(a)** D-aspartate levels were measured by HPLC in the hippocampus of C57BL/6J mice treated for 3 (H_2O, n = 7; D-aspartate, n = 6) or 12 months (H_2O, n = 3; D-aspartate, n = 3) with a 20 mM D-aspartate solution. Shorter and longer chronic treatments started on 45 days old mice. Both treatments significantly increased the hippocampal levels of the D-amino acid, compared to respective controls. However, no differences appeared within groups at different ages. * = $p < 0.05$, ** = $p < 0.01$, compared with control groups (Student's *t* test). Values are expressed as mean ± SEM. Treatments are as indicated. **(b)** Pooled data (mean ± SEM) showing the relationship between fEPSP slope and their corresponding presynaptic fiber volley amplitudes in C57BL/6J D-aspartate treated *vs* untreated mice. Treated mice continuously drank 20 mM D-aspartate for 3 (left panel) or 12 months (right panel). A regression fit of fEPSP versus presynaptic fiber volley at a stimulating intensity that produces 35% of the maximum response shows a similar pattern of response between the two groups. The trendlines for I-O curves in three-month treated mice were: y = 2.13x, R^2 =0.96 for treated *vs* y = 2.70x, R^2 =0.91 for untreated animals ($p > 0.1$). The following trendlines were observed following one year chronic treatment: y = 1.56, R^2 =0.89 in treated *vs* y = 2.15x, R^2 = 0.96 in untreated animals ($p > 0.1$). **(c)** Pooled data showing the paired-pulse ratio ± S.E.M., against the paired-pulse interval following three- and twelve-month D-aspartate treatments *vs* respective H_2O groups. No significant differences in paired-pulse facilitation were observed between the two treatments, at every tested interval ($p > 0.1$ for all comparisons). **(d)** Superimposed pooled data showing the normalized changes in field potential slope (± SEM) induced by HFS in three-month treated mice *vs* H_2O (left panel). Sixty min after application of HFS, the magnitude of LTP was 52.9 ± 10.1% above baseline in untreated (n = 8) but 74.5 ± 11.5% in three-month treated mice (n = 6) ($p < 0.001$). This potentiating effect was transient (middle panel) since LTP returned to control levels (45.1 ± 8.1% above baseline, n = 6, $p > 0.05$) following a three-week D-aspartate withdrawal, and was re-inducible (right panel) after one-month re-administration (62.2 ± 5.9% above baseline, n = 5, $p < 0.001$). **(e)** Superimposed pooled data showing the effects of continuous one-year D-aspartate administration on NMDAR-dependent LTP. The degree of potentiation was 47.15 ± 11.9% above baseline in untreated (n = 7) compared to 29.09 ± 2.8% in treated mice (n = 8) ($p < 0.001$). **(f)** Summary graph (mean ± SEM) showing the modulatory effects of three- *vs* twelve-month treatment on the fEPSP slopes (% of baseline) quantified 50-60 min after HFS.

Figure 6. Chronic D-aspartate treatment enhances spatial memory in C57BL/6J adult mice. **(a)** C57BL/6J mice treated for 3 months with D-aspartate (n = 10) and age-matched untreated animals (n = 10) were trained for 5 consecutive days in a submerged platform version of the Morris water

maze (acquisition phase). As shown in the figure, platform was subsequently moved to the opposite location and mice further trained for 5 days (reversal phase). As indicated by two-way ANOVA with repeated measures, treated and untreated mice displayed similar learning profiles, both during acquisition [days effect, $F_{(4, 72)} = 29.186$, $p < 0.0001$; treatment effect, $F_{(1, 72)} = 0.153$, $p = 0.7001$; genotype x days interaction, $F_{(4, 72)} = 0.545$, $p = 0.7034$] and reversal phase [days effect, $F_{(4, 72)} = 39.970$, $p < 0.0001$; treatment effect, $F_{(1, 72)} = 0.534$, $p = 0.4744$; genotype x days interaction, $F_{(4, 72)} = 1.408$, $p = 0.2398$]. **(b)** In the first probe test performed after 3 days of training, both groups preferentially searched the platform in the proper quadrant (untreated: $p < 0.05$, compared to others; treated: $p < 0.01$, compared to opposite, $p < 0.0001$, compared to right and left; Fisher's *post-hoc* comparison) but treated mice spent significantly more time in this location ($p < 0.05$, between genotypes; Student's *t* test). **(c)** After a five-day training, both D-aspartate treated and untreated animals similarly remembered the correct position of the platform (untreated: $p < 0.05$, compared to others; treated: $p < 0.05$, compared to left, $p < 0.01$, compared to right, $p < 0.0001$, compared to opposite). **(d)** Differently to untreated mice, after 3 days of reversal training, D-aspartate treated mice were already able to discriminate the new goal quadrant from others ($p < 0.05$, compared to left and old goal, $p < 0.01$, compared to right) while, **(e)** after 5 days, both groups displayed a similar spatial search (untreated: $p < 0.05$, compared to others; treated: $p < 0.05$, compared to left and old goal, $p < 0.01$, compared to right). **(f)** After twelve-month D-aspartate treatment, both C57BL/6J treated (n = 11) and untreated (n = 10) mice similarly learned the correct location of the platform, both during acquisition [days effect, $F_{(4, 76)} = 63.430$, $p < 0.0001$; treatment effect, $F_{(1, 76)} = 1.264$, $p = 0.2748$; genotype x days interaction, $F_{(4, 76)} = 0.630$, $p = 0.6424$] and reversal phase [days effect, $F_{(4, 76)} = 16.685$, $p < 0.0001$; treatment effect, $F_{(1, 76)} = 0.031$, $p = 0.8618$; genotype x days interaction, $F_{(4, 76)} = 0.391$, $p = 0.8144$]. **(g)** In the first probe test, both treated and untreated mice were not able to remember the position of the platform. **(h)** However, after two additional days, both D-aspartate- and H_2O-treated animals spent significantly more time in the goal quadrant (untreated: $p < 0.05$, compared to left, $p < 0.01$, compared to right and opposite; treated: $p < 0.01$, compared to others). Similarly, **(i)** in the first probe test of the reversal phase, both groups displayed a random search of the platform while, **(j)** in the second reference memory task, treated and untreated animals showed a bias spatial search in the correct new goal quadrant (untreated: $p < 0.05$, compared to right, $p < 0.01$, compared to left and old goal; treated: $p < 0.05$, compared to right and left, $p < 0.01$, compared to old goal). Escape time, expressed in seconds, was used as dependent variable in the acquisition and reversal phases. Search quadrant, expressed as percentage of time, was used as dependent variable in the probe tests. The dashed lines in panels **b-e** and **g-j** indicate

the chance level (25%) of search in the four quadrants. * = $p < 0.05$ in **b** and **d**, between treatments (Student's *t* test). All values are expressed as mean ± SEM. Treatments are as indicated.

Figure 7. Beneficial effects of D-aspartate in hippocampus-related functions during aging. All experiments were carried out on female C57BL/6J mice. **(a)** D-aspartate levels were measured by HPLC in the hippocampus of thirteen-month old C57BL/6J mice which drank H_2O (n = 4) or a 20 mM D-aspartate solution (n = 3) between their 12^{th} and 13^{th} month of life. One-month D-aspartate administration consistently increased the hippocampal levels of this D-amino acid, compared to the control. ** = $p < 0.01$, compared to untreated mice (Student's *t* test). Values are expressed as mean ± SEM. Treatments are as indicated. **(b)** Superimposed pooled data showing input-output curves in two-month (n = 7), thirteen-month old (n = 6) untreated mice and in thirteen-month old D-aspartate treated mice (n = 8). No differences were observed among the three groups under observation. **(c)** Superimposed pooled data showing the normalized changes in field potential slope (± SEM) induced by HFS in two-month old (58.07 ± 3.1% above baseline, n = 8) *vs* thirteen-month old untreated mice (32.22 ± 6.5% above baseline, n = 6). A treatment with D-aspartate between the 12^{th} and the 13^{th} month of life was able to reverse the potentiation decay observed in controls to 82.25 ± 4.6% above baseline (n = 8). **(d)** Summary graph (mean ± SEM) showing the fEPSP slopes (% of baseline) quantified 50-60 min after HFS (100 Hz for 1 s) in the three groups under observation. Notably, the degree of potentiation was significantly higher in treated *vs* age-matched controls ($p < 0.001$). Also the age-dependent effect on the decay of LTP in control animals was significant ($p < 0.001$). **(e)** Thirteen-month old untreated (n = 10) and age-matched D-aspartate treated animals (n = 10) were employed for 5 consecutive days in a submerged platform version of the Morris water maze (acquisition phase). Platform was subsequently moved to the opposite location (as shown in the figure) and mice further trained for 5 days (reversal phase). As revealed by two-way ANOVA with repeated measures, both groups revealed a significant decrease in the latency to reach the platform during the acquisition days [$F_{(4, 72)} = 31.347$, $p < 0.0001$], even if treated mice displayed improved learning abilities, compared to untreated animals [$F_{(1, 72)} = 7.774$, $p = 0.0121$]. During reversal phase, both untreated and one-month treated old mice evidenced similar learning abilities [days effect, $F_{(4, 72)} = 13.232$, $p < 0.0001$; treatment effect, $F_{(1, 72)} = 0.251$, $p = 0.6222$; genotype x days interaction, $F_{(4, 72)} = 1.850$, $p = 0.1287$]. Nevertheless, D-aspartate treatment slightly ameliorated learning abilities of mice at day 10 ($p < 0.05$). In the probe tests performed during acquisition phase, however, treated and untreated mice did not display to remember the correct location of the platform, both after a **(f)** shorter and a **(g)** longer training. During reversal phase, **(h)** both groups displayed a random search of the platform after 3 days of training while, **(i)** after a

longer training period, both treated and untreated mice displayed a significant bias search in the proper quadrant (treated and untreated: $p < 0.05$, compared to others). Escape time, expressed in seconds, was used as dependent variable in the acquisition and reversal phases. Search quadrant, expressed as percentage of time, was used as dependent variable in the probe tests. The dashed lines in panels **f-i** indicate the chance level (25%) of search in the four quadrants. All values are expressed as mean ± SEM. Treatments are as indicated.

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AUTHOR CONTRIBUTIONS

A.U. was the project leader and wrote the manuscript together with R.N. and F.E.

F. E. and F. N. performed behavioral studies (**Fig. 3, Fig. 6, Fig. 7**) and prepared animal models used in this work. All electrophysiological experiments were carried out by R. N. (**Fig. 2, Fig. 5, Fig. 7**), except those reported in **Fig 1**, performed by M. G.. HPLC experiments included in **Fig. 2, Fig. 5, Fig. 7** were performed by A. D. and E. T.. Morphological analysis showed in **Fig. 4** was performed by S. S. under the supervision of L. C. who prepared such figure.

N. B. M. and G. B. participated in the study design and revised the draft manuscript.

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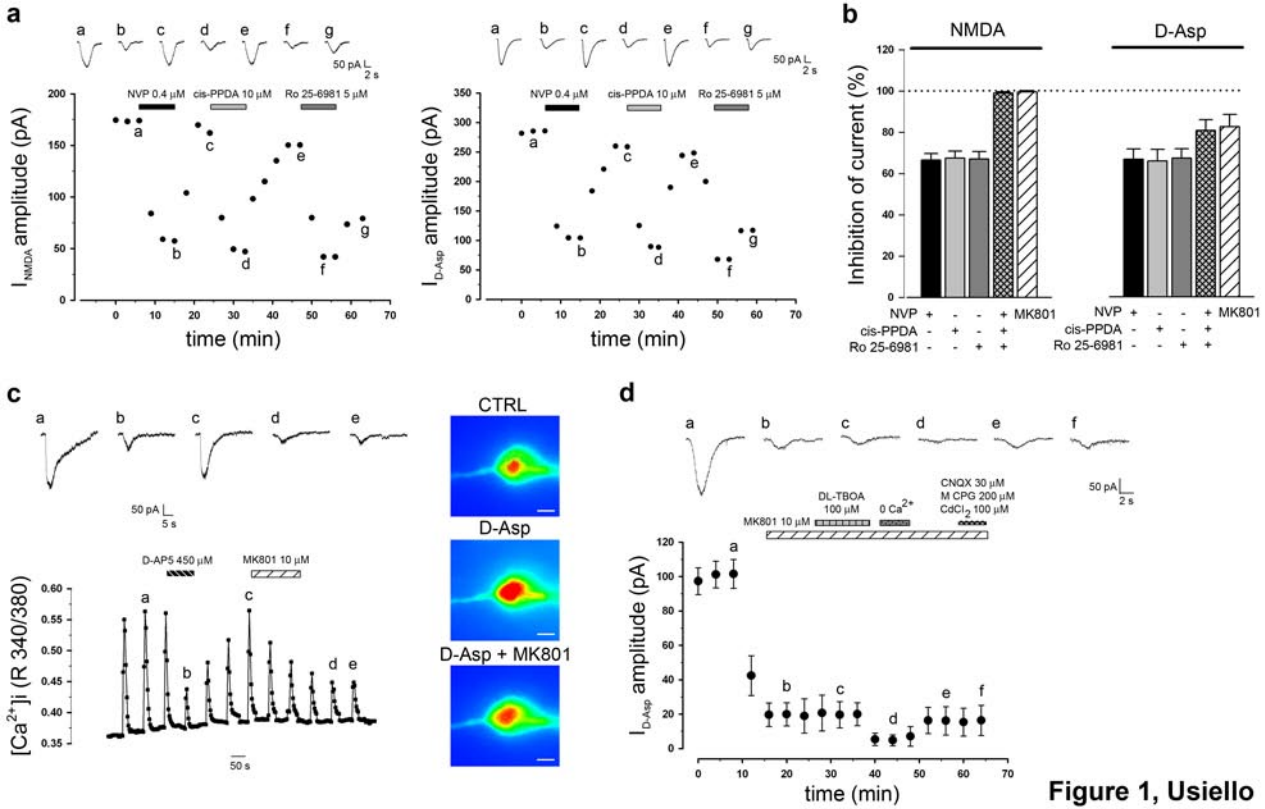

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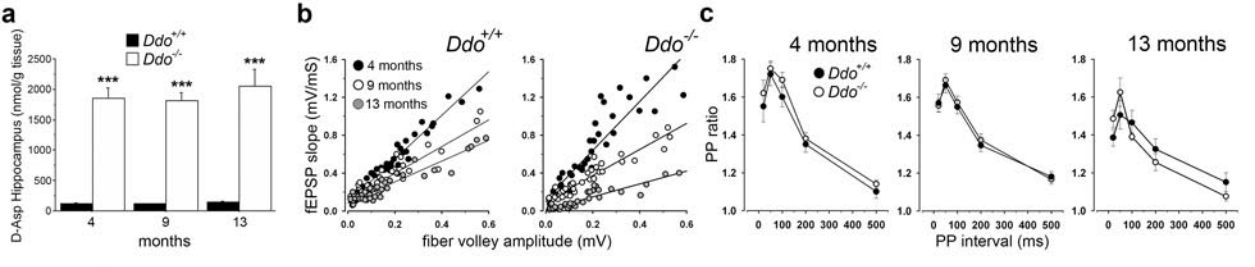

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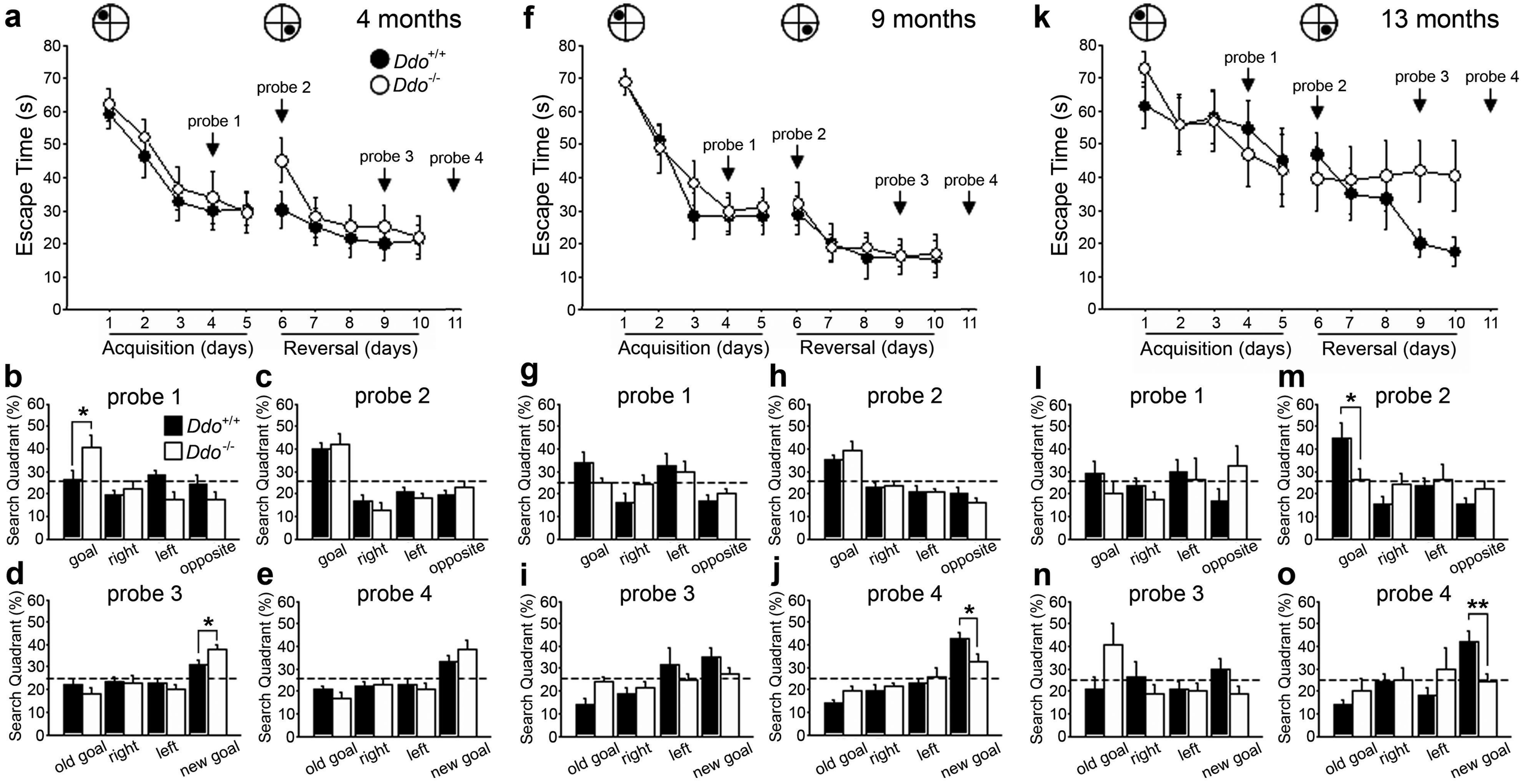

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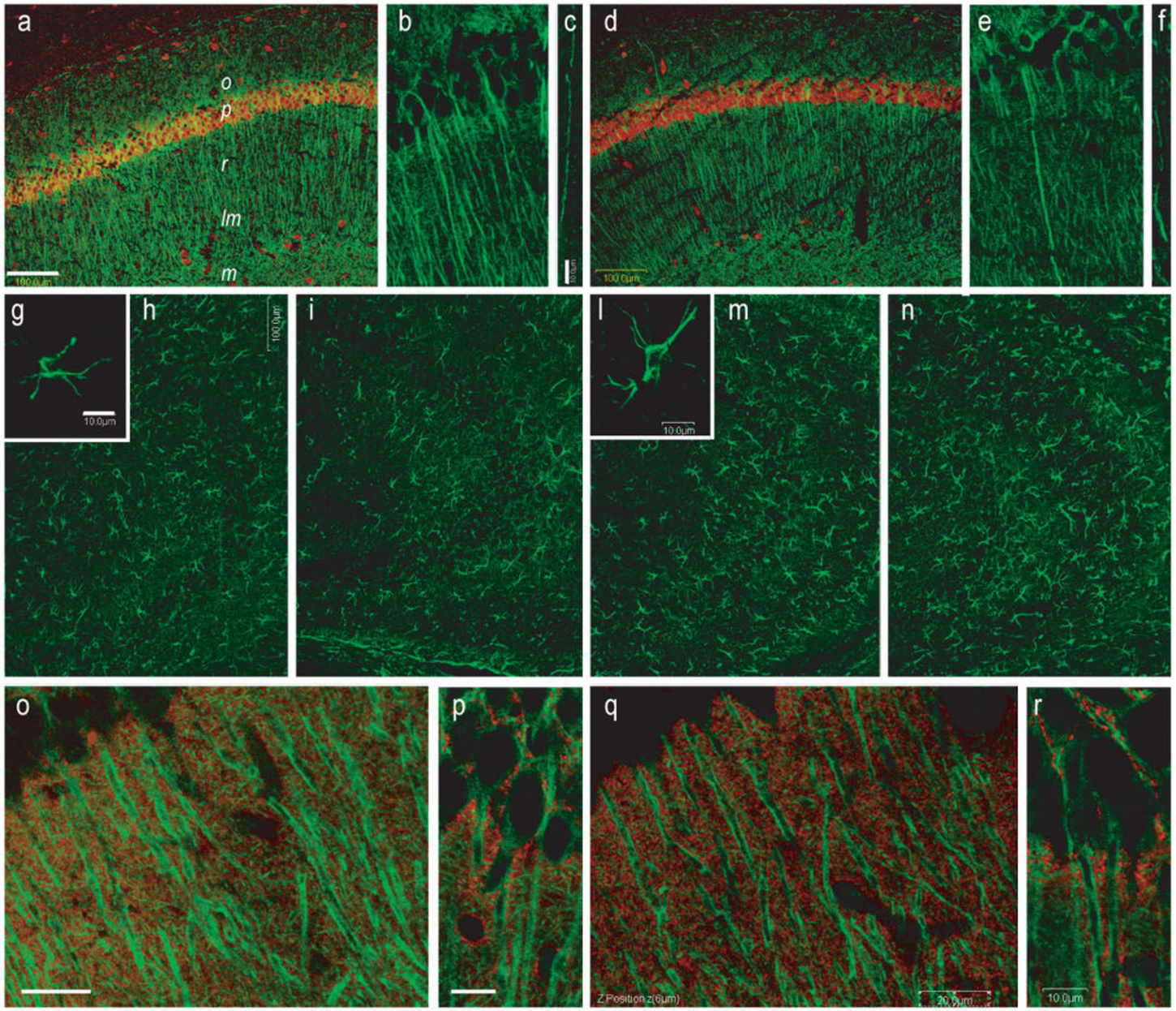

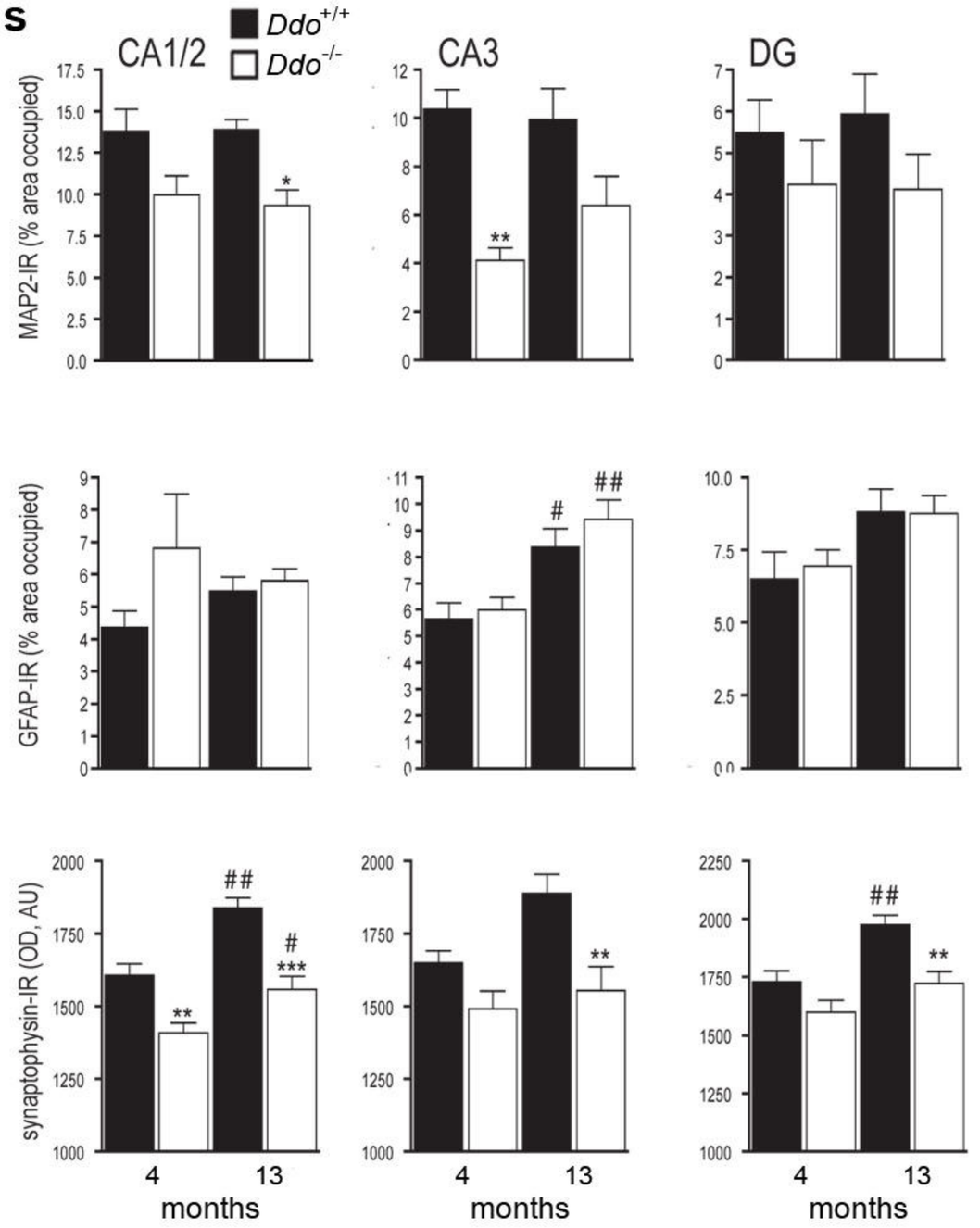

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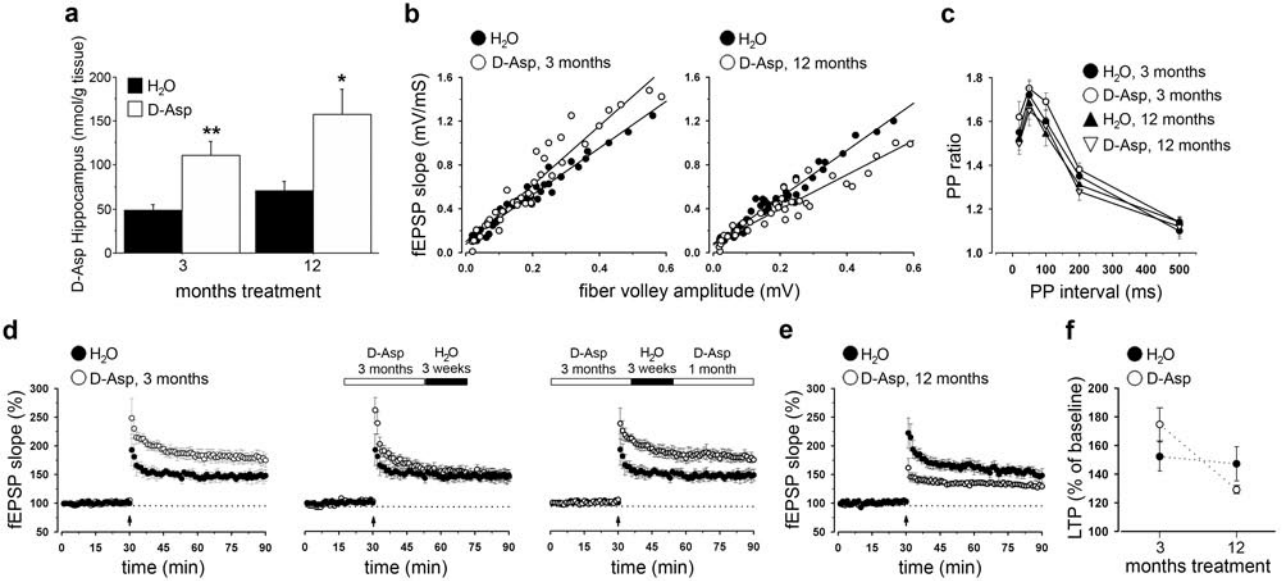

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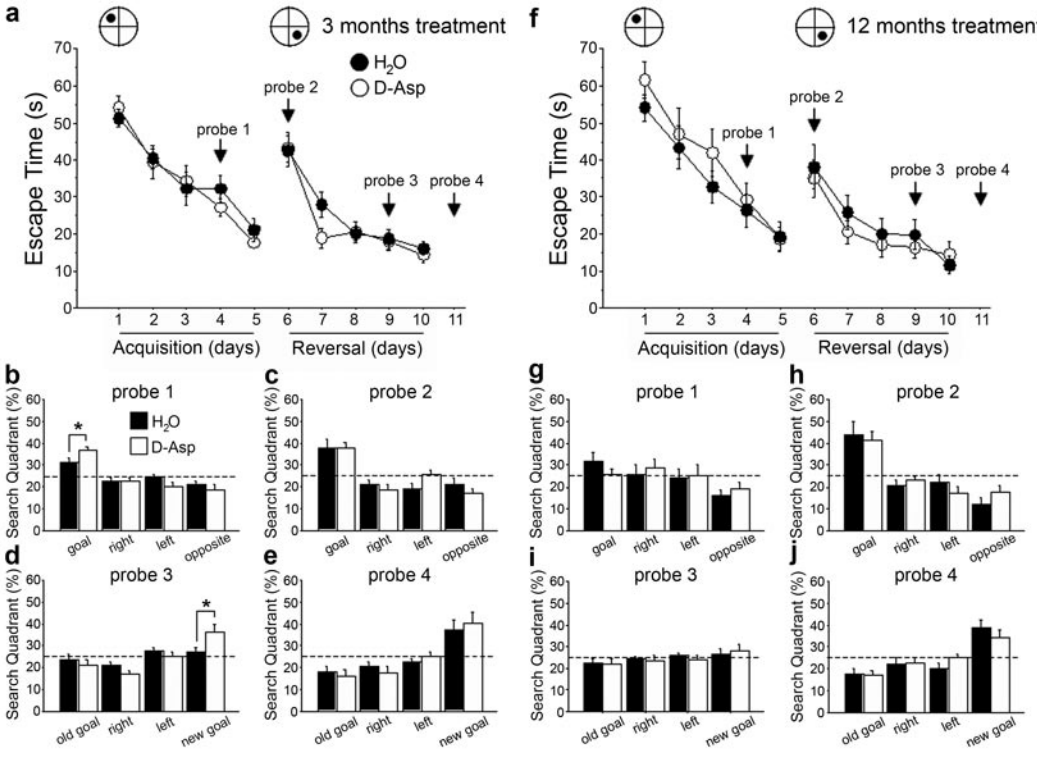

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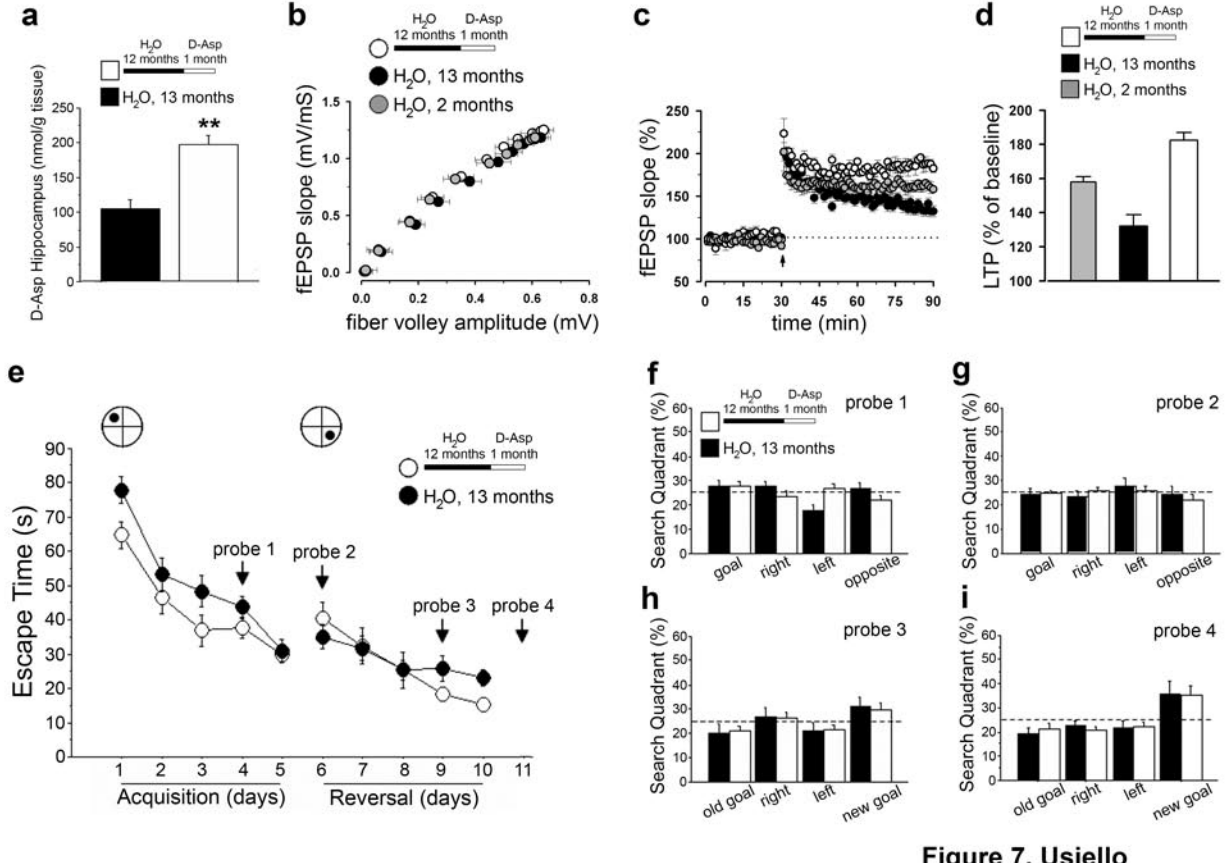

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