International PhD Program in Neuropharmacology

XXIII Cycle

NEUROPHARMACOLOGY AND BEHAVIURAL ANIMAL MODELS

Doctorate Thesis

Carmen Mazzola

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Introduction

The study of human disease often involves performing physiological and pharmacological experiments in animal models.

Generally, experimental results obtained in these models are extrapolated to the human situation, providing new insights into disease mechanisms and treatment options. To be able to reliably extrapolate results obtained in animal experiments, it is important to consider the validity of the animal model used, i.e., the extent to which the model mimics the disease. This validity is often characterized by *1*) the resemblance in symptoms (face validity), *2*) shared etiology and underlying pathophysiological mechanisms (construct validity), and *3*) similarity of pharmacological responses (predictive validity). Hence, the analysis of face, construct, and predictive validity of animal models constitutes a very important aspect in the study of disease physiology and pharmacology.

TIN CHLORIDE ENHANCES HIPPOCAMPAL NEURONAL SURVIVAL BY MODULATING HEME METABOLISM IN A MODEL OF CEREBRAL ISCHEMIA

$SnCl_2$ has been reported to increase the expression level of heme-oxygenase 1 (HO-1) and to decrease ischemic injury, in not-nervous tissues. This study was aimed to examine the neuroprotective effect of $SnCl_2$ in the hippocampus of rats submitted to cerebral ischemia. $SnCl_2$ was administered 18 hours before bilateral carotids obstruction. Changes in HO-1 expression and activity, heme content, inducible nitric oxide synthase (iNOS) expression and interneuronal cells survival was studied. Thereafter both behavior and memory were tested. The administration of $SnCl_2$ increased the expression of HO-1 protein and HO activity in the hippocampus and concomitantly decreased heme content at both mitochondrial and nuclear level. Furthermore, ischemized animals showed a strong increase in iNOS expression in the hippocampus, where a loss of interneurons also occurred. Pre-treatment with $SnCl_2$, decreased both iNOS expression in ischemized rats and increased survival of hippocampal interneurons. The beneficial effects of $SnCl_2$ were prevented by concomitant treatment with SnMP, a strong inhibitor of HO activity. $SnCl_2$ also caused an improvement in short term memory. Our results showed that following $SnCl_2$ administration, HO-1 is strongly induced in hippocampus and that it can modulate heme metabolism and iNOS expression, resulting in strong neuroprotective effect following ischemic injury.

INTRODUCTION

Cerebral ischemia leads to brain injury through a complex series of pathophysiological events leading to neuronal death and subsequent neurological

dysfunction. There is a wealth of evidence to suggest that this acute neuronal damage is followed by a second round of neuronal injury, called delayed neuronal death, in the bystanding areas of the ischemic core [1,2]. Ischemia leads to the elevation of extracellular glutamate and activation of glutamate receptors, with a subsequent increase in intracellular calcium, resulting in generation of free radicals and overproduction of nitric oxide (NO) from excessive or inappropriate stimulation of NO synthase enzymes (NOS), which appears to mediate a major component of excitotoxic damage [3]. In particular, iNOS, the inducible isoform, is barely detectable in healthy tissue, but under pathological conditions it can be expressed in most tissues, including neurons, astrocytes, and endothelial cells [4]. Clarification of the role of iNOS in late neuronal injury accompanying cerebral ischemia is provided by the observation that mice lacking the gene for iNOS have significantly reduced infarct volumes compared with wild-type controls [5].

The heme oxygenase system (HO), the rate limiting enzyme in heme catabolism, has been associated with strong protective effects in various experimental models [6]. The neuroprotective effect of HO is related to the conversion of the free heme, a prooxidant molecule, into anti-oxidadant end products. Two HO isoforms have been shown to be catalytically active in heme degradation [6]. HO-2 is constitutively expressed, whereas HO-1 is inducible.

This research was aimed to study if the administration of $SnCl_2$, a pharmacological inducer of HO-1, could result in a neuroprotective effect.

MATERIALS AND METHODS

Animals

Animals were subdivided into 4 groups (a group of $SnCl_2$-treated plus a group of saline-treated rats underwent to cerebral ischemia and a group of $SnCl_2$-treated plus a

group of saline-injected rats were sham-operated). $SnCl_2$ (10 mg/100g b.w.) was administered 18h before induction of cerebral ischemia. Tin mesoporphyrin (SnMP) (Frontier Scientific Europe, Carnforth, UK) a potent HO activity inhibitor, was used at a dose of 10 mg/kg in a separate set of experiments in order to elucidate the effects of HO activity. Rats were anesthetized by an intraperitoneal injection of ethyl urethane (1.2 g/kg body weight) and then subjected to transient ischemia by bilateral clamping of the common carotid arteries for 20 minutes and reperfused for 72 hours.

Immunohistochemistry

Immunohistochemical studies were performed as previously described using anti-HO-1 or anti-iNOS (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA). In a separate set of experiments, parvalbumin was used in order to evaluate specific hippocampal interneuron loss following ischemia.

HPLC Analysis of Heme Content in mitochondrial and nuclear fractions

Mitochondrial and nuclear fractions were obtained using a commercially available kit (Pierce Biotechnology, Rockford, IL) which results in a high grade purity preparations, as demonstrated by low cytoplasm contamination (data not shown). Heme was extracted from 40 µl of organelles fraction with 300 µl of HCl/acetone (250 µl of HCl in 10 ml of acetone).

Measurement of HO-1 content and activity

A commercially available enzyme linked immunosorbent assay (ELISA) kit (Stressgen, Victoria, Canada) was used to measure HO-1 protein concentration in the hippocampus. HO activity was assayed by extracting bilirubin with chloroform and determining its concentration spectrophotometrically (Perkin-Elmer Dual UV/VIS Beam Spectrophotometer Lambda 25) using the difference in absorbance at a wavelength from 460 to 530 nm with an absorption coefficient of 40 $mmol^{-1}cm^{-1}$.

Behavioural Tests

Open Field

Test was performed as previously described [7]. Briefly, spontaneous motor activity was scored in a circular open field arena. Animals were placed in a circular litup arena, with the bottom divided into 27 areas of equal size. The behavior of each animal was observed for a period of 5 min and the occurrence of these items was recorded: locomotion; rearing and grooming.

Passive avoidance test

The apparatus for the step-through passive-avoidance test was an automated shuttle-box (Ugo Basile, Milan, Italy) divided into an illuminated compartment and a dark compartment of the same size by a wall with a guillotine door, and the floor was a grid capable of delivering a controlled foot-shock (0.5 mÅ intensity, 2 seconds duration).

Adaptation, training trial, and retention test

Retention of the passive avoidance response was measured during the retention tests performed 1 and 7 days after the learning trial. Each animal was again placed into the illuminated compartment, the door was opened, and the latency to re-enter the dark compartment was recorded. No foot-shock was delivered when retention tests were performed. If the rat failed to enter the dark compartment within 300 s during retention testing, the trial was terminated and the latency was recorded as 300 s.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Bonferroni's *t* test was performed in order to estimate significant differences among groups. Data were reported as mean values ± SD. Regarding behavioural tests, the results (time in sec employed for re-entering the dark box in the first or second retention test) were

expressed as mean ± s.e.m.. The data for each experiment was analyzed using one-way ANOVA followed by Tukey's *post hoc* procedure for multiple comparisons. Differences between groups were considered to be significant at $p<0.05$.

RESULTS

Protein content and functional activity of HO-1 in the hippocampus of $SnCl_2$ treated rats

HO-1 protein content, sampled by ELISA test in extracts of the hippocampus of animals treated with $SnCl_2$, increased up to 3.2 ± 0.4 folds compared to control (Figure 1A). The increased level of HO-1 protein was also followed by a significant induction of HO activity. In particular, $SnCl_2$ treatment increased HO activity up to 3.8 ± 0.2 folds compared to control basal activity (Figure 1B). Consistently with these results, we observed a concomitant reduction of heme content in both nuclear (-50%, Figure 1C) and mitochondrial (-60%, Figure 1D) compartments following $SnCl_2$ treatment. These results were further confirmed by immunohistochemical analysis showing that $SnCl_2$ increased HO-1 expression in all hippocampal regions (Figure 2A and B).

$SnCl_2$ controls iNOS expression following ischemia via heme metabolism regulation

The hippocampus of ischemized animals showed strong iNOS staining (Figure 2D) compared to sham operated animals (Figure 2C), whereas $SnCl_2$ treatment lowered the expression of the iNOS (Figure 2E) thus suggesting that $SnCl_2$ plays a strong inhibitory role on the expression of iNOS and, as consequence, on the generation of NO. To further confirm the role of the HO system in such regulation, in a separate set of experiments, animals were also treated with SnMP, a strong inhibitor of HO activity. These set of experiment showed that SnMP completely reversed the inhibitory effect of $SnCl_2$ on iNOS expression (Figure 2F).

We further tested the cellular density of the hippocampus, 72 hours after transient

ischemia in vehicle treated and $SnCl_2$ treated animals. Vehicle treated animals submitted to transient ischemia showed a dramatic decrease of PV-positive neuronal cells throughout the hippocampus (62% of PV positive cells) (Figures 3B and 4B) when compared to sham operated animals (Figures 3A and 4A). The decrease was more evident in the CA1, where PV-positive cells of transient ischemia animals corresponded to 38.1 % in respect of the cells of the CA1 of sham operated animals. Interestingly, rats pretreated with $SnCl_2$ (Figures 3C and 4C), showed a less severe depletion of hippocampal PV positive cells than vehicle treated animals. In fact, CA1 of $SnCl_2$ treated animals showed PV positive cells corresponding to 75% compared to 38.1% of untreated animals whereas, the overall hippocampal PV positive cells of $SnCl_2$ treated animals corresponded to 73.7% of survival interneuronal cells with respect to 62% of vehicle treated animals.

In order to test whether $SnCl_2$ neuroprotection could be mediated by the involvement of the HO pathway, we planned a separate set of experiments with the concomitant treatment of $SnCl_2$ and SnMP. These animals showed a loss of PV positive cells in the CA1 (39.5% of survival interneuronal cells with respect to 75% of $SnCl_2$ treated animals) and of PV positive cells throughout hippocampus (58.4% of survival interneuronal cells with respect to 73.7% of $SnCl_2$ treated animals) (Figures 3D and 4D). These values are similar to those observed in transient ischemia of vehicle treated animals.

Behavioral correlates following $SnCl_2$ treatment

$SnCl_2$ treatment increased locomotion activity up to +51% ($p<0.05$; Figure 5A). Furthermore, it also induced a slight increase of rearing behavior (Figure 5B) and impaired grooming behavior (Figure 5C). $SnCl_2$ and co-treatment with $SnCl_2$/SnMP in sham operated rats slightly increased and respectively decreased locomotion compared to

vehicle treated rats (Figure 5D). Transient ischemia induced low locomotion level (Figure 5D), which was significantly increased up to +254% by $SnCl_2$ ($p<0.05$) when compared to vehicle. Consistent with previous results, $SnCl_2$/SnMP co-treatment in transient ischemia rats prevented the increment of locomotion activity mediated by $SnCl_2$ (Figure 5D). Rearing and grooming behavior in transient ischemia rats showed no significant differences in all animals groups (Figure 5E and F).

Our results further showed that $SnCl_2$ treatment did not significantly affects the latency time of rats to re-enter the dark compartment in both Rt1 and Rt2 compared to vehicle treated rats (Figure 5G). In sham operated rats, $SnCl_2$ treatment increased up to +71% ($p<0.05$) the performance, while $SnCl_2$/SnMP co-administration prevented (+29.3%) the improved behavior induced by $SnCl_2$ when compared to vehicle (Figure 5H).

Following transient ischemia**,** passive avoidance test in vehicle treated rats showed a significant reduction (-64,2%) ($p<0.05$) of latency time when compared to the corresponding sham operated group. By contrast, $SnCl_2$ dramatically improved (+467%; $p<0.01$) the performance with respect to vehicle treated rats. Consistent with our previous biochemical data, the co-administration of $SnCl_2$/SnMP strongly antagonized the positive effect induced by $SnCl_2$ (Figure 5H).

Regarding Rt2**, v**ehicle treated rats of both sham operated and transient ischemia groups showed quite similar scores. The latency time of transient ischemia rats was higher that of the corresponding group of transient ischemia rats in Rt1 (Figure 5H). This finding showed that the retention of long term memory improved spontaneously up to normal values after 12 days recovery following transient ischemia injury. Treatment with $SnCl_2$ in both sham operated and transient ischemia rats did not change significantly the latency time of the passive avoidance test compared to the respective controls (Figure

5H). However, co-treatment with $SnCl_2$ and SnMP in both sham operated and transient ischemia groups strongly reduced the latency time with respect to both vehicle and $SnCl_2$ treated rats (Figure 5H).

DISCUSSION

The study showed that $SnCl_2$ strongly increased the HO-1 expression in neuronal cells and that $SnCl_2$ treatment prevented hippocampal neuronal cell loss due transient ischemia and that this resulted in improved performance of short term memory

HO-1 induction was associated with significant variations in mitochondrial and nuclear heme pool. In fact, significant decrement of heme content, expression of imbalance between degradation and synthesis, at mitochondrial level was observed in $SnCl_2$-treated animals. This finding is consistent with previous studies of Converso et al. [8]showing that administration of hemin, a potent inducer of HO-1 expression and activity, results in significant translocation of HO-1 protein to mitochondrial compartment, and this accounted for the increased heme degradation. Also nuclear heme pool is decreased following $SnCl_2$ treatment. This observation is noteworthy since nuclear HO-1 has been shown under various experimental conditions including excitotoxic injury [9].

The involvement of the HO system in iNOS regulation following transient ischemia is corroborated by the prevention of $SnCl_2$ effects by SnMP, a potent inhibitor of HO activity. Several mechanisms may concur to the decrease of iNOS regulation following HO-1 overexpression: 1) reduced heme availability which is required for iNOS assembly; 2) HO-1 derived CO may act as anti-inflammatory molecule and inhibiting iNOS expression at nuclear level, regulating transcriptional factors [10]; 3) bilirubin/biliverdin directly inhibits iNOS expression in various experimental models [11]; 4) HO-1 overexpression leads to decreased expression of CAT-2 transporter which

is required for cellular intake of arginine, the substrate of NOS [12].

Our results also clearly showed that $SnCl_2$ pretreatment significantly decreased transient ischemia mediated neuronal cell loss and that this mechanism is related to increased HO activity. In fact, treatment with SnMP completely prevented the protective effects of $SnCl_2$. Finally, our results showed that increased neuronal cell survival also translated into an improved functional performance of ischemized animals. In particular, we observed that $SnCl_2$ prevents short term memory impairment (Rt1) and also in this case the mechanism is related to HO-1 induction. In fact, SnMP treatment reversed the beneficial effect of $SnCl_2$ on short term memory retention. Interestingly, as far as long term memory (Rt2) is concerned, we did not observe any further recovery of memory performance following $SnCl_2$ treatment, thus suggesting that HO-1 did not further improve long term memory. However, inhibition of HO activity by SnMP impaired the physiological recovery of long tem memory in ischemized rats, suggesting that HO-1 is required in the physiological mechanism underlying such recovery.

CONCLUSIONS

Taken all together, our data showed that it is possible to regulate the expression and functional activity of HO-1 by $SnCl_2$ and this effect leads to a down-regulation of iNOS, thus preventing CA1 hippocampal neuronal cell loss and improving memory retention. Furthermore, our results may provide the molecular basis for the neuroprotective mechanism/s of important drugs used in daily clinical practice to attenuate or prevent cerebral injury due to transient ischemia.

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FIGURE LEGENDS

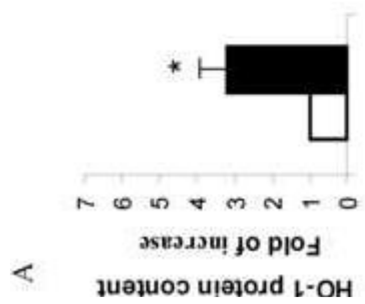
Figure 1: **(A)** ELISA detection of HO-1 protein in the hippocampus of vehicle and $SnCl_2$ treated animals. **(B)** HO activity measurement in the hippocampus of vehicle and $SnCl_2$ treated animals. **(C)** Nuclear and mitochondrial **(D)** levels of heme content in the hippocampus of vehicle and $SnCl_2$ treated animals. Data are presented as the mean ± SEM. (* $p<0.01$ vs vehicle).

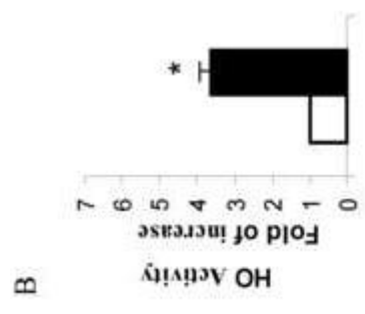
Figure 2: Photomicrographs of HO-1 distribution in hippocampus of vehicle **(A)** and $SnCl_2$ treated **(B)** rats. Transient ischemia injury **(C)** resulted in a significant increase of iNOS positive neurons when compared to sham operated animals **(D)**. Our data also showed that $SnCl_2$ (10 mg/Kg bw i.p.) treatment **(E)** significantly reduced iNOS positive neurons in the CA1 region following transient ischemia injury. The involvement of the HO system in this mechanism was confirmed by the coadministration of $SnCl_2$ and SnMP (10 mg/kg) **(F)**.

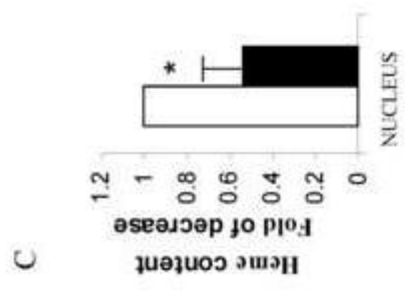
Figure 3: Photomicrographs of Parvalbumin positive interneurons in CA1 hippocampal region following transient ischemia injury. **(A)** sham operated animals. **(B)** Parvalbumin positive cells in the CA1 region. **(C)** prevented hippocampal interneurons cell loss following transient ischemia. The involvement of the HO system in this mechanism was confirmed by the co-administration of $SnCl_2$ and SnMP, a potent HO activity inhibitor, which completely prevented the effects of $SnCl_2$ **(D)**.

Figure 4: Drawings representing Parvalbumin positive interneuron distribution in different hippocampal regions in sham operated animals **(A)** and following transient ischemia injury **(B)**. Our data showed that $SnCl_2$ **(C)** selectively attenuated CA1 hippocampal interneuron loss following transient ischemia injury. The beneficial effects of SnCl2 were prevented by the concomitant treatment with SnMP (10 mg/kg) **(D)**.

Figure 5: Effects of $SnCl_2$ on intact rats tested in the Open Field Test. Our data showed that $SnCl_2$ (10 mg/Kg bw i.p.) increased locomotion activity of rats compared to vehicles (*$p<0.05$) (**A**). No significant changes were observed for rearing (**B**) and grooming (**C**). Our data also showed that $SnCl_2$ improved locomotion of transient ischemia rats compared to vehicle group (*$p<0.05$) (**D**). Similarly, no significant changes were observed for rearing (**E**) and grooming (**F**) following transient ischemia injury or pharmacological treatments. **(G)** Effects of $SnCl_2$ (10 mg/Kg bw i.p.) on intact rats in the passive avoidance test. No significant changes were observed following $SnCl_2$ treatment. **(H)** Effects of SnCl2 on ischemic rats tested in the passive avoidance test. During the first retention, vehicle transient ischemia rats showed a lower latency to reenter the dark box compared to vehicle sham operated rats (*$p<0.05$). $SnCl_2$ improves the latency to re-enter the dark compartment of sham operated rats compared to vehicle shamed operated rats (**$p<0.05$). $SnCl_2$ also improved the latency to reenter the dark compartment of transient ischemia rats compared to vehicle transient ischemia rats (***$p<0.01$) and its effect was prevented by SnMP (10 mg/kg) (#$p<0.05$). In the second retention test, $SnCl_2$+SnMP transient ischemia group showed a significantly decreaselatency compared to vehicle and S nCl_2 transient ischemia groups (##$p<0.01$).







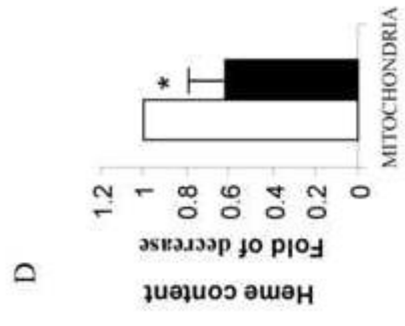


Figure 1

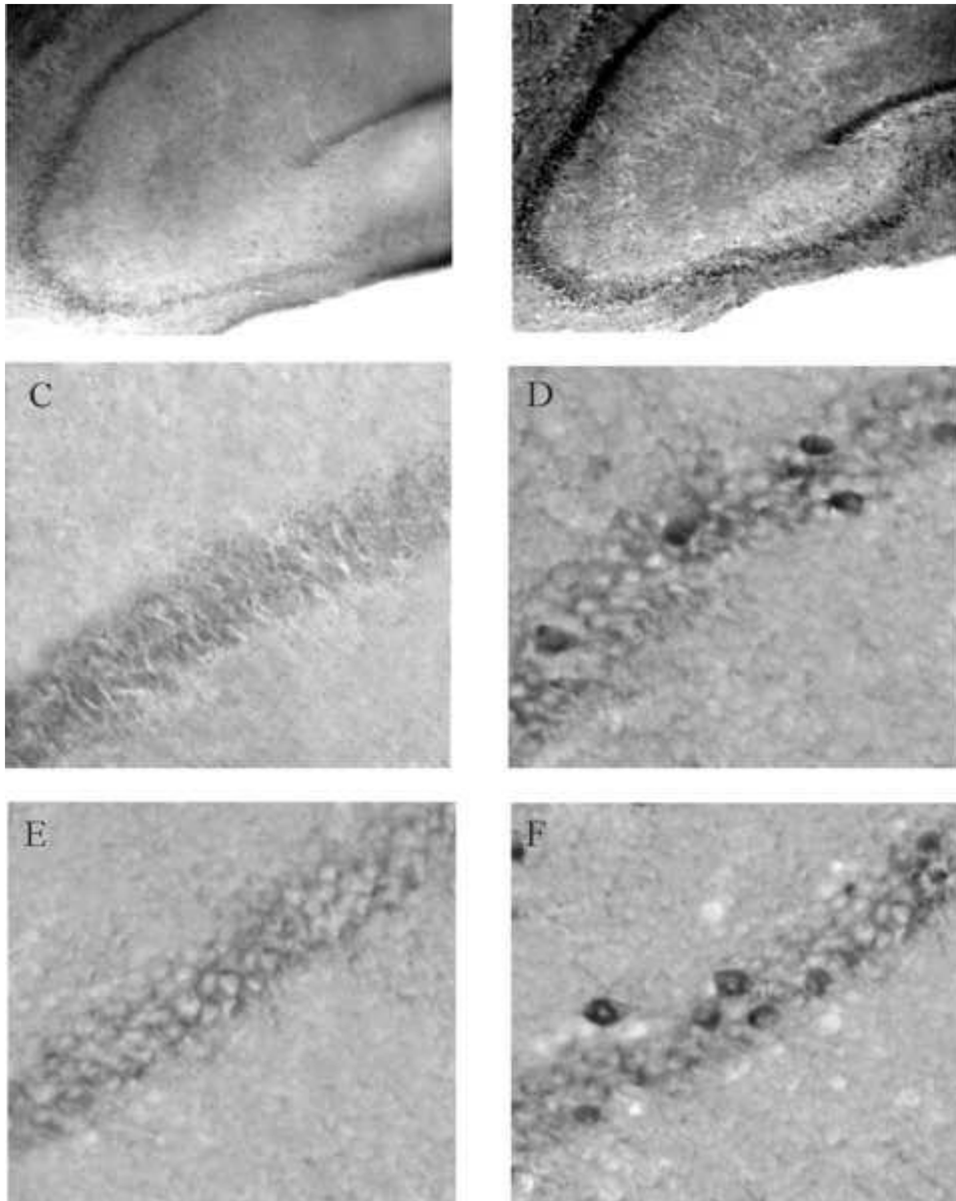


Figure 2

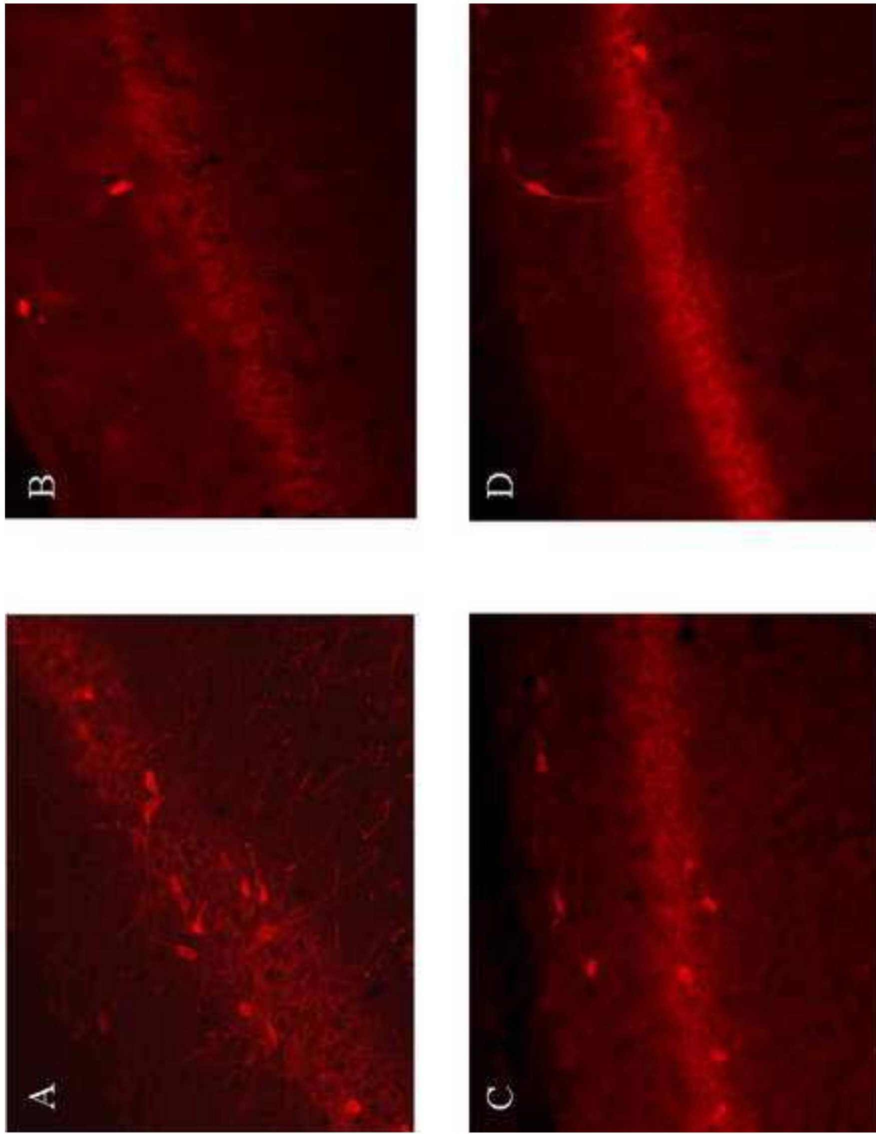
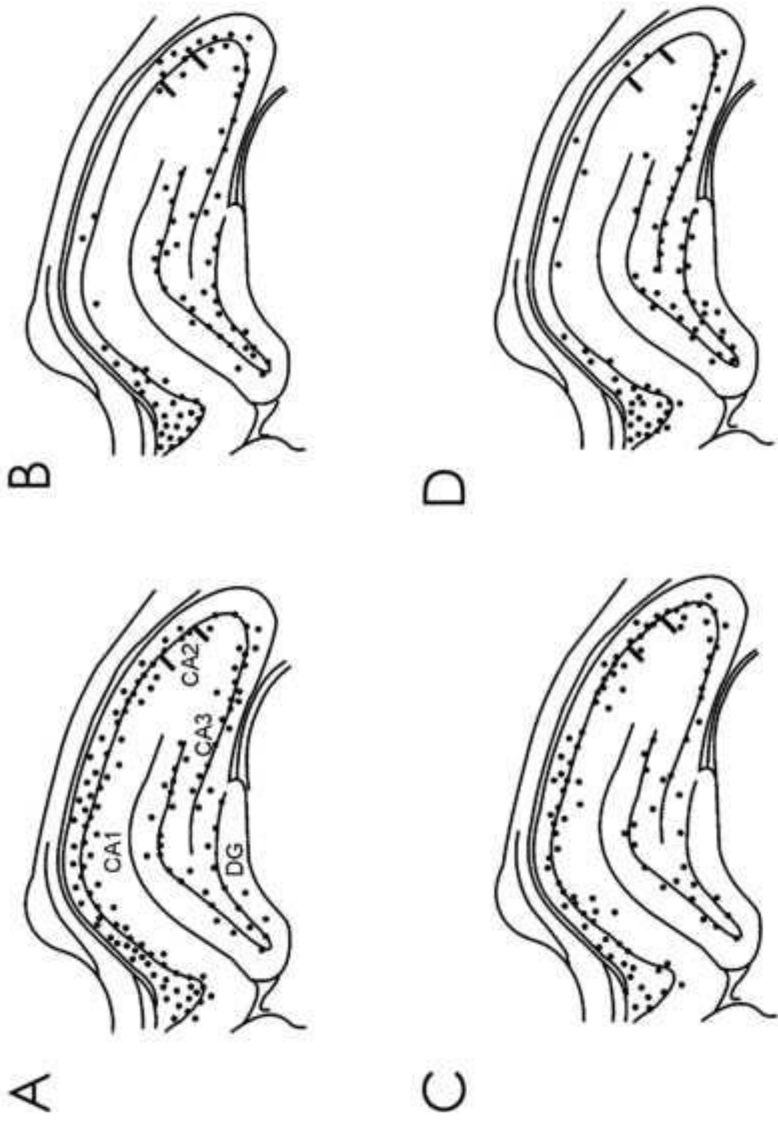
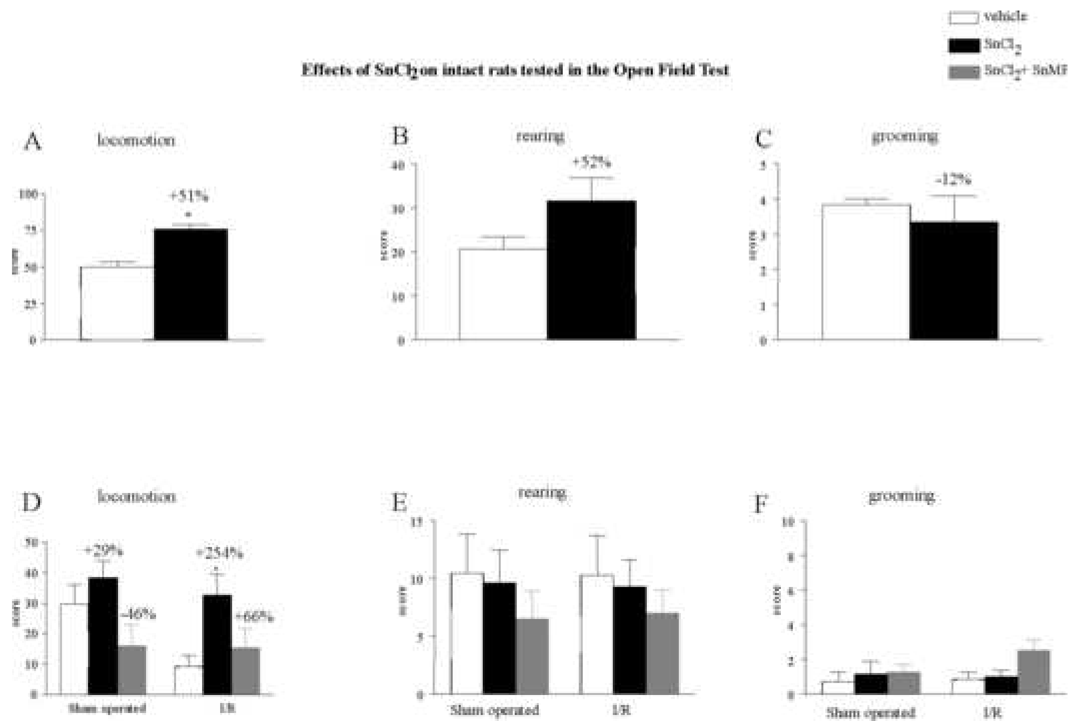


Figure 3



• = Pv positive cells

Figure 4

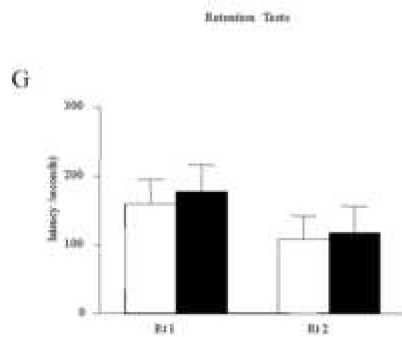


Effects of $SnCl_2$ on intactrats tested in the passive avoidance test

Effectsof $SnCl_2$ on ischemic rats tested in the passive avoidance test

Retention Tests

Retention Tests



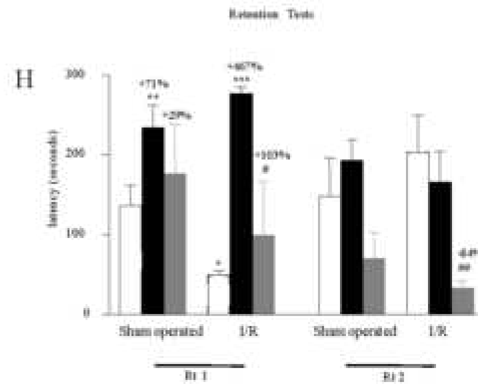


Figure 5

Dopamine D3 receptor knock-out mice exhibit increased behavioral sensitivity to the anxiolytic drug, diazepam

Dopamine D3 receptors (DRsD3) seem to have a pivotal role in mood disorders. Using the elevated plus-maze (EPM) and the novelty induced grooming test (NGT), we assessed the responses of DRD3-deficient (D3-/-) mice to the acute treatment (different testing time) with the anxiolytic drug, diazepam. D3-/- mice treated with diazepam (0.1 or 0.5 mg/kg) exhibited a better behavioral response in the EPM than their wild type (WT). Furthermore, in D3 -/- mice, but not in WT, 1 mg/kg diazepam induced anxiolytic effects at all testing times. The contribution of DRsD3 in the anxiolytic effects of diazepam was confirmed by similar results obtained in EPM by using the selective DRD3 antagonist U99194A (10 mg/kg) in combination with diazepam, in WT animals. D3-/- mice treated with diazepam (all doses), also showed a decrease in grooming behavior. However, the [3H]flunitrazepam autoradiographic analysis revealed no significant changes in D3 -/- mice compared to WT, suggesting that if γ- aminobutyric acid receptor GABAA changes are involved, they do not occur at the level of binding to benzodiazepine site. These data suggest that D3 -/- mice exhibit low baseline anxiety levels and provide the evidence that the DRD3 is involved in the modulation of benzodiazepine anxiolytic effects.

1. Introduction

Increasing evidence indicates that dopamine (DA) neurotransmission is involved in the pathophysiology of anxiety. In vitro and in vivo methods have shown that acute exposure to a range of stressors activates the mesocorticolimbic DA system and increases extracellular DA levels in the nucleus accumbens (NAc) and medial prefrontal cortex, inducing anxiolytic-like behavioral changes (Cabib and Puglisi-Allegra, 1994; Dunn, 1998). Further evidence has been

provided that stress-induced increases in DA metabolism can be attenuated by anxiolytic drugs such as diazepam (Finlay et al, 1995).

The action of DA in the central nervous system is mediated by five G protein-coupled receptors divided in two classes, the “D1-like”(D1, D5) and the “D2-like” (D2, D3, D4) receptors

(Seeman et al, 1994). While experimental findings on the involvement of dopamine D1 receptors (DRsD1) in anxiety are inconsistent, animal studies show anxiolytic-like effects of drugs acting as antagonists on dopamine D2 receptors (DRD2) (Pich and Samanin, 1986; Rodgers et al, 1994). Furthermore, biochemical studies have indicated that these compounds also possess affinity for dopamine D3 receptors DRD3 (Sokoloff et al, 1990).

The DRD3 subtype shares significant sequence homology with DRD2 but display a much more restricted distribution, with relatively high levels in limbic brain areas (Bouthenet et al, 1991). DRsD3 are also expressed at low levels in DAergic neurons within the substantia nigra, suggesting that they could have an autoreceptor function (Diaz et al, 2000). Thus, although DRsD2 are the major functional presynaptic autoreceptors (Mercuri et al, 1997), DRsD3 could inhibit DA transmission by modulating DA impulse flow, synthesis and release (Levant et al, 1997). Interestingly, while no change in basal extracellular dopamine levels has been detected in DRD2-deficient mice (Schmitz et al, 2002), D3

-/- mice have extracellular DA

levels twice as high as their wild type (WT) littermates (Joseph et al, 2002; Koeltzow et al, 1998).

It has been proposed that this enhanced DA-ergic tone and the resulting adaptations may reflect the removal of the inhibitory role for DRD3 in the control of basal extracellular DA levels (Le Foll et al, 2005), giving further support to an autoreceptor role for the DRD3. Due

to the increased DA-ergic activity, D3-/- mice are described phenotypically as hyperactive in various behavioral paradigms, exhibiting higher basal levels of grooming behavior, locomotion and reactivity to drug-paired environmental cues (Accili et al, 1996; Le Foll et al, 2002; 2005). Moreover, our previous findings have shown that D3 -/- mice are more sensitive to different antidepressant drugs in the forced swim test (FST) paradigm than their WT counterparts (Leggio et al, 2008). Higher levels of DA in mesolimbic areas of D3
-/- mice could explain, in fact ,the better behavioral performance of D3 -/- mice in the FST in comparison to WT mice, after treatment with doses of SSRIs that are usually ineffective (David et al, 2003; Renard et al, 2001). D3 -/- mice also exhibited reduced “anxiety-like behavior” in the open field and in the EPM test (Accili et al, 1996; Steiner et al, 1998) although this finding was not confirmed in other studies (Betancur et al, 2001; Xu et al, 1997). Consistent with these observations on the role of DRsD3 in anxiety, putative DRD3 antagonists have shown anxiolytic-like effects in rodents (Rogòz et al, 2000).
It is well established that the GABA system plays a pivotal role in emotional processes such as anxiety. However, the contribution of GABAergic innervation of particular brain structures involved in the organization and expression of emotions is still unclear (Plaznik et al, 1995). To confirm the interaction between DAergic and GABAergic systems, DRsD3 activation has been found to inhibit GABAergic neurotransmission in the Nac neurons by increasing the phospho-dependent endocytosis of GABAA receptors (Chen et al, 2006).
Based on the above premises, this study was undertaken to assess the behavioral response of D3 -/- and WT mice, tested in experimental models of anxiety (EPM and novelty induced grooming test, NGT), to the acute treatment with the classical benzodiazepine and standard anxiolytic drug, diazepam (Lister, 1990). We used two different tests since growing evidence indicates that measures of anxiety from different tests could reflect different states of anxiety (File, 1992). Diazepam was administered at different doses (0.1, 0.5 and 1 mg/kg) and testing

times (30, 60, 90 and 120 min), since natural strain differences exist in mice both for anxiety level and for sensitivity to diazepam (Griebel et al, 2000; Lepicard et al, 2000). Furthermore, to confirm and extend the contribution of the DRD3 in the anxiolytic effects of diazepam, we studied the effects of the selective D3 antagonist U99194A (10 mg/kg) alone or in combination with diazepam (0.5 mg/kg) in WT animals. Finally, a quantitative ligand binding study was performed to assess whether changes in GABAA receptors in different brain structures may be associated to behavioral responses of D3
-/- mice to diazepam.

2. Materials and methods

2.1. Animals

All experiments were carried out on D3 -/- mice and WT littermates (male mice 8-12 weeks old). The animals were housed four per cage and fed standard laboratory food and allowed free access to water ad libitum, in an air-conditioned room with a 12-h light-dark cycle. All the experimental procedures were performed during the light cycle. D3 -/- mice used in these experiments were 5th-8th generation of congenic C57BL/6J mice, and generated by a backcrossing strategy. The genotypes of the DRD3 mutant and WT mice were identified by a PCR method with two pairs of primers flanking either exon 3 of the wild-type DRD3 or the PGK (phosphoglycerate kinase 1 gene promoter) cassette of the mutated gene (Accili et al, 1996). All animals were used only once in experiments, which were carried out according to the European Community Council Directive 86/609/EEC. Efforts were made to minimize animal suffering and to reduce the number of animals used. The rationale, design and methods of this study were approved by the Ethical Committee for Animal Research, University of Catania.

2.2. Drugs and treatments

All drugs were purchased from Sigma (USA). Diazepam (0.1, 0.5 or 1 mg/kg), prepared

as a suspension in physiological saline containing Tween 80 (0.1%), or vehicle, was injected intraperitoneally (IP) 30, 60, 90 or 120 min prior to the EPM test. The selective DRD3 antagonist U99194A was diluted in physiological saline containing Tween 80 (0.1%) and injected (IP) alone or in combination with 0.5 mg/kg diazepam, 30 min prior to the EPM test. In mice subjected to NGT, diazepam (0.1, 0.5 and 1 mg/kg) or vehicle were administrated IP 30 min prior to the test. All animals were gently manipulated by experienced facilities keepers to avoid any environmental or physical stress. Mice subjected to drugs or vehicle administration received an injection of a 0.1ml/100g volume of solution with a 23-gauge stainless steel needle of 31 mm length. The animals were randomly assigned to treatment groups (n=6-7) and were used only once in the behavioral experiments.

2.3. Behavioral tests

2.3.1. Elevated plus maze (EPM) test

The apparatus consisted of two opposite open arms, (30 x 5 cm) and two arms with walls (30 x 5 x 14 cm) that were attached to a central platform (5 x 5 cm) to form a cross. The maze was elevated 50 cm from the floor (Pellow et al, 1985). Illumination measured at the center of the maze was 40 lx. After treatment, each animal was placed at the center of the maze with its nose in the direction of one of the closed arms, and observed for 5 min, according to the following parameters: number of entries in the open and closed arms, and time of permanence in each of them. The time of permanence measures the time spent by the animal in the open and closed arms. An arm entry was defined as all four paws having crossed the dividing line between an arm and the central area. The anxiolytic effect of a drug treatment is indicated by a significant increase in parameters in open arms (time and/or number of entries). Increased percentage of entries in open arms in proportion to total entries in both arms is another good indicator of anxiolytic-like behavior, while entries in closed arms and total entries reflect the motor component of the exploratory activity. On removal of

each mouse, the maze floor was carefully wiped with a wet towel. The behavior of animals was recorded using a video camera (Hitachi Videocam) and then scored from the monitor display by an independent observer.

2.3.2. Novelty-induced grooming sampling test (NGT)

Novelty-induced grooming behavior was observed between 15:00 and 18:00 hr, under the same environmental conditions according to the method described elsewhere (Drago et al, 1980). Mice were placed individually into Plexiglas boxes (24x12x24 cm) in a low noise room. After a minute of adaptation, behaviour was sampled every 15 sec, and the occurrence of grooming was recorded in a 30-min session. The occurrence of the following single elements of grooming was scored as grooming: washing (vibrating movements of the fore paws in front of the snout and liking of the same paws leading to a series of strokes along the snout and semicircular movements over the top of the head), scratching (scratching of the body by one of the limbs), licking (licking of the body fur, limbs and tail), and genital grooming (licking of genital area). Stretching and yawning episodes were notrecorded. Grooming behavior of all animals was recorded using a video camera (Hitachi Videocam) and then scored from the monitor display by two independent observers. The mean score of the two observations was used for the statistical analysis.

2.4. Benzodiazepine receptor binding

Twenty-micron coronal sections were prepared on a cryostat at -20°C and stored at -80°C until assay time. On the day of the assay slide-mounted sections were first preincubated for 3 x 10 minutes in 50mM Tris.HCl buffer at room temperature, and then incubated in 3 nM [3H]-flunitrazepam (85.2 Ci/mmol) in buffer for 80 minutes at 23°C. Adjacent sections were incubated with the radioligand in the presence of 1.0 μM clonazepam in order to define nonspecific binding. Slides were then rinsed 2 x 2 minutes in Tris.HCl

buffer at 4□C, blown dry and exposed to Kodak Biomax MR film for 4 weeks along with calibrated radioactivity standards. Densitometric film analyses were performed with an MCID AIS/C system (Imaging Research, St. Catharines, Ont., Canada) on coded films.

2.5. Statistical analysis of data

Data were analyzed using two-way analysis of variance (ANOVA) with genotype and treatment as independent variables. Furthermore, one-way ANOVA, followed by Newman-Keuls Test for multiple comparisons was performed to allow adequate multiple comparisons between groups. A *P* level of 0.05 or less was considered as indicative of a significant difference. Autoradiographic binding data were analyzed by Bonferroni-adjusted Student t tests.

3. Results

3.1.Effects of 0.1 mg/kg diazepam in the elevated plus maze test.

In the EPM (Fig 1, panel A), D3-/- untreated mice exhibited a low baseline anxiety level, walking out onto the open arms more often and staying there longer than untreated WT (data not shown). Thirty and 60 min after the acute treatment with 0.1 mg/kg diazepam, D3 -/- mice showed a low level of “anxiety-like behavior” as well. Indeed, two-way ANOVA analyses revealed a main effect of genotype/treatment interaction in the behavioral response derived from activity in the EPM after 30 (percentage of time spent in open arms [$F(1,24)=40.73$, $p<0.01$], percentage of entries in open arms [$F(1,24)=38.95$, $p<0.01$]) and 60 min (percentage of time spent in open arms [$F(1,24)=37.42$, $p<0.01$], percentage of entries in open arms [$F(1,24)=25.71$, $p<0.01$]) from the drug injection. Thirty and 60 minutes after the injection of 0.1 mg/kg diazepam, post-hoc analyses revealed a significant drug effect for time spent ($P<0.01$, Newman-Keuls test), and entries ($P<0.01$, Newman-Keuls test) in open arms in comparison to D3 -/- mice treated with vehicle. Furthermore D3 -/- mice treated with vehicle exhibited increased behavioral responses in the EPM (percentage of time spent in

open arms and percentage of entries in open arms; $P<0.01$, Newman-Keuls test) in comparison to the vehicle injected WT . No differences in behavioral responses were observed between WT groups. Furthermore, no differences in the total number of entries were observed between different experimental groups (data not shown).

3.2. Effects of 0.5 mg/kg diazepam in the elevated plus maze test.

The injection of 0.5 mg/kg diazepam (Fig. 1, panel B) caused an “anxiolytic-like” effect in both D3 -/- and WT mice as indicated by the increased percentage of time spent in open arms after 30 (genotype/treatment interaction [$F(1,24)=75.06$, $p<0.01$]) and 60 min(genotype/treatment interaction [$F(1,24)=63.64$, $p<0.01$]). Post-hoc analyses revealed that D3 -/- and WT mice treated with 0.5 mg/kg diazepam after 30 and 60 min spent more time in the open arms of the maze in comparison with their respective controls ($P<0.01$, Newman-Keuls test). Furthermore, D3-/- diazepam-treated mice, but not WT animals, exhibited a significant increase in the percentage of entries in open arms ($P<0.01$, Newman-Keuls test) when compared with their controls (D3-/- and WT given vehicle). No differences in the total number of entries were observed between different experimental groups (data not shown).

3.3. Effects of 1 mg/kg diazepam in the elevated plus maze test.

Figure 1, panel C, shows the effects of 1 mg/kg diazepam on behavioral responses of D3 -/- and WT mice tested in the EPM. Two-way ANOVA revealed a genotype/treatment interaction in the behavioral responses derived from activity in the EPM after 30 (percentage of time spent in open arms [$F(1,24)=1022.1$, $p<0.0001$] and percentage of entries [$F(1,24)=667.22$, $p<0.0001$]), 60 (percentage of time spent in open arms [$F(1,24)=7.66$, $p<0.05$] and percentage of entries [$F(1,24)=9,34$, $p<0.05$]), 90 min (percentage of time spent in open arms [$F(1,24)=164.03$, $p<0.0001$] and percentage of entries [$F(1,24)=198.43$, $p<0.0001$]) and 120 min (percentage of time spent in open arms [$F(1,24)=161.54$, $p<0.0001$] and percentage of entries [$F(1,24)=81.87$, $p<0.0001$]) after the i.p injection of 1 mg/kg

diazepam. Post-hoc analyses revealed that D3 -/- treated mice exhibited an increased percentage of time spent ($P<0.001$, Newman-Keuls test) and percentage of entries ($P<0.001$, Newman-Keuls test) in open arms at all testing times (30, 60, 90 and 120 min) when compared to D3-/- vehicle injected controls. Diazepam-treated WT mice showed a better performance than WT vehicle-treated mice in the percentage of time spent ($P<0.001$, Newman-Keuls test) and percentage of entries in open arms ($P<0.001$, Newman-Keuls test) only at 30 and 60 min after the acute treatment. No differences in the total number of entries were observed between different experimental groups (data not shown).

3.4. Effects of 10 mg/kg U 99194A injected alone or in combination with 0.5 mg/kg diazepam in the elevated plus maze test.

Figure 2 reports the effects of the selective DRD3 antagonist U99194A (10 mg/kg) in combination to the diazepam (0.5 mg/kg) in WT mice tested in the EPM 30 min after the injection. As expected, 0.5 mg/kg diazepam alone elicited an increase in the percentage of time spent in open arms ($p<0.05$, Newman-Keuls test) as compared with the vehicle treated group. The injection of 10 mg/kg U99194A alone had no effect both in the percentage of time and the percentage of entries in open arms. On the other hand, WT mice treated with 10 mg/kg U99194A in combination with 0.5 diazepam, showed better behavioral responses in the behavioral measures derived from activity in the EPM such as the percentage of time spent in open arms ($p<0.01$, Newman-Keuls test) and percentage of entries in open arms ($p<0.001$, Newman-Keuls test) as compared to diazepam treated group. No differences in the total number of entries were observed between different experimental groups.

3.5. Effects of diazepam in the Novelty Iinduced Grooming Test.

In the NGT (Fig 3), two-way ANOVA analyses revealed a main effect of genotype/treatment interaction [$F(4,60)=81.66$, $p<0.0001$]. WT untreated mice and WT vehicle-treated mice exhibited a significant decrease in grooming response in comparison to both D3

-/- mice groups (P<0.001). Acute treatment with different doses of diazepam (0.1, 0.5 and 1 mg/kg) induced a decrease in grooming response in D3 -/- mice. Statistical analysis revealed a significant drug effect (P<0.001, Newman-Keuls test) in comparison to WT groups.

3.6. [3H]flunitrazepam binding in different brain areas of WT and D3 -/- mice.

The results of benzodiazepine receptor binding assays using [3H]flunitrazepam in D3 -/-and WT mice are shown in Table 1. Although D3 -/- mice exhibited trends towards decreased binding in some brain areas (e.g. an 8.73% decrease in the CA1 area of hippocampus) none of the differences between D3 -/- and WT mice reached statistical significance when Bonferroniadjusted independent t tests were used.

4. Discussion

This results demonstrate that D3 -/- mice exhibit a high sensitivity to the acute treatment with the anxiolytic drug diazepam than their WT littermates both in the EPM and the NGT, thereby suggesting the implication of DRsD3 in the expression of “anxiety-like” behavior.

In the present study, the functional role of DRsD3 on “anxiety-like” behavior was assessed by using two of the most used animal models to assess the behavioral effects of anxiolytic drugs, the EPM and the NGT. Several pieces of data, in fact, indicate that parameters recorded in different anxiety models produce distinct anxiety factors, reflecting different kind of emotional states (File, 1992; Lister, 1987). The EPM reflects the conflict between exploration and avoidance of a novel environment and the inhibition of exploratory behavior is commonly associated with high emotionality or anxiety, while the novel environment in the NGT only influences the emotionality of rodents (Archer, 1973).

We found here that D3 -/- mice, tested in the EPM, exhibit lower baseline anxiety levels in comparison with their WT littermates. The present results are in agreement to the results obtained by Accili et al. (1996) and Steiner et al. (1998) showing that D3 -/- mice entered the center of the open field more often and walked out onto open arms of the EPM more often

and for a longer time than their WT littermates, both parameters indicating lower anxiety levels. However, these results were not confirmed by other groups (Betancur et al, 2001; Waddington et al, 2001; Xu et al, 1997).

Progress in understanding the DRD3 has long been limited by the lack of well-characterized selective ligands, so in vitro and in vivo studies have been characterized by conflicting data (Boulay et al, 1999a; 1999b; Mercuri et al, 1997)**.** Moreover, although the use of D3 -/- animal has scientific merit, it also has limitations defined by potential compensatory adaptations such as expression of other receptors in place of DRsD3 during nervous system development, the different strategies used to create these D3 -/- lines, or specific environmental conditions (Pritchard et al, 2003).

Biochemical and pharmacological research in anxiety has been focused on serotonergic, GABAergic and noradrenergic neurotransmitter systems but DA has also been proposed to play a significant role in pathophysiology of anxiety. The two distinct classes of DA receptors, the “D1-like” and “D2-like” are involved in anxiety differently. The DRD1 seem not to be involved, since DRD1 agonists and antagonists are devoid of anxiogenic or anxiolyticlike activity in different animal models (Bartoszky, 1998; Rodgers et al, 1994). In contrast, DRD2 antagonists such as haloperidol or sulpiride showed anxiolytic-like effect in conflict and exploration models, suggesting a more consistent involvement of “D2-like” receptors. The DRD2-family consists of DRD2, DRD3 and DRD4. There is no clear indication on the role of DRD4 on anxiety. Indeed, a study by Navarro et al. (2003), has found no effect of DRD4 antagonist on elevated plus maze. Therefore, it appears that the anxiolytic effects mediated by “D2-like” receptors are probably mediated either by the DRD2 or the DRD3. Activation of DRD2 by agonists may play a role in anxiety because low doses of apomorphine or quinpirole exhibited anxiolytic-like effects whereas higher doses produced anxiogenic-like effects (Costal et al, 1987; Gao et al, 1993; Pich and Samanin, 1986; Simon

et al, 1993). However, biochemical experiments have pointed out that these compounds show appreciable affinity for DRD3 as well, suggesting a possible role of these receptors in the behavioral effects of these drugs (Sokoloff et al, 1990). Indeed, a large body of data support the potential role for DRsD3 in several functions, including mood disorders (Leggio et al, 2008; Micale et al., 2009; Sokoloff et al, 2006). Furthermore, several DRD3 agonists exert a modulatory action on "anxiety-like behavior" (Bartoszyk, 1998; Genderau et al, 1997; Rogòz et al, 2003).

In contrast to the low baseline anxiety level recorded in the EPM, D3 -/- mice tested in the NGT have shown an higher basal level of grooming behavior than their WT littermates, as previously demonstrated by Le Foll et al (2005). Grooming is a "maintenance" behavior, a common species-characteristic movement pattern with readily definable components (Bolles, 1960, Fentress, 1973). In rodents, spontaneous grooming behavior may occupy as much as 25%–40% of the awake time, but is specifically elicited in situations in which an animal is in a state of stress-induced conflict or frustration. A typical condition of this type is the NGT where grooming may play a deactivating role in restoring homeostasis (Gispen et al, 1981). Thus, while the higher baseline grooming score showed by D3 -/- mice could be considered as an index of anxiety (as it decreases after anxiolytic treatment), it may alternatively be a phenotypic peculiarity of the hyperdopaminergic status of D3 -/- mice (Le Foll et al, 2005).

A point of our results deserving attention is the difference in sensitivity to the acute treatment with various doses of diazepam in D3-/- mice and WT littermates, tested in the EPM and the NGT. Surprisingly, the acute treatment with 0.1 mg/kg diazepam, which is known to be ineffective in rodent models of anxiety (Griebel et al, 2000; Lepicard et al, 2000), induced an "anxiolytic-like" effect in D3 -/- but not in their WT mice in both behavioral tests.

When diazepam was administered at the dose of 0.5 mg/kg, D3 -/- mice, tested in the EPM, remained for a longer time in open arms of the maze and showed an higher percentage of

entries in open arms when compared to their WT littermaze. In the NGT, D3
-/- mice injected with 0.5 mg/kg diazepam also showed a significant decrease of the grooming score than their WT littermates.

At the dose of 1 mg/kg, diazepam induced strong "anxiolytic-like" effects in both the EPM and the NGT, and generated a more pronounced response in D3 -/- than in WT animals.

The present results confirm that D3 -/- animals are less anxious than their WT littermates, but also indicate that the DRD3 may modulate, but is not needed to mediate acute anxiolytic effects of benzodiazepines (BZP). This conclusion is supported by the data showed in figure 2. Indeed, to provide evidence for the contribution of DRD3 in the anxiolytic effects induced by diazepam, we tested in the EPM, WT mice treated with the selective DRD3 antagonist U99194A (10 mg/kg) (Gendreau et al., 1997) in combination with 0.5 mg/kg diazepam. As previously shown by Gendreau et al. (1997), the selective DRD3 antagonist U99194A (10 mg/kg) alone did not show any effect on the behavioral measures derived from activity in the EPM. On the other hand, WT animals treated with 10 mg/kg U99194A in combination with 0.5 mg/kg diazepam, have shown a better "anxiolytic-like" effect than diazepam treated animals. Hence, it is noteworthy that the strongest "anxiolytic-like" effect showed by animals treated with 10 mg/kg U99194A plus 0.5 mg/kg diazepam may be due to the blockade of DRsD3. The different sensitivity to diazepam shown by D3 -/- versus WT mice could be related to the influence of DA on the GABAergic system. Previous studies have shown that activation of DRD1 or DRD2 is followed by opposite effects on GABA release. Although DRD1 activation increases GABA release (Floran et al, 1990), activation of DRD2 by the selective agonist quinpirole inhibits GABA release (Mayfield et al, 1996). Since quinpirole also shows affinity for DRD3 (Sokoloff et al, 1990), its inhibitory effects on GABA release could be linked to the action of this subtype of DA receptors. To give further support to the role of DRD3 on GABAergic system, Chen et al. (2006) have shown that application of the

DRD3 agonist, PD128907 reduces GABAergic neurotransmission in nucleus accumbens by increasing the phospho-dependent endocytosis of GABAA receptors.

The role of the GABA system and GABAA receptor complex in mediation of emotional processes is very well established (Clemet et al, 2002; Metha and Ticku, 1999). However, the contribution of the GABAergic innervation of particular brain structures to regulate anxiety is not satisfactorily recognized. The mechanism of action of typical anxiolytic drugs such as BZD is related to an enhancement in the affinity of the recognition site of GABAA receptors, thus potentiating the GABA inhibitory action in the brain limbic structures. Local administration of selective receptor ligands showed that GABAergic innervation of the hippocampus, amygdala, septal nuclei, and some hypothalamic nuclei also influence the expression of emotions (Stefanski et al, 1993; Plaznik et al, 1995).

Thus, although, the behavioral effects of diazepam observed in our experiments pointed to possible alterations in the GABAA receptor complex in D3 -/- mice, the absence of significant differences in [3H]flunitrazepam binding in these animals indicates that if alterations in GABAA neurotransmission exist in D3 -/- mice these alterations do not occur at the level of binding to the benzodiazepine site of the receptor complex. Other possibilities need to be further investigated.

In conclusion, these data confirm the implication of DRD3 in expression of “anxiety-like behavior” and point out the critical influence of DRD3 mediating action on the acute effects of BZD in rodents. Further studies are needed to elucidate which signaling pathway is involved and to reveal the potential utility of DRD3 ligands in the pharmacological treatment of anxiety.

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Tab.1

[3H] flunitrazepam binding in different brain areas of WT and D3-/- mice.

	WT	D3 (-/-)
Nucleus accumbens (Core)	2.7±3.3	3.01±0.2
Nucleus accumbens (Shell)	2.86±0.13	2.95±0.13
Dentate Gyrus	5.55±0.2	5.4±0.1
Hippocampus CA1	3.93±0.09	3.59±0.1
Hippocampus CA3	2.62±0.06	2.51±0.08
Amygdala lateral	4.76±0.14	4.53±0.06

Amygdala basolateral 3.88±0.19 3.77±0.17

Data are presented as means ± S.E.M of specific binding in uCi/g tissue for n=6 wild type (WT) mice and for n=6 dopamine D3 receptors knock-out (D3-/-) mice.

No significant differences were seen between D3-/- and WT mice (Bonferroni-adjusted student's ttests).

FIGURE LENGENDS

Figure 1

Effects of diazepam in WT and D3-/- mice tested in the elevated plus maze (EPM) test. (A) Effects of 0.1 mg/kg diazepam in D3-/- mice and WT littermates (n=7). The percentage of time spent on open arms (upper panel) and the percentage of entries in open arms (lower panel) with all four paws during the 5-min test. Data are presented as means ± S.E.M.

* Significantly different as compared to WT mice vehicle (VHC) treated group (*P*<0.01, Newman-Keuls post-hoc test). ** Significantly different as compared to D3

-/- mice vehicle

(VHC) treated group (*P*<0.01, Newman-Keuls post-hoc test). § Significantly different as compared to WT mice diazepam (DZP) treated group (*P*<0.01, Newman-Keuls post-hoc test).

(B) Effects of 0.5 mg/kg diazepam in D3-/- mice and WT littermates (n=7).

* Significantly different as compared to WT mice vehicle (VHC) treated group (*P*<0.01, Newman-Keuls post-hoc test). ** Significantly different as compared to D3

-/- mice vehicle

(VHC) treated group (*P*<0.01, Newman-Keuls post-hoc test). § Significantly different as compared to WT mice diazepam (DZP) treated group (*P*<0.01, Newman-Keuls post-hoc test).

(C) Effects of 1 mg/kg diazepam in D3-/- mice and WT littermates (n=7).

* Significantly different as compared to WT mice vehicle (VHC) treated group (*P*<0.01, Newman-Keuls post-hoc test).** Significantly different as compared to D3

-/- mice vehicle

(VHC) treated group (*P*<0.001, Newman-Keuls post-hoc test). § Significantly different as compared to WT mice vehicle (VHC) treated group (*P*<0.001, Newman-Keuls post-hoc test).

Figure 2

Effects of 10 mg/kg U99194A *per se* or in association with 0.5 mg/kg diazepam in WT mice tested in the elevated plus maze (EPM). Behavioral measures derived from activity in the EPM such as time spent on open arms (A), the percentage of entries in open arms (B) and the total number of entries in arms (C) with all four paws during the 5-min test were recorded 30 min after the treatment (n=6). Data are presented as means ± S.E.M.

* Significantly different as compared to vehicle treated group (*P*<0.05, Newman-Keuls posthoc test). ** Significantly different as compared to 0.5 mg/kg diazepam treated group (*P*<0.01, Newman-Keuls post-hoc test). *** Significantly different as compared to vehicle treated group (*P*<0.001, Newman-Keuls post-hoc test) and 0.5 mg/kg diazepam treated group (*P*<0.001, Newman-Keuls post-hoc test).

Figure 3

Effects of diazepam (0.1, 0.5 and 1 mg/kg) in WT and D3-/- mice tested in the noveltyinduced grooming sampling test (NGT). Diazepam or its vehicle (VHC) were injected IP to D3 -/- and WT littermates (n=7) 30 min prior the behavioral test. Data are presented as means ± S.E.M.

Increased D-aspartate brain content rescues hippocampal age-related synaptic plasticity deterioration of mice

Until recently, free D-amino acids were thought to be involved only in bacterial physiology. Nevertheless, today there is evidence that D-serine, by acting as co-agonist at NMDARs, plays a role in controlling neuronal functions in mammals. Besides D-serine, another D-amino acid, Daspartate (D-Asp), is found in the mammalian brain with a temporal gradient of occurrence: high in embryo and low in adult. In this study, we demonstrate that D-Asp acts as an endogenous NMDAR agonist, since it triggers currents via interaction with each of NR2A-D receptor subunits. According to its pharmacological features, we showed that oral administration of D-Asp strongly enhances NMDAR-dependent LTP in adulthood and, in turn, completely rescues the synaptic memory decay observed in the hippocampus of aged animals. Therefore, our findings suggest a tantalizing hypothesis for which this in-embryo-occurring D-amino acid, when "forced" at late stages of life, may disclose plasticity windows inside which it counteracts the physiological reduction of NMDAR signaling.

1. Introduction

Cognitive processes are believed to depend on changes in synaptic efficacy in key brain regions, including the hippocampus (Bliss and Collingridge, 1993). 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 (Bliss and

Collingridge, 1993,Doyere and Laroche, 1992,Lynch, 2004,Malenka and Nicoll, 1999). Accordingly, antagonists of NMDARs blocked the induction of LTP in hippocampal slices (Bashir, et al., 1991,Collingridge, 1987,Harris, et al., 1984) and impaired reference memory abilities in rats (Morris, 1989,Morris, et al., 1986). Similarly, synaptic memory and cognition are influenced by genetically manipulated NMDARs. Indeed, hippocampus-related synaptic plasticity and learning were improved in mice overexpressing NR2B subunit of these receptors (Tang, et al., 1999). Intriguingly, up-regulation of this subunit has been also described beneficial for improving cognitive functions in aged mice (Cao, et al., 2007). On the other hand, extensive evidence suggests that hippocampus is the main brain region sensitive and vulnerable to age-related loss of functional synapses and NMDAR-mediated responses (Rosenzweig and Barnes, 2003). Accordingly, aged rats exhibit reduced NMDAR binding sites, which correlate with their impairments in synaptic plasticity and cognition (Magnusson, 1998).

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 (Snyder and Kim, 2000). Specifically, the D-amino acid D-serine, by acting as an endogenous co-agonist at the glycine-binding-site of NMDARs, has been shown to rescue age-related impairment of NMDAR-mediated synaptic potentials in old mice (Yang, et al., 2005). Besides D-serine, today well characterized for its pharmacological properties and clinical implications in schizophrenia treatment (Martineau, et al., 2006,Mothet, 2001), another D-amino acid, namely D-aspartate (D-Asp), occurs in the mammalian brain. In contrast to the 4 former, the central role of the latter remains, so far, an issue of controversy. However, neuroanatomical and biochemical analyses indicated that, both in humans and rodents, D-Asp occurs at abundant levels in the developing brain to

decrease at low amounts during postnatal life (Schell, et al., 1997,Wolosker, et al., 2000). This has been linked to the concomitant postnatal expression of DAspartate Oxidase (DDO), the only enzyme known so far to be responsible for its degradation (Errico, et al., 2006,Huang, et al., 2006). According to binding studies, which indicated the ability of D-Asp to bind to NMDARs (Olverman, et al., 1988), also the analysis of *Ddo* knockout animals demonstrated that when the endogenous content of this D-amino acid exceeds physiological levels, NMDAR-dependent LTP is increased in the hippocampus while Long-Term Depression (LTD) is suppressed in the striatum (Errico et al., 2008b; Errico et al., 2008a). However, its *in vivo* relevance in modulating glutamatergic neurotransmission remains unclear, since the affinity of these receptors for D-Asp is 10-fold lower than that for glutamate (Olverman, et al., 1988). Based on these findings, in the present work, we challenged the hypothesis by which “forcing” higher D-Asp 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.

2. Materials and Methods

2.1. Animals. C57BL/6J male mice were used to test the effects of chronic 3- and 12-month oral administration of D-Asp in neurochemical, cognitive and electrophysiological studies. D-Asp was delivered in drinking water at the concentration of 20 mM to 45-day-old mice until they were used for experiments. Thirteen-month-old C57BL/6J female mice were used to test the effects of chronic 1-month D-Asp oral administration, delivered between the 12th and the 13th month of their life. To minimize the interference of hormonal influence on synaptic plasticity and spatial memory responses, electrophysiological experiments and Morris water maze were performed on treated and untreated females synchronized at the same phase of estrous cycle. Mice were housed in groups (n5 = 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.

2.2. HPLC analysis. Mice were killed and the hippocampus dissected and stored at -80°C. The determination of D-Asp was performed by HPLC technique, based on the diastereomeric separation of D-Asp from the L-form and other L-amino acids, as previously described (D'Aniello, et al., 2000). Data were analysed using ANOVA or Student's *t* test and data are expressed as means ± standard error of the mean (SEM).

2.3. Morris water maze. Morris water maze was performed according to a modified version of the protocol previously described (Errico, et al., 2008a). 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/day for a week before the experiment. The acquisition phase consisted of 2 sessions/day (3 h interval between sessions) over a 5-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. Four 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 4th 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 9th 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 6 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 (treatment x days) with repeated measures. Data obtained in the probe trials were analysed by one-way ANOVA followed by appropriate *post-hoc* comparison.

2.4. Novelty-induced exploration. The novelty-induced exploratory task was performed as previously described (Usiello, et al., 2000). Locomotor activity (expressed in cm) was performed in 35x25x30 cm experimental cages and recorded over a 60-min period by using a computerized video tracking system (Videotrack, Viewpoint S.A. Champagne au Mont d'Or, France). Data from this study were analysed, for each considered treatment-length group (3 or 12 months), by two-way ANOVA (time x treatment) with repeated measure.

2.5. Elevated plus-maze test. The elevated plus-maze task was performed as previously described (Shum, et al., 2005). The experimental apparatus is 55 cm lifted from the floor and consists of two open arms and two closed arms situated opposite each other and separated by a 6 cm squared center platform. Animals were placed in the center square and allowed to move freely for 5 min. Test sessions were videotaped and the time spent in each arm recorded by a blind experimenter. All four paws had to cross the entry of the open, closed arm or the center, to be considered an entry. Percentage of time spent in the open arms was used as dependent variable. Data were analysed, for each considered treatment-length group (3 or 12 months), by one-way ANOVA (treatment effect).

2.6. Object recognition test. Mice were gently handled 5 min/day for a week before the experiment. The experimental procedure was in accord to a previous protocol (Bevins and Besheer, 2006). In order to provide mice the familiarity to the testing environment, a 3-day habituation phase was conducted by exposing each animal to the experimental cage (40 × 40 cm Plexiglas chamber with white walls and black floor) for at least 20 min/day. On the training session (day 4), the box was enriched with two identical plastic, green, squared Lego objects (3 × 6 × 6 cm), positioned in the back left and right corners of the apparatus. Each animal was placed in the middle point of the wall,
opposite to the sample objects, and left to freely explore and familiarize with the objects for 15 min. Twenty-four h later (testing session), one of the two familial objects was substituted by a novel one, different in color, material and shape (a grey, metal pyramid, 5 x 5 x 6 cm). Each mouse was placed in the apparatus and left free to explore it for 5 min. Both training and testing phases were videorecorded and the time spent exploring each of the two objects was measured by a blind experimenter. Interaction with the objects was scored when the mouse nose was in contact with the object or directed at the object within a distance ≤ 2 cm. Two measures were considered: 1) the total exploration time (s) that animals spent interacting with the two familial objects during the training phase; 2) the exploration time (%) that animals spent interacting with the novel object over the total
exploration time (e.g. [novel/(familial + novel)] x 100) during the testing phase. Mice that had less than 8 s of exploration during the training phase were excluded from the analysis. Data were analysed, for each considered treatment-length group (3 or 12 months), by one-way ANOVA (treatment effect).

2.7. Contextual fear conditioning. Mice were gently handled 5 min/day for a week before the experiment. Contextual fear conditioning protocol was performed as previously described (Errico, et al., 2008b). On the training day, animals were placed individually in a

conditioning shock chamber (SDI's Freeze Monitor™, San Diego Instruments) for 2 min before the onset of an 85-dB tone (conditioned stimulus, CS) that lasted for 30 s. The last 2 s of the CS were paired with a 0.7- mA continuous foot-shock (unconditioned stimulus, US). CS-US pairing was then repeated with an inter-trial-interval of 30 s. One min after the second foot-shock, mice were returned to their homecages.

Twenty-four h later, testing session was performed in the context in which mice were trained, by scoring freezing behavior for 3 consecutive min. Both training and testing sessions were videotaped and analysed ethologically by an experimenter blind to the treatment.

Conditioning was assessed immediately and 24 h after training (retention session) by scoring freezing behavior, defined as the complete lack of movement (with the exception of respiratory movements). Time spent in freezing was analysed, for each considered treatment-length group (3 or 12 months), by one-way ANOVA (treatment effect).

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).

2.8. Patch-clamp recordings. Experiments aimed at investigating the effects of D-Asp were performed in whole-cell path-clamp recordings on CA1 pyramidal neurons obtained from juvenile (13- to 15-day-old) Wistar rats. All recordings were obtained in voltage-clamp mode (at –60 mV holding potential), in the presence of TTX (1 μM) and nifedipine (10 μM). Vibratome-cut parasagittal slices (300 μm) were prepared from hippocampi and were then placed in a recording chamber on the stage of an upright microscope (Axioscope FS, Carl Zeiss, Germany), submerged in a continuously flowing (2.5 ml/min) solution at 30°C (± 0.5°C). Borosilicate glass electrodes (3-4

MΩ) were filled with (in mM): K-Gluconate 135; KCl 10; MgCl2 2; CaCl2 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). Pressure applied (10 psi, 0.5-1.0 s) NMDA (300 μM), and D-Asp (2 μM) were used to obtain inward currents. The puff electrode was positioned above the slice, in close proximity of the recorded neuron. Drugs were also bath-applied by switching the solution to one containing known concentrations of drugs.

2.9. Extracellular recordings. Hippocampal slices (400 μm thick) were prepared from halothaneanesthetized mice as previously described (Errico, et al., 2008a). A single slice was then placed on a nylon mesh, completely submerged in a small chamber (0.5 ml) and superfused with oxygenated ACSF (30-31 °C) at a constant flow rate of 3 ml min-1. 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 pathway. To record fEPSPs from the CA1 region of the hippocampus, recording microelectrodes (2–10MΩ) filled with 3M NaCl were placed within the stratum radiatum. Data were collected and analysed on-line using WinLTP software (Anderson and Collingridge, 2007). All data are normalised with respect to a 30 min baseline. LTP was induced by a high frequency stimulation (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, unless indicated otherwise. Data were assessed for significance using the Student's t test or ANOVA, as appropriate.

2.10. Drugs. D-Asp, D-(-)-2-amino-5-phosphonoeptanoic acid (AP5), 5S,10R-(+)-5-Methyl-10,11- dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801), Tetrodotoxin (TTX), Nifedipine and N-Methyl-D-aspartic acid (NMDA) from Sigma-Aldrich, Milan, Italy; (2S*,3R*)-1- (Phenanthren-2-carbonyl)piperazine-2,3-dicarboxylic acid (cis-PPDA) and (aR,bS)-a-(4- Hydroxyphenyl)-b-methyl-4-(phenylmethyl)-1 -piperidinepropanol maleate

(Ro 25-6981) from Tocris Cookson Ltd, Bristol, UK. NVP-AAM077 was a generous gift from Novartis Pharma AG, Switzerland.

3. Results

3.1 D-Asp triggers NMDAR-dependent and NMDAR-independent inward currents.

The pharmacological features of D-Asp were studied on CA1 pyramidal neurons. NMDA (300 μM) or D-Asp (2 μM) were pressure applied (10 psi, 0.5-1.0 s) through a glass pipette positioned in close proximity of the recorded neurons. NMDA induced an inward current (INMDA 173.6 ± 47.2 pA, n = 7), which remained constant upon repeated application of the agonist. Perfusion with NVPAAM077 (NVP, 0.4 μM), at a concentration that completely blocks NR2A/NR1 receptors, although producing a partial inhibition of NR2B/NR1 receptors (Bartlett, et al., 2007), and cis-PPDA (10 μM), a selective NR2C-D/NR1 antagonist, reversibly reduced INMDA amplitude by 66.47± 3.18% and 67.40 ± 3.43%, respectively. Subsequent application of Ro 25-6981 (5 μM), a selective
NR2B/NR1 antagonist, irreversibly reduced in the same neurons INMDA amplitude by 66.99 ± 3.55% (n = 7, $p < 0.05$; Fig. 1A and 1B). Similarly, NVP, cis-PPDA and Ro 25-6981 reduced the inward current induced by D-Asp (ID-Asp 260 ± 84.4 pA, n = 9) by 66.68 ± 4.88%, 65.79 ± 5.61% and 67.11 10 ± 4.62% (n = 9, $p < 0.05$; Fig. 1A and 1B), respectively. Interestingly, while INMDA was totally blocked by the simultaneous combination of all NR2 selective antagonists (reduced by 98.98 ± 0.67%), as well as by MK801 (10 μM), (99.54 ± 5.18%; n = 7, $p < 0.05$; Fig. 1B); on the contrary, D-Asp was still able to excite CA1 pyramidal neurons under the same conditions. In fact, ID-Asp amplitude was reduced by 80.65 ± 0.55% when pharmacologically blocking all NR2 subunits (n = 7, $p < 0.05$; Fig. 1B), and by 82.43 ± 5.92% when MK801 was applied (n = 7, $p < 0.05$; Fig. 1B).

3.2 Hippocampal D-Asp levels influence NMDAR-dependent LTP amplitude.

To study the *in vitro* and *in vivo* consequences associated to increased levels of D-Asp, we recently developed a mouse model based on the oral administration of this D-amino acid (Errico, et al., 2008a,Errico, et al., 2008b). Herein, we further examined the neurochemical and electrophysiological consequences of 3-month chronic oral administration of this D-amino acid. In line with our previous report (Errico, et al., 2008a), we found that oral treatment with 20 mM DAsp resulted in a significant increase of its hippocampal content (H2O: 40.6 2.4 nmol/g tissue; DAsp: 99.5 5.4 nmol/g tissue, $p < 0.0001$; Fig. 2A). We then examined the time course of D-Asp clearance by replacing D-Asp with water in the drinking solution of mice. The results showed that the endogenous content of D-Asp in the hippocampus returned to its physiological levels within 7- day withdrawal (one-way ANOVA: 1 day: 64.0 2.5 nmol/g tissue, $p < 0.0001$; 2 days: 53.2 2.1 nmol/g tissue, $p < 0.01$; 3 days: 49.0 1.7 nmol/ g tissue, $p < 0.05$; 7 days: 42.0 1.5 nmol/g tissue, $p > 0.1$; 21 days: 44.2 2.7 nmol/g tissue, $p > 0.1$, compared to H2O-treated mice; Fig. 2A). To study the *in vitro* effects of increased D-Asp levels, we then analysed hippocampal basal synaptic transmission and synaptic plasticity. Our data indicated that, in consequence of 3-month oral administration, C57BL/6J mice showed comparable input/output (I/O) curves (H2O: y = 2.70x, R2 = 0.91; D-Asp: y = 2.13x, R2 = 0.96, $p < 0.1$; Fig. 2B). Next, we investigated short-term synaptic plasticity by analysing Paired Pulse Facilitation (PPF). Our data indicated that, at all interpulse intervals, PPF was indistinguishable between groups ($p > 0.05$; Fig. 2C). Finally, we examined the effects of D-Asp on long-term synaptic plasticity. We showed that around two-fold increase of DAsp (Fig. 2A) induced a robust enhancement in NMDAR-dependent LTP at CA1 synapses. In fact, while LTP was 52.9 ± 10.1% above baseline (n = 8) in untreated animals, it reached 74.5 ± 11.5% (n = 6) in treated mice ($p < 0.001$; Fig. 2D, left panel). Interestingly, this plastic augmentation

appeared to be strictly related to endogenous D-Asp content since 3-week withdrawal, which reestablish physiological hippocampal levels of D-Asp (Fig. 2A), was able to completely reverse effect on LTP to 45.1 ± 8.1% above baseline (n = 6) (Fig. 2D, middle panel). Then, to verify the reversibility of D-Asp effect on the modulation of NMDAR-dependent LTP, we re-administered DAsp to mice for 1 month after 3-week withdrawal. Notably, our data indicated that this D-amino acid is able to regulate LTP magnitude in a reversible manner since its re-administration produced an enhancement of LTP (62.2 ± 5.9% above baseline, n = 5; Fig. 2D, right panel) similar to that observed before withdrawal.

3.3 Effect of 12-month chronic exposure to D-Asp on hippocampal synaptic functions.

To explore the synaptic effects of more prolonged exposure to D-Asp, we examined the consequences of its 12-month oral administration. First, we measured the hippocampal content of this D-amino acid. HPLC analysis indicated that continuous treatment for 1 year resulted in around two-fold increase of D-Asp content in this brain region (H2O: 70.7 10.7 nmol/g tissue; D-Asp: 157.7 28.9 nmol/g tissue, $p < 0.05$, Student's *t* test; Fig. 3A).

We then explored the effects of such long-term exposure on basal glutamatergic transmission and short-term synaptic plasticity. Overall, we found that increased hippocampal DAsp content did not affect either I/O curves (H2O: y =2.15x, R2 = 0.96; D-Asp: y = 1.56, R2 = 0.89, $p > 0.1$; Fig. 3B) or PPF responses ($p > 0.1$; Fig. 3C). Conversely, hippocampal slices from 12- month chronically treated mice were significantly less responsive to HFS, compared to age-matched controls. In fact, LTP at 60 min was 47.15 ± 11.9% above baseline (n = 7) in untreated, compared to 29.09 ± 2.8% (n = 8) in D-Asp-treated mice ($p < 0.001$; Fig. 3D, left panel). Similarly to what done on 3-month treated mice, to test the potential reversible effect of 1-year D-Asp treatment on LTP, we replaced D-Asp with H2O for 3 weeks. Interestingly, such interruption was sufficient to fully

restore the synaptic long-term memory (48.17 ± 8.1% above baseline, n = 6) at levels recorded in untreated controls (49.1 ± 6.4% above baseline, n = 6) ($p > 0.05$; Fig. 3D, right panel).

3.4 D-Asp oral administration slightly improves spatial memory in adulthood without perturbing cognitive functions in elderly phases.

Spatial learning and memory abilities of D-Asp-treated C57BL/6J mice were analysed after its 3- month or 12-month chronic oral administration. As revealed by two-way ANOVA with repeated measures, 3-month D-Asp treatment did not produce any visible change in spatial learning aptitudes of mice both during acquisition [days effect, $F(4, 72) = 29.186$, $p < 0.0001$; treatment effect, $F(1, 72) = 0.153$, $p > 0.1$; genotype x days interaction, $F(4, 72) = 0.545$, $p > 0.1$] and reversal phase [days effect, $F(4, 72) = 39.970$, $p < 0.0001$; treatment effect, $F(1, 72) = 0.534$, $p > 0.1$; genotype x days interaction, $F(4, 72) = 1.408$, $p > 0.1$] (Fig. 4A). Importantly, in the probe test performed after a short training, even though both groups preferentially searched the platform in the proper quadrant (H2O: $p < 0.05$, compared to others; D-Asp: $p < 0.01$, compared to opposite, $p < 0.0001$, compared to right and left; Fisher's *post-hoc* comparison), D-Asp-treated mice evidenced a mild improvement in spatial memory, as indicated by longer search in the goal area, compared to controls ($p < 0.05$, between genotypes; Student's *t* test; Fig. 4B). However, such slight difference between groups disappeared after a 2-day additional training (H2O: $p < 0.05$, compared to others; D-Asp: $p < 0.05$, compared to left, $p < 0.01$, compared to right, $p < 0.0001$, compared to opposite; Fig. 4C). 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 ($p < 0.05$, compared to left and old goal, $p < 0.01$, compared to right), compared to non-treated mice (Fig. 6D), while both groups were similarly able to remember the position of the

new target quadrant after a 5-day training (H2O: $p < 0.05$, compared to others; D-Asp: $p < 0.05$, compared to left and old goal, $p < 0.01$, compared to right; Fig. 4E).

Like the shorter treatment, also 12-month D-Asp administration did not modify spatial learning performances of C57BL/6J mice, as revealed by two-way ANOVA analysis [acquisition phase: days effect, $F(4, 76) = 63.430$, $p < 0.0001$; treatment effect, $F(1, 76) = 1.264$, $p > 0.1$; genotype x days interaction, $F(4, 76) = 0.630$, $p > 0.1$; reversal phase: days effect, $F(4, 76) = 16.685$, $p < 0.0001$; treatment effect, $F(1, 76) = 0.031$, $p > 0.1$; genotype x days interaction, $F(4, 76) = 0.391$, $p > 0.1$] (Fig. 4F). Nevertheless, this long-term treatment induced a lack of the previous memory enhancement seen in 3-month D-Asp-treated mice. Indeed, both groups needed longer training periods to target the correct quadrant (probe 2, H2O: $p < 0.05$, compared to left, $p < 0.01$, compared to right and opposite; D-Asp: $p < 0.01$, compared to others; probe 4, H2O: $p < 0.05$, compared to right, $p < 0.01$,
compared to left and old goal; D-Asp: $p < 0.05$, compared to right and left, $p < 0.01$, compared to old goal) but no differences were detected between D-Asp-treated and untreated mice in each of the retention tests performed (Fig. 4G-J).

3.5 Chronic D-Asp administrations do not produce behavioral deficits.

After examination of hippocampus-dependent spatial reference memory, we extended the *in vivo* analysis on 3- and 12-month D-Asp-treated mice to other behavioral paradigms. First, we analysed motor activity of animals in the novelty-induced exploration paradigm over a 60-min session (Fig. 5A). Our data indicated no main influence of D-Asp treatment on motor response. Statistical analysis revealed a comparable locomotor profile of habituation between D-Asp and H2O-treated mice after both 3-month [two-way ANOVA: time effect, $F(5, 120) = 52.085$, $p < 0.0001$; time x treatment interaction, $F(5, 120) = 2.215$, $p > 0.05$] and 12-month administration [two-way ANOVA: time effect, $F(5, 110) = 32.017$, $p < 0.0001$; time x treatment interaction, $F(5, 120) = 1.702$, $p > 0.1$].

We next analysed anxiety-like responses of mice in the elevated plus-maze task (Fig. 5B). In this test, the time spent in the open arms of the apparatus is regarded as an index of anxiety-like behavior (Pellow, et al., 1985). D-Asp treatment did not produce any significant effect on anxietyrelated behavior. Indeed, one-way ANOVA revealed no difference between treatments in the time spent in open arms, after both 3-month [$F(1, 40) = 1.581$, $p > 0.1$] and 12-month chronic administration [$F(1, 23) = 2.689$, $p > 0.1$].

Then, we assessed the effect of abnormal higher D-Asp brain levels in other cognitive tasks, such as object recognition test (Winters, et al., 2008) and contextual fear conditioning (Phillips and LeDoux, 1992). In the object recognition test, our behavioral analysis failed to reveal differences between groups. Indeed, in the retention session performed 24 h after the first exposure to the objects, D-Asp- and H2O-treated mice showed similar preference towards the novel object [oneway ANOVA: 3-month treatment: $F(1, 37) = 3.353$, $p > 0.05$; 12-month treatment: $F(1, 18) = 2.068$, $p > 0.1$] (Fig. 5C). This result indicates that continuative higher endogenous levels of D-Asp do not interfere with the discriminative ability of mice and their retention for the familiarized object.

Similarly, in the contextual fear conditioning paradigm, D-Asp administration did not produce any deficit. Indeed, one-way ANOVA indicated that, 24 h after the training session, animals displayed a similar freezing response, regardless to treatment, either the administration endured 3 [$F(1, 30) = 0.029$, $p > 0.1$] or 12 months [$F(1, 19) = 0.205$, $p > 0.1$] (Fig. 5D).

3.6 D-Asp rescues CA1 synaptic plasticity decline associated with aging.

We investigated whether a short-term treatment with D-Asp may be beneficial to counterbalance the age-related decay of hippocampus-dependent functions at senescence. Since it is known that rodent females exhibit a faster deterioration of synaptic plasticity and reference memory (Frick, et al., 2000,Spencer, et al., 2008,Williams, et al., 1990), we utilized

for this study 13-month-old C57BL/6J females that were treated with D-Asp for 1 month before experimental analyses. In line with previous detections, animals treated for 1 month with D-Asp exhibited a significant two-fold increase of this D-amino acid levels in their hippocampi, compared to untreated mice (H2O: 104.2 12.7 nmol/g tissue; D-Asp: 196.7 13.2 nmol/g tissue, $p < 0.01$, Student's *t* test; Fig. 6A).

Moreover, electrophysiological experiments indicated no main differences in basal glutamatergic transmission and short-term plasticity, as measured by I/O curves (Fig. 6B) and PPF (data not shown), respectively. In contrast, we found a strong enhancement of LTP in treated mice (82.25 ± 4.6% above baseline, n = 8), compared to both age-matched controls (32.22 ± 6.5% above baseline, n = 6) and 2-month old C57BL/6J females (58.07 ± 3.1% above baseline, n = 8) (Fig. 6C and 6D).

Finally, we explored spatial cognitive abilities of aged females treated with D-Asp. We found that treated animals displayed a slight improvement of spatial learning, compared to untreated controls (Fig. 6E). In fact, two-way ANOVA with repeated measures indicated significant days effect [$F(4, 72) = 31.347$, $p < 0.0001$] and treatment effect [$F(1, 72) = 7.774$, $p < 0.05$] and a nonsignificant days x treatment interaction [$F(4, 72) = 0.766$, $p > 0.1$]. In this regard, it is important to note that the difference in the escape latency seen after the first training day derives from the second daily session since, in the first test session, mice exhibited comparable escape latencies (H2O: 84.2 2.3 s; D-Asp: 76.5 4.2 s; $p > 0.1$, Fisher's *post-hoc* comparison). However, according to a more relevant age-dependent mnemonic decay in females, likely due to hormonal influences (Frick, 2009,Spencer, et al., 2008), even after a 5-day training exposure, both treated and untreated mice

did not remember the correct location of the platform (Fig. 6F and 6G). During reversal phase, both groups showed a comparable profile of learning [two-way ANOVA: days effect, $F(4, 72) = 13.232$, $p < 0.0001$; treatment effect, $F(1, 72) = 0.251$, $p > 0.1$; genotype x days

interaction, $F(4, 72) = 1.850$, $p > 0.1$] (Fig. 6E). Nevertheless, a mild improvement was detected at day 4 and 5 in D-Asp-treated animals ($p < 0.05$, Fisher's *post-hoc* comparison). Conversely, no differences were found in the retention tests (Fig. 6H and 6I).

4. Discussion

It is widely assumed that an established role for D-Asp is mainly confined to the endocrine system (D'Aniello, 2007), while its involvement in central functions remains largely unknown. In the current work, we extended previous findings on the ability of D-Asp to bind to and activate NMDARs (Errico, et al., 2008a,Fagg and Matus, 1984,Olverman, et al., 1988), by further demonstrating that D-Asp triggers NMDAR-dependent inward currents via interaction with each of the NR2A-D subunits. Moreover, according to previous studies (Errico, et al., 2008a,Errico, et al., 2008b), we confirmed that this D-amino acid elicits also NMDAR-independent currents, since its local application in CA1 pyramidal neurons evokes electrophysiological responses not completely blocked even in the presence of MK-801. Nevertheless, the main pharmacological feature of D-Asp to act as an endogenous NMDAR agonist is in line with its role in modulating synaptic plasticity at hippocampal CA1 synapses. In fact, it has been recently demonstrated that non-physiological increase of D-Asp in the hippocampus of mice, achieved either by targeted deletion of *Ddo* gene or by 1-month D-Asp treatment, are able to enhance NMDAR-dependent LTP (Errico, et al., 2008a). Based on this, here we explored the consequences of more prolonged oral administration of D-Asp on hippocampusrelated functions. The reason to focus our studies on this brain area is because the hippocampus displays low D-Asp levels along with high DDO activity, thus suggesting that a tight physiological control of this catabolic enzyme over its substrate must occur in this region (Schell, et al., 1997). On the other hand, the hippocampus is highly enriched with NMDARs, known to play an important role in learning and memory processes (Lynch, 2004,Martin, et al., 2000). Notably, here we demonstrate that minimal variations of

hippocampal D-Asp levels, in the range of nanomol/g tissue, are able to regulate in a reversible manner NMDAR-dependent LTP magnitude. Indeed, around two-fold increase of D-Asp levels induce higher NMDAR-dependent synaptic memory in 3-monthtreated mice. The direct implication of D-Asp on modulation of synaptic plasticity is further confirmed by the fact that 3-week treatment interruption is sufficient to wash-out the excess of this D-amino acid and, consequently, to normalize LTP amplitude at physiological levels. Intriguingly, we also found that D-Asp regulates NMDAR-dependent synaptic memory in a reversible manner since 1-month re-administration of this D-amino acid, after 3-week withdrawal, reinstates LTP amplitude to previous potentiated levels.

Interestingly, treatment for 12 consecutive months produces in mice similar increase in DAsp levels, as measured after its 3-month administration. Therefore, we argue that the absence of consistent increase in D-Asp amount after further 9-month administration is due to the buffering activity of DDO enzyme that may prevent potential neurotoxicity produced by deregulation of this NMDAR agonist. Surprisingly, despite comparable D-Asp levels, 1-year D-Asp administration consistently reduces LTP amplitude at CA1 synapses. Although the molecular mechanisms underlying this bimodal NMDAR-dependent effect are still unclear, we show that the temporal exposure to this D-amino acid, rather than differences in its concentration, is responsible for the direction of synaptic plasticity modulation. In addition, similarly to what observed after 3-month DAsp administration, we found that 3-week treatment interruption following its 1-year administration, is able to fully reverse the synaptic memory reduction seen at CA1 area. Despite the evident effect of D-Asp on NMDAR-related synaptic plasticity, it is still obscure the route by which

this D-amino acid exerts its activity at neuronal level. Previous *in vitro* studies have proposed different mechanisms to explain D-Asp cellular efflux, including vesicular Ca2+-mediated

exocytosis (Nakatsuka, et al., 2001) or Ca2+-independent spontaneous outflow (Homma, 2007) and L-glutamate transporters-dependent heteroexchange mechanism (Bak, et al., 2003). Also the subcellular localization of D-Asp in neurons does not clarify this topic since this D-amino acid appears both in cytoplasm and fiber tracks (Schell, et al., 1997,Wolosker, et al., 2000). Elucidation on mechanism of D-Asp release would also help to clarify whether D-Asp action occurs via synaptic NMDARs, as suggested by the D-Asp-dependent modulation of LTP, or also through an extrasynaptic NMDAR-dependent route of action. NMDARs are known to play a key role in age-related hippocampal cognitive decline (Rosenzweig and Barnes, 2003). Indeed, previous studies indicated that the hippocampus of aged animals sustains the most prominent loss of functional synapses and decrease of NMDAR-mediated responses, including induction and maintenance of LTP. Based on the observations that aged females manifest a more prominent hippocampal cognitive and synaptic memory decline (Frick, 2009,Spencer, et al., 2008), compared to males, we decided to test whether this embryonic D-amino acid may produce a rejuvenation influence on neuronal plasticity in this aging-sensitive gender. Importantly, we showed that a 30-day D-Asp treatment to one-year old C57BL/6J females confers considerable stronger plastic properties, compared to age-matched untreated controls. The entity of this amelioration in synaptic memory is even more striking if it is compared to LTP levels observed
in younger 2-month old naïve females. Besides its ability in modulating LTP magnitude, D-Asp supplementation also slightly improves cognitive abilities of old females. However, the discrepancy between the consistent synaptic memory enhancement and the modest cognitive beneficial effects induced by D-Asp oral administration may be explained by the influence of hormonal deregulations
occurring in senescent female rodents (Spencer, et al., 2008). Altogether, these results further

extend to aged brains the ability of this endogenous D-amino acid to modulate NMDAR-dependent functions and, in turn, provide an attractive possibility when considering its clinical potential. In this regard, it is remarkable that long-term chronic exposures to D-Asp do not affect either AMPAR-related basal synaptic transmission or behavioral traits associated with motor and sensoricognitive- motivational responses. These observations overall indicated the lack of detrimental *in vivo* effects associated to long-term D-Asp exposure which, together with the beneficial effects of this molecule in attenuating schizophrenia-like symptoms induced by amphetamine and MK801
(Errico, et al., 2008b), support a valuable interest for this atypical amino acid.
On the other hand, our data pointed out also a direct involvement of the specific genetic background on which our pharmacological manipulation is studied (Gerlai, 1996,Nguyen and Gerlai, 2002). In fact, C57BL/6J animals, characterized to display very high performances in hippocampus-dependent functions, represent a “difficult” mouse strain for easily detecting improvements in cognitive tasks (Nguyen and Gerlai, 2002). Thus, even though D-Asp induces very strong influences on hippocampal synaptic memory in treated animals, only mild differences, if any, are found in their spatial memory aptitudes. In this regard, the use of animal models with different genetic background or manifesting NMDAR-mediated cognitive impairments would certainly represent a useful tool to confirm, in future studies, the potential therapeutic effects of D-Asp administration. In this scenario, we cannot rule out that increased levels of this embryonic molecule may reactivate “developmental windows” of plasticity in the aging brain able to counteract the physiological or pathological reduction of NMDAR signaling.

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Figure Legends

Fig. 1. D-Asp triggers NMDAR-dependent and NMDAR-independent inward currents in CA1 pyramidal neurons. (A) Plot of EPSC amplitude *vs* time, showing the effect of pressure applied NMDA (left) and D-Asp (right) on CA1 pyramidal neurons. 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 or D-Asp on, at least, seven separate cells.

Fig. 2. Oral 3-month D-Asp administration modulates hippocampal synaptic functions at CA1 synapses. (A) D-Asp levels were detected by HPLC in the hippocampus of C57BL/6J mice treated for 3 months with a 20 mM D-Asp solution (H2O, n = 5; D-Asp, n = 4). Treatment, that started on 45-day-old mice, significantly increased the levels of the D-amino acid, compared to respective controls. A time-course of hippocampal D-Asp clearance was also measured after withdrawal that followed 3-month D-Asp treatment (n = 4 per each time point). This study indicated that D-Asp returns to basal levels within a 7-day withdrawal. (B) I/O curves (mean ± SEM) showing the relationship between fEPSP slope and their corresponding presynaptic fiber volley amplitudes in C57BL/6J D-Asp-treated *vs* untreated mice. A regression fit of fEPSP *vs* presynaptic fiber volley shows a similar pattern of

response between the two groups ($p > 0.05$). Each data point indicates at least 18 separate recordings. (C) Paired-pulse ratio ± SEM, against the paired-pulse interval following 3-month D-Asp treatment *vs* H2O control group. No significant differences in pairedpulse facilitation were observed at every tested interval. (D) Superimposed pooled data showing the normalized changes in field potential slope (± SEM) induced by HFS in 3-month D-Asp treated mice *vs* H2O (left), following 3-week D-Asp withdrawal (middle) and after 1-month of its readministration (right). Three-month treatment with D-Asp is able to enhance LTP (left). Successive 3-week withdrawal bring back LTP magnitude to control levels (middle). Finally, 1-month D-Asp re-administration restores the potentiated state of synaptic plasticity (right). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.0001$, compared with control H2O-treated group (one-way ANOVA). Values are expressed as mean SEM. Treatments are as indicated.

Fig. 3. Prolonged 12-month D-Asp exposure reduces hippocampal synaptic plasticity. (A) D-Asp levels were detected by HPLC in the hippocampus of C57BL/6J mice treated for 12 months with a 20 mM D-Asp solution (H2O, n = 3; D-Asp, n = 3). Long-term treatment, that started on 45-day-old mice, significantly increased the levels of this D-amino acid, compared to respective controls. (B)
Basal synaptic transmission was normal even at 12-month D-Asp-treated synapses. Each data point indicates at least 13 separate recordings. (C) PPF was comparable at every tested interval between both treated and untreated groups. (D) Superimposed pooled data showing the effects of 1-year DAsp administration on LTP (left) and following one-month withdrawal (right). Chronic long-term administration of D-Asp reduced NMDAR-dependent synaptic plasticity at CA1 synapses (left) while successive 1-month removal of D-Asp from drinking solution was able to reinstate LTP at control levels (right). * = $p < 0.05$, compared to control

$H2O$-treated group (Student's *t* test). Values are expressed as mean SEM. Treatments are as indicated.

Fig. 4. Chronic 3-month D-Asp treatment enhances spatial memory in C57BL/6J mice. (A) Mice treated for 3 months with D-Asp (n= 10) and age-matched untreated animals (n = 10) were trained in a submerged platform version of the Morris water maze during acquisition phase (days 1-5), followed by a reversal phase (days 6-10), in which the submerged platform was moved to the opposite position of the pool (as indicated in the figure). Sixty-second probe tests were performed along training (B) after a 3-day (probe 1) and (C) 5-day (probe 2) acquisition phase and (D) after a 3-day (probe 3) and (E) 5-day (probe 4) reversal phase. (F)-(J) correspond to tasks shown in (A)- (E), but performed on 12-month D-Asp treated (n = 11) and untreated (n = 10) animals. Escape time, expressed in seconds, was used as dependent variable in the acquisition and reversal phases.

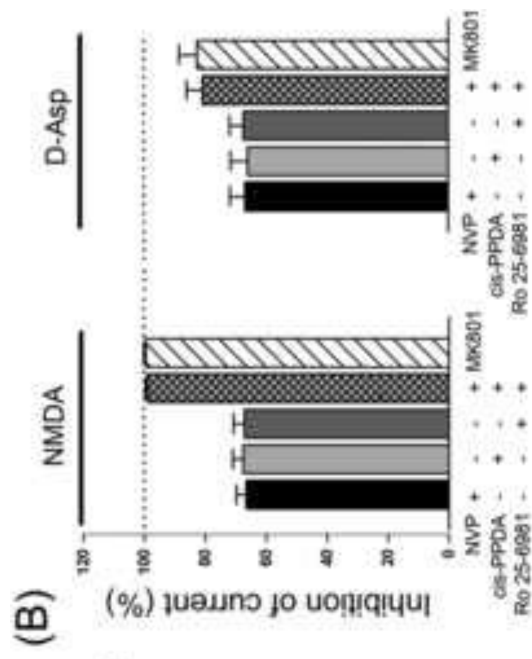
Search quadrant, expressed as percentage of time, was used as dependent variable in the probe tests. The dashed lines in (B)-(E) and (G)-(J) indicate the chance level (25%) of search in the four quadrants. * = $p < 0.05$ in (B) and (D), between treatments (Student's *t* test). All values are expressed as mean SEM. Treatments are as indicated.

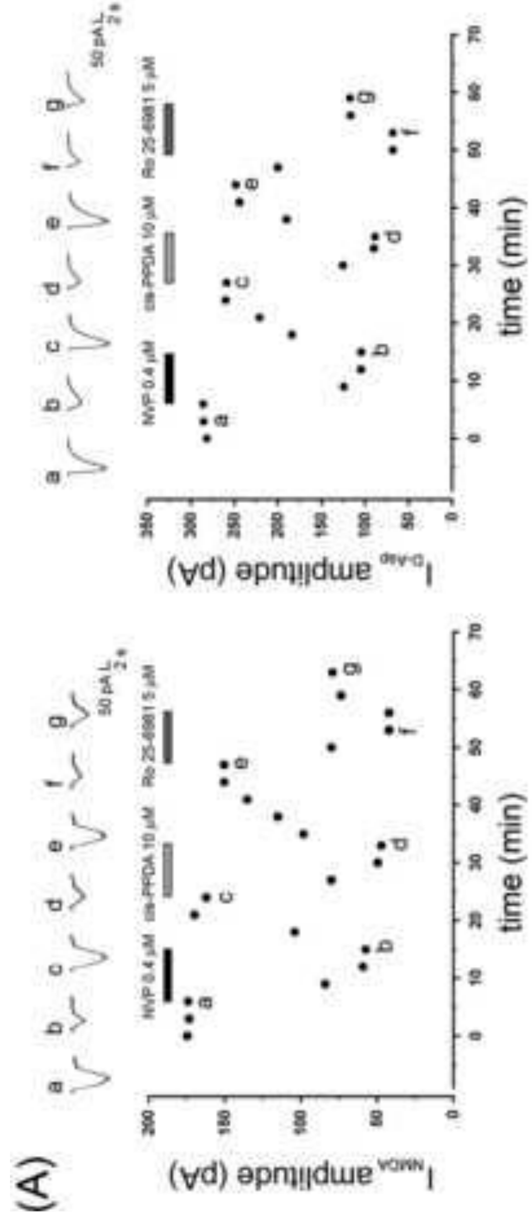
Fig. 5. Absence of locomotor, anxiety-like and cognitive alterations in 3- and 12-month D-Asptreated mice. (A) D-Asp treatment does not induce motor effects. Mice treated for 3 (n = 13, per treatment) or 12 months with D-Asp (n = 12, per treatment) were submitted to a novelty-induced exploration task. Locomotor activity is expressed as distance travelled (cm) during 10-min intervals, over a 60-min period. (B) D-Asp treatment does not affect anxiety-like behavior in mice. Three- (n = 21, per treatment) and 12-month D-Asp treated mice (H2O, n = 13; D-Asp, n = 12) were submitted to the elevated plus-maze task. Time spent in each arm of the apparatus over a 5-min test session, expressed as percentage, was similar between treatments, at each considered administration period. (C) D-Asp treatment does not affect

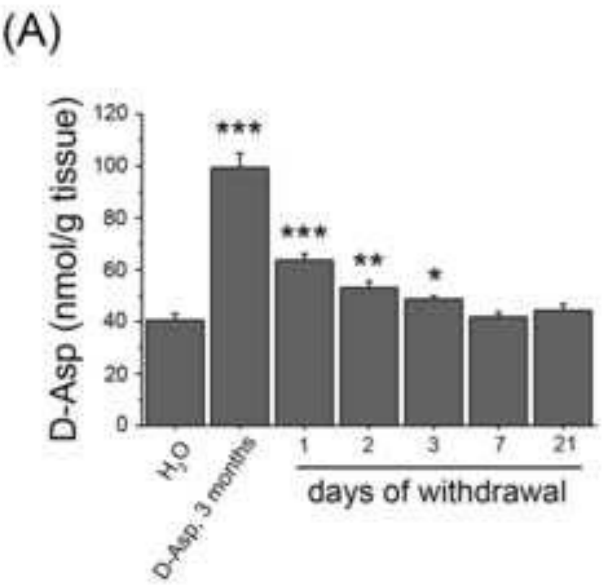
object recognition memory. During retention session, performed 24 h after the exposure to the to-be-familiarized objects, mice from both treatment groups display a clear preference for the novel object. However this preference does not significantly change between D-Asp and H2O-treated animals, both after 3-month (H2O, n = 19; DAsp, n = 20) or 12-month administrations (H2O, n = 12; D-Asp, n = 8). Exploratory preference, expressed as percentage of time spent on the familial or novel object, was used as dependent variable. (D) D-Asp treatment does not affect hippocampus-dependent emotional memory. In the hippocampus-dependent contextual fear conditioning, D-Asp-treated and untreated mice showed a similar freezing response, after both 3-month (n = 16, per treatment) or 12-month administration (H2O, n = 13; D-Asp, n = 8). All data are expressed as mean + SEM. Treatments are as indicated.

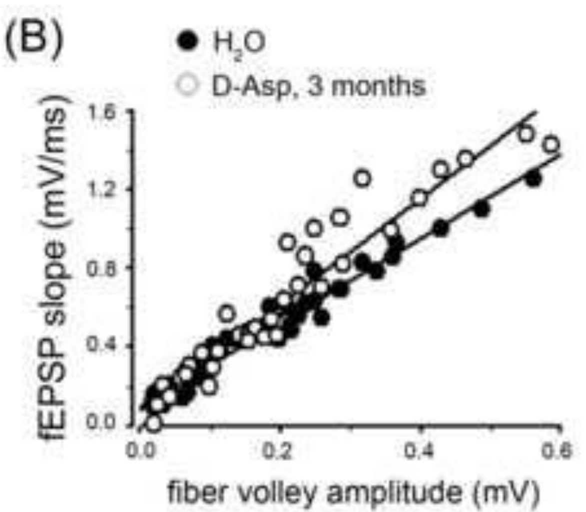
Fig. 6. Beneficial effects of D-Asp in hippocampus-related functions during aging. All experiments were carried out on female C57BL/6J mice. (A) D-Asp levels were measured by HPLC in the hippocampus of 13-month-old C57BL/6J mice which drank H2O (n = 4) or a 20 mM D-Asp solution between their 12th and 13th month of life (n = 3). One-month D-Asp administration consistently increased the hippocampal levels of this D-amino acid, compared to controls. (B) Superimposed pooled data showing I/O curves in 2-month-, 13-month-old untreated mice and in 13- month-old mice treated with D-Asp for 1 month. No differences were observed among the three 28 groups under observation. (C) Superimposed pooled data showing the normalized changes in field potential slope (± SEM) induced by HFS in 13-month-old mice treated with D-Asp for 1 month *vs* 13-month-old and 2-month-old untreated mice. D-Asp administration between the 12th and the 13th month of life was able to reverse the potentiation decay observed in age-matched controls. (D) Summary bar graph (mean ± SEM) showing the fEPSP slopes (% of baseline) quantified 50-60 min after HFS in the three groups under observation. Notably, the degree of potentiation was

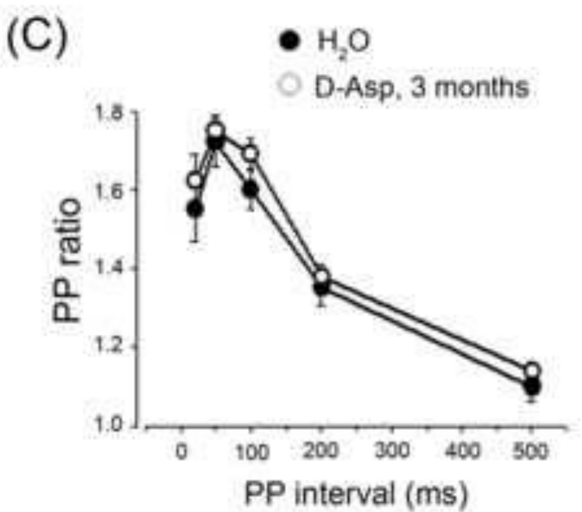
significantly higher in treated *vs* age-matched controls ($p < 0.001$). Also the age-dependent effect on the decay of LTP in control animals was significant ($p < 0.001$). (E) Thirteen-month-old untreated (n = 10) and age-matched D-Asp-treated animals (n = 10) were trained in a submerged platform version of the Morris water maze during acquisition phase (days 1-5), followed by a reversal phase (days 6-10), in which the submerged platform was moved to the opposite position of the pool (as indicated in the figure). Sixty-second probe tests were performed along training (F) after a 3-day (probe 1) and (G) 5-day (probe 2) acquisition phase, and (H) after a 3-day (probe 3) and (I) 5-day (probe 4) reversal phase. 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 (F)-(I) indicate the chance level (25%) of search in the four quadrants. ** = $p < 0.01$, compared to untreated mice (Student's *t* test). Values are expressed as mean SEM. Treatments are as indicated.

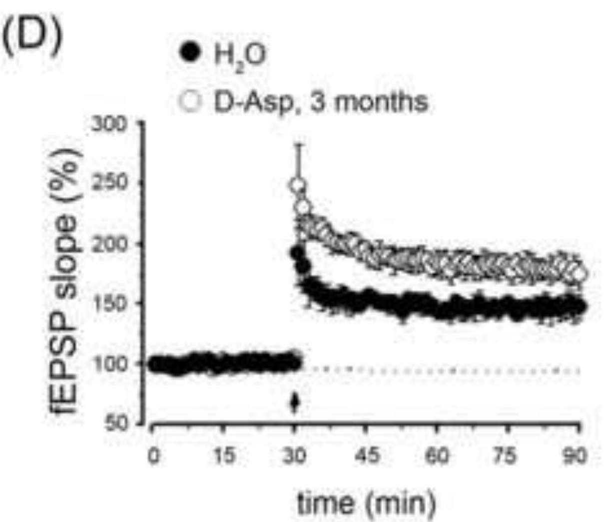

(B)
NMDA
D-Asp
Inhibition of current (%)
0
20
40
60
80
100
120
NVP + - - + MK801
cis-PPDA - + - +
Ro 25-6981 - - + +
NVP + - - + MK801
cis-PPDA - + - +
Ro 25-6981 - - + +

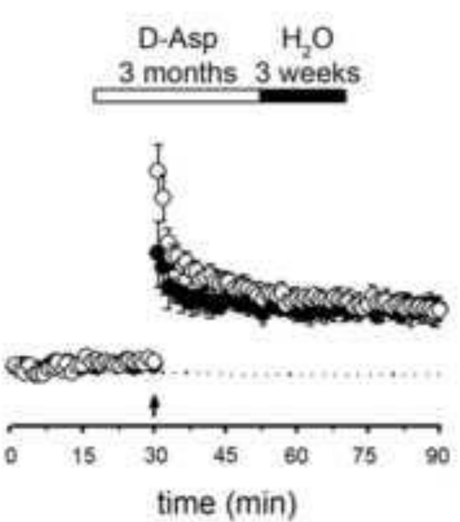

(A)
a b c d e f g
50 pA
2 s
NVP 0.4 μM
cis-PPDA 10 μM
Ro 25-6981 5 μM
I NMDA amplitude (pA)
I D-Asp amplitude (pA)
time (min)
0
10
20
30
40
50
60
70

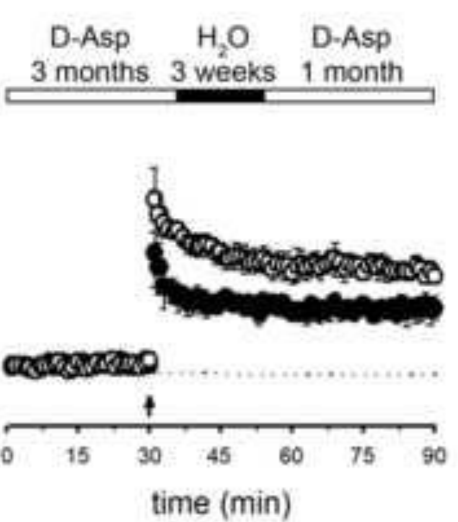

(A)
D-Asp (nmol/g tissue)
0
20
40
60
80
100
120
***
***
**
*
H_2O
D-Asp, 3 months
1
2
3
7
21
days of withdrawal

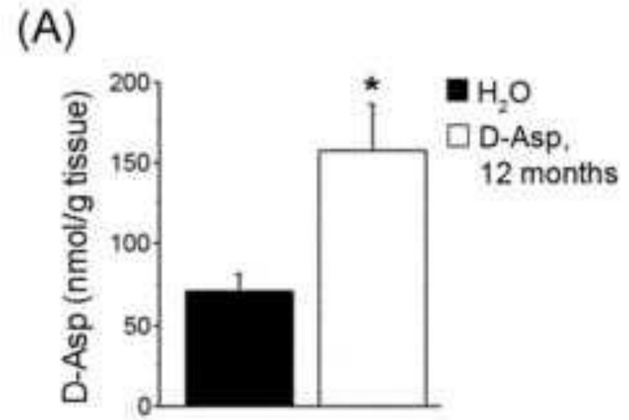

(B)
H_2O
D-Asp, 3 months
fEPSP slope (mV/ms)
0.0
0.4
0.8
1.2
1.6
0.0
0.2
0.4
0.6
fiber volley amplitude (mV)

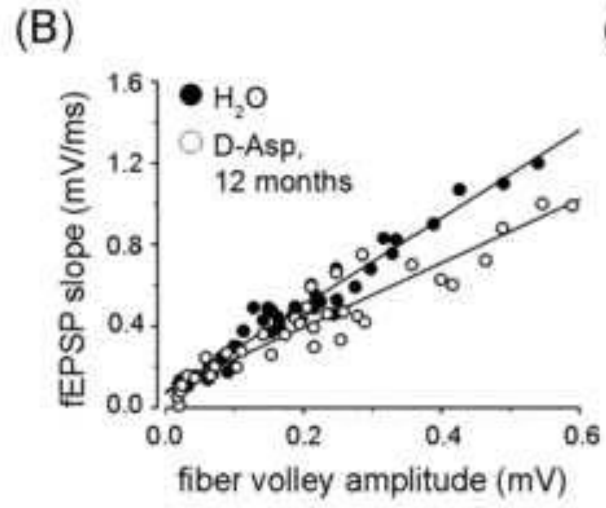

(C)
H_2O
D-Asp, 3 months
PP ratio
1.0
1.2
1.4
1.6
1.8
0
100
200
300
400
500
PP interval (ms)

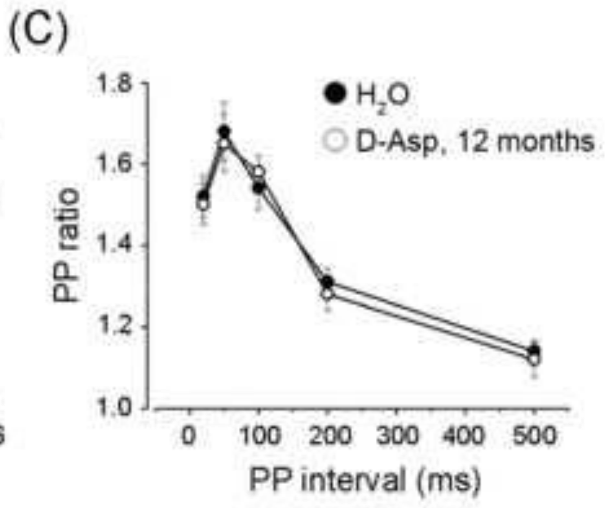

(D)
H_2O
D-Asp, 3 months
fEPSP slope (%)
50
100
150
200
250
300
0
15
30
45
60
75
90
time (min)

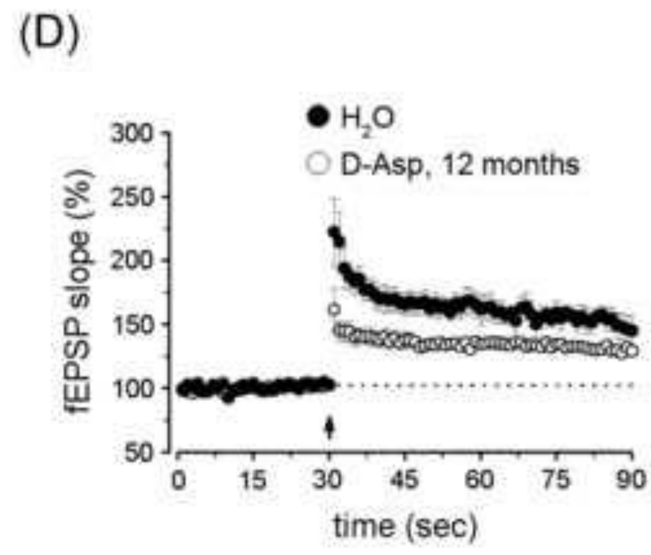

D-Asp
3 months
H_2O
3 weeks
0
15
30
45
60
75
90
time (min)

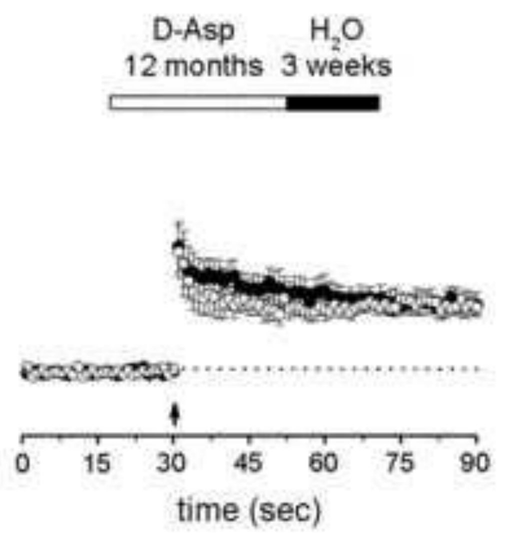

D-Asp
3 months
H_2O
3 weeks
D-Asp
1 month
0
15
30
45
60
75
90
time (min)

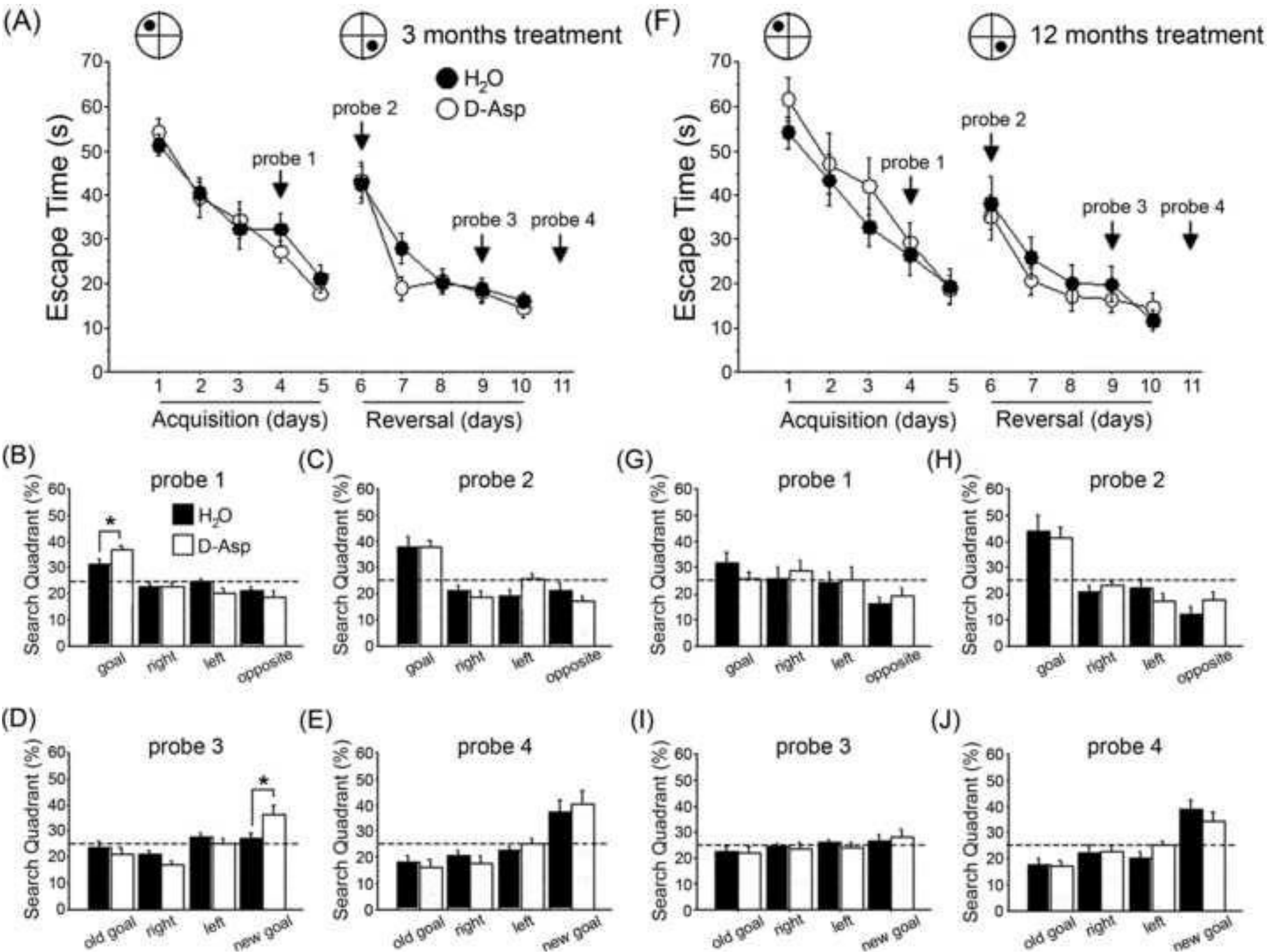

(A)
D-Asp (nmol/g tissue)
200
150
100
50
0
*
H_2O
D-Asp,
12 months

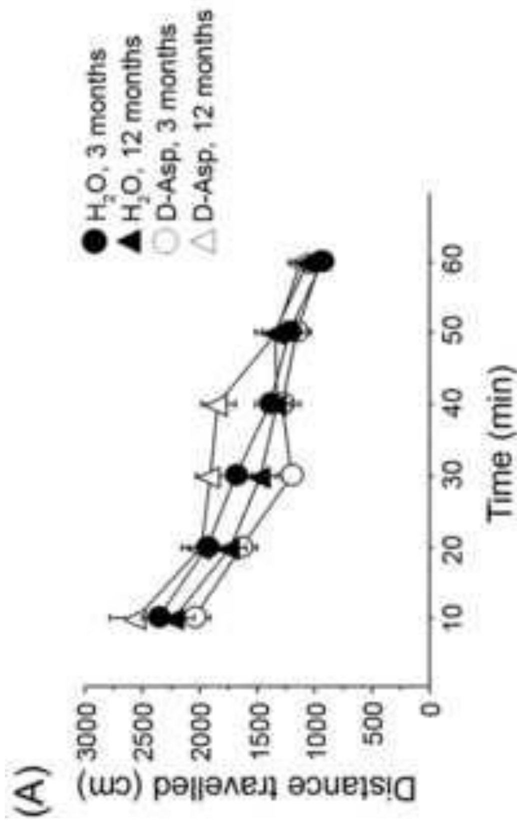

(B)
fEPSP slope (mV/ms)
1.6
1.2
0.8
0.4
0.0
0.0
0.2
0.4
0.6
fiber volley amplitude (mV)
H_2O
D-Asp,
12 months

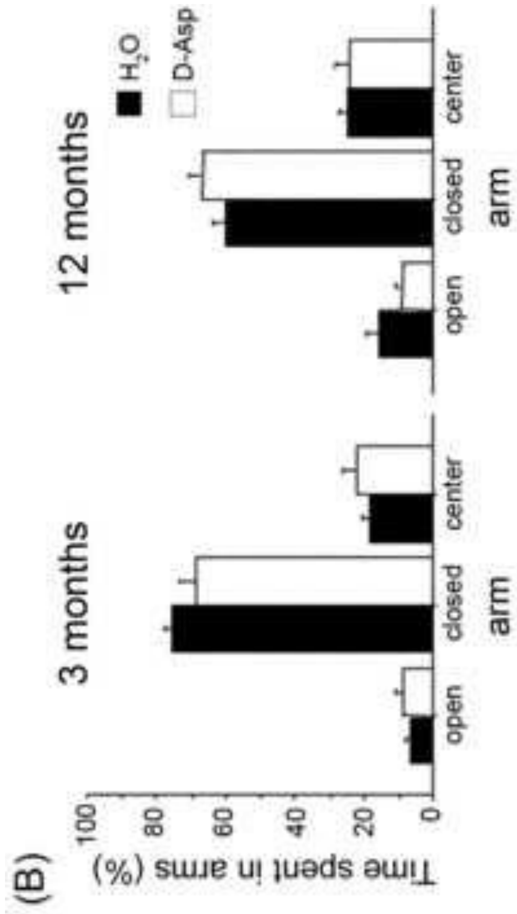

(C)
PP ratio
1.8
1.6
1.4
1.2
1.0
0
100
200
300
400
500
PP interval (ms)
H_2O
D-Asp, 12 months

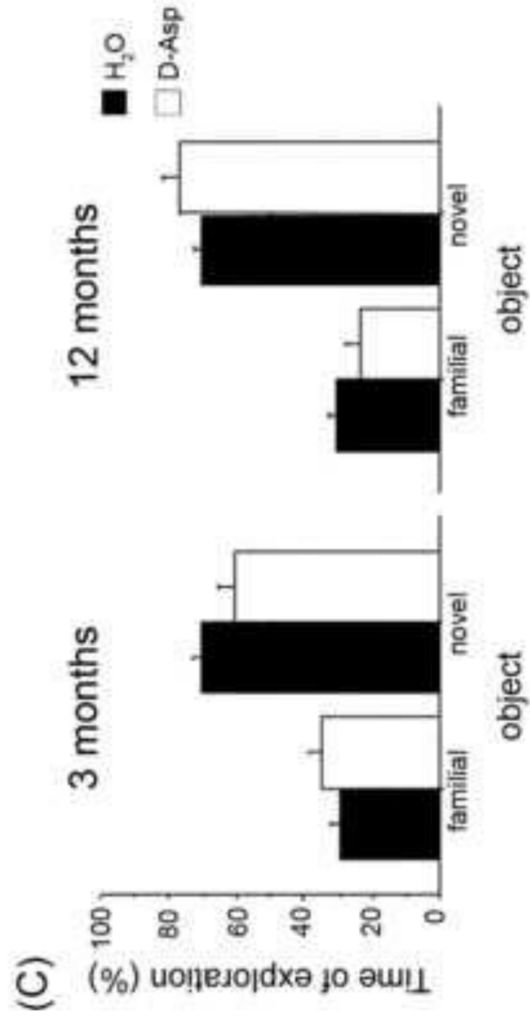

(D)
fEPSP slope (%)
300
250
200
150
100
50
0
15
30
45
60
75
90
time (sec)
H_2O
D-Asp, 12 months

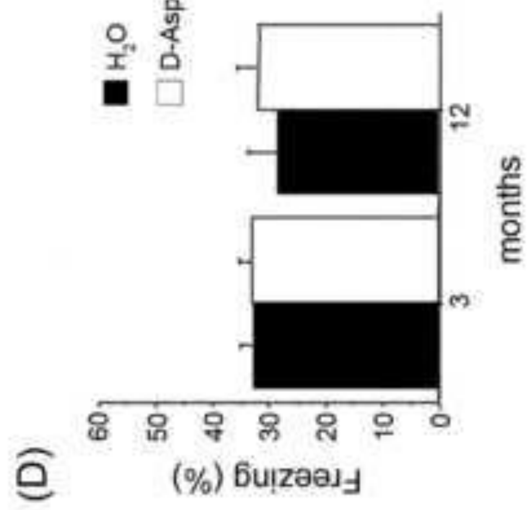

D-Asp
12 months
H_2O
3 weeks
0
15
30
45
60
75
90
time (sec)


(A)
3 months treatment
H2O
D-Asp
Escape Time (s)
probe 1
probe 2
probe 3
probe 4
Acquisition (days)
Reversal (days)
(B)
probe 1
(C)
probe 2
(D)
probe 3
(E)
probe 4
Search Quadrant (%)
goal
right
left
opposite
old goal
new goal
*
(F)
12 months treatment
(G)
probe 1
(H)
probe 2
(I)
probe 3
(J)
probe 4


(A)
Distance travelled (cm)
3000
2500
2000
1500
1000
500
0
10
20
30
40
50
60
Time (min)
H2O, 3 months
H2O, 12 months
D-Asp, 3 months
D-Asp, 12 months


(B)
3 months
12 months
Time spent in arms (%)
100
80
60
40
20
0
open
closed
center
arm
H2O
D-Asp


(C)
3 months
12 months
Time of exploration (%)
100
80
60
40
20
0
familial
novel
object
H2O
D-Asp


(D)
Freezing (%)
60
50
40
30
20
10
0
3
12
months
H2O
D-Asp