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ORIGINAL ARTICLE

Evidence for the involvement of D-aspartic acid in learning and memory of rat

Enza Topo · Andrea Soricelli · Angela Di Maio · Enrico D'Aniello · Maria Maddalena Di Fiore · Antimo D'Aniello

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Abstract D-Aspartic acid (D-Asp) is an endogenous amino acid present in neuroendocrine systems. Here, we report evidence that D-Asp in the rat is involved in learning and memory processes. Oral administration of sodium D-aspartate (40 mM) for 12–16 days improved the rats' cognitive capability to find a hidden platform in the Morris water maze system. Two sessions per day for three consecutive days were performed in two groups of 12 rats. One group was treated with Na-D-aspartate and the other with control. A significant increase in the cognitive effect was observed in the treated group compared to controls (two-way ANOVA with repeated measurements: $F_{(2,\ 105)} = 57.29$; P value < 0.001). Five further sessions of repeated training, involving a change in platform location, also displayed a significant treatment effect [$F_{(2,\ 84)} = 27.62$; P value < 0.001]. In the hippocampus of treated rats, D-Asp increased by about 2.7-fold compared to controls (82.5 ± 10.0 vs. the 30.6 ± 5.4 ng/g tissue; $P < 0.0001$). Moreover, 20 randomly selected rats possessing relatively high endogenous concentrations of D-Asp in the hippocampus were much faster in reaching the hidden platform, an event suggesting that their enhanced cognitive capability was functionally related to the high levels of D-Asp. The correlation coefficient calculated in the 20 rats was $R = -0.916$ with a df of 18; $P < 0.001$. In conclusion, this study provides corroborating evidence that D-aspartic acid plays an important role in the modulation of learning and memory.

Keywords D-Aspartic acid · Learning and memory · Rat · Hippocampus · Brain · Morris water maze system

E. Topo (✉) · E. D'Aniello · A. D'Aniello
Laboratory of Animal Physiology and Evolution,
Stazione Zoologica Anton Dohrn, Villa Comunale,
80121 Naples, Italy
e-mail: enza.topo@szn.it

A. Soricelli
Fondazione IRCCS-SDN, Via Gianturco 113,
80143 Naples, Italy

A. Di Maio · M. M. Di Fiore
Department of Life Sciences,
Second University of Naples, Via Vivaldi, 43,
81100 Caserta, Italy

Introduction

D-Aspartic acid (D-Asp) is an endogenous amino acid occurring in free form in endocrine and nervous tissues of all the animal phyla investigated thus far (for a review see Takemitsu and Homma 2005; D'Aniello 2007). This amino acid has been found in endocrine glands, particularly in those associated with the reproductive system of some invertebrates, including the *Octopus vulgaris* (D'Aniello et al. 1996a, b) and *Ciona intestinalis* (D'Aniello et al. 2003), and of several vertebrates, including mammals (Di Fiore et al. 1998; Assisi et al. 2001; Hashimoto et al. 1993a; D'Aniello et al. 1996a, b; Imai et al. 1995; Lee et al. 1997; Nagata et al. 1999; D'Aniello et al. 1998a, b; A. D'Aniello et al. 2000; Wang et al. 2002; Boni et al. 2006). For instance, in rats (D'Aniello et al. 1996a, b), in sheep (Boni et al. 2006), and in humans, D-Asp has been found in the seminal fluid and spermatozoon (G. D'Aniello et al. 2005), as well as in the follicular fluid (D'Aniello et al. 2007).

In the nervous tissues, D-Asp was first found in the brain and optic lobes of the mollusk *O. vulgaris* (D'Aniello and Giuditta 1977) and later in the nervous systems of other animal phyla such as the Amphioxus *Branchiostoma lanceolatum* (D'Aniello and Garcia-Fernàndez 2007) and

the Opistobranks *Aplysia limacina* (Spinelli et al. 2006), as well as in the bird *Gallus gallus* (Neidle and Dunlop 1990) and in mammals, namely, in the rat (Dunlop et al. 1986, D'Aniello et al. 1993; Hashimoto et al. 1995; Schell et al. 1997; Wolosker et al. 2000) and human (Fisher et al. 1991, 1994; Hashimoto et al. 1993b). In the early stages of the embryonic life of rat and chicken, D-Asp occurs at high concentrations in the brain (Dunlop et al. 1986; Neidle and Dunlop 1990), indicating a role of D-Asp in the development of the nervous system. In the adult animal, instead, several findings indicate that D-Asp has also a role in neurotransmission, either as a neurotransmitter or as a neuromodulator, indicative of its involvement in cognitive processes. Concerning the role of D-Asp in the nervous system, several studies have reported its role in neurotransmission. For instance, in the retina of the goldfish, D-Asp is capable of enhancing the effect of L-glutamate in response to light (Ishida and Gordon 1981) by as much as 15-fold. Similarly, the presence of D-Asp in the retina of *Sepia officinalis* suggests a direct correlation between D-Asp concentration and the animal's response to light exposure (D'Aniello. et al. 2005b). Depolarization of rat cerebral and hippocampal slices by K^+ ions has been found to induce D-Asp release by a calcium channel-dependent mechanism (Davies and Johnston 1976; Holopainen and Kontro 1990; Malthe-Sorenssen et al. 1979). Furthermore, a specific transporter for D-Asp, which carries it from synaptic clefts to postsynaptic nerve cells, has been detected (Kanai and Hediger 1992). Similarly, in *Aplysia limacine,* D-Asp is present at very high concentrations in nerve endings (synaptosomes) and in synaptic vesicles (Spinelli et al. 2006). Interestingly, evidence that D-Asp is able (1) to modulate AMPA-like glutamate receptors (Brown et al. 2006), (2) to enhance the long-term potentiation LTP in mouse hippocampus (Errico et al. 2008a), and (3) to prevent corticostriatal long-term depression (LTD) (Errico et al. 2008b) fully confirms its role in cognitive processes. In addition, D-aspartate oxidase (D-AspO), the enzyme which specifically oxidizes D-Asp, is present in the neuronal postsynaptic membrane (Spinelli et al. 2006). Lastly, D-Asp is stored in secretory granules of PC12 cells and is secreted through a Ca^{2+}-dependent exocytotic mechanism (Nakatsuka et al. 2001). Most important, it has been reported that in the brain of Alzheimer's patients, D-Asp occurs at a significantly lower concentration than in the brain of healthy subjects belonging to the same age group (control). A similar phenomenon has been detected in other parts of the brain associated with higher cognitive functions, particularly in the frontal cortex (Fisher et al. 1991), in the parietal, in the occipital, in the cerebellum, as well as in the amygdale and hippocampus (D'Aniello et al. 1998a, b).

Taken altogether, these studies strongly suggest that D-Asp, in addition to playing an important role in the endocrine regulation of hormonal activity and in gametogenesis, is also highly involved in the nervous system, either as a possible neurotransmitter or as a possible neuromodulator, thereby suggesting its particular role in learning and memory. In this study, to validate even further this last hypothesis, two important experiments were carried out on rats. Rats were first treated with D-Asp and then subjected to the Morris water maze test to assess whether the exogenous administration of this amino acid was able to improve spatial memory and whether there existed a relationship between the endogenous concentration of D-Asp in rat hippocampus and the actual ability of the rat to learn the position of the platform in the water maze system.

Materials and methods

Animals, chemicals and D-Asp treatment

Adult male rats 120 days old (320–340 g body weight; Wistar rats from Charles River Laboratories, Italy) were housed 2 per cage in a controlled environmental animal facility with 12 h light/dark cycle. All animal procedures were carried out in accordance with the principle of laboratory animal care (NIH publication N. 85-23, revised in 1985, the European Communities Council Directive of 24 November 1986 (86/609/EEC) and Italian legislation). Adequate measures were taken to minimize pain and suffering and to minimize the number of animals used. D-aspartic acid and all other amino acids used as standards for HPLC were from Sigma Chemical Company, USA. D-AspO from beef kidney was obtained by overexpression in bacteria, as previously described (S. D'Aniello. et al. 2005). All chemical substances used were of a reagent grade. Two groups of rats were used for this study. The rats in the first group ($n = 18$) received a solution of 40 mM sodium D-aspartate for 12 days. The ones in the second group ($n = 18$) received 40 mM NaCl (2.3 g/L) for the same period of time. All rats were allowed food ad libitum. Throughout the entire duration of the experiments, each rat drank 9–11 ml of sodium D-aspartate or NaCl solution which corresponded to about 60 mg of Na-D-Asp/rat per day (0.19 mg/g body weight) and about 23 mg of NaCl/rat per day (0.069 mg/g body weight). These doses were chosen on the basis of preliminary experiments, as they were sufficient to examine their effects on learning and memory and caused no adverse effects on the animals even after 1 month of treatment. In addition, no body weight changes were detected in the treated animals.

We have also reported that the intake of NaCl or Na-D-Asp was not much different from that of tap water. Indeed, the rather low salinity contained in these two chemicals did not alter either the effects or concentration

levels in serum. In particular, when we analyzed the common clinical metabolites—glucose, urea, creatinine, electrophoresis of the proteins, sodium, potassium and calcium, and of specific enzymes, i.e, aspartate aminotransferase (EC 2.6.1.1.), alanine aminotransferase (EC 2.6.1.2.), alkaline phosphatase (EC 3.1.3.1.), cholinesterase (EC 3.1.1.8.), and lactate dehydrogenase (EC 1.1.1.27.)—in treated rats (NaCl or Na-D-Asp) and in those of the same age that received only tap water to verify serum concentrations of the two chemicals in the treated rats, no significant differences were found between control and treated rats. For both analyses, we adopted the clinical methods used for human blood analysis with automation technology.

Morris water maze experiments

The Morris water maze test is well known and is the most widely used behavioral test for learning and memory (Morris 1984). It consists of a circular plastic tank (180 cm diameter, 36 cm wall height) surrounded by two dimensional visual cues, containing opaque water at 24 ± 1°C with a circular platform (10 cm diameter) submerged 1 cm beneath the water surface. Preliminarily, all rats were subjected to a pretreatment in the Morris water maze. Each rat was left to swim in the water tank until it found the platform hidden in the center of the NW quadrant. Once it reached the platform, the rat was left for 20 s to memorize the position of the platform. After that, it was removed from the platform and put into a cage to rest. Rats who did not find the platform within 3 min were excluded. Twelve rats from each group were chosen to perform the Morris water maze test. Two training sessions per day were carried out at 11 am and 4 pm. Each session consisted of two trials with an interval of about 10 min. The time it took rats to reach the platform was recorded and was used as the dependent variable during the acquisition phase. Each rat treated with D-Asp and control was subjected to water maze acquisition for three consecutive days using the platform in the center of the NW quadrant. After this training session, the platform was moved from the NW to the NE quadrant, and the training phase was continued for the following 2 days and up until the morning of the third day. Throughout the duration of all the experiments, rats continued to drink sodium D-aspartate or NaCl. A probe test was conducted at the end of each training phase (acquisition and reversal). During the probe test, the animals were allowed to swim for 60 s in the absence of the platform. The percentage of time spent in each quadrant was recorded. A video tracking system (Videotrack, Viewpoint) was used for all Morris water maze tasks performed to collect data during the learning phase and probe tests.

The second experiment was designed to determine whether there existed an inverse relationship between the amounts of D-Asp present endogenously in the hippocampus and the time it took rats to reach the platform. To this aim, 20 male rats (120 days old) were subjected to the Morris water maze experiments twice a day for three consecutive days. The time it took each rat to reach the platform was recorded in seconds. Then, at the end of the treatment and training sessions, the rats were decapitated and their brains dissected to determine D-Asp levels in the hippocampus. Finally, these values were correlated with the amount of time it took rats to reach the platform.

Determination of D-Asp by HPLC associated with D-aspartate oxidase

D-Asp was determined by HPLC associated with D-AspO, as previously described (S. D'Aniello et al. 2005). In brief, each rat tissue (total brain or hippocampus) was homogenized 1:20 in 0.2 M TCA and centrifuged at 10,000*g* for 10 min. Next in two Eppendorf tubes was added 10 µl of supernatant and mixed with 9 µl of 0.2 M NaOH (to neutralize TCA), and 50 µl of 0.2 M borate buffer, pH 8.2 (optimum pH for D-AspO activity). Then, 5 µl H_2O was added to the first tube (sample) and 5 µl of purified kidney D-AspO (300 U/ml; 1 mg/ml) was added to the second tube (blank sample). Afterwards, both tubes were incubated at 37°C for 20 min. Following incubation, 200 µl of 0.2 M sodium pyrophosphate buffer, pH 10, and 20 µl of OPA-NAC reagent (20 mg *O*-phthaldialdehyde and 10 mg of *N*-acetyl-L-cysteine in 2 ml 50% methanol) were supplemented to the first tube. Two minutes later, distilled water was added to a final volume of 1 ml and mixed. Then, 100 µl of sample tissue (0.05 mg of original tissue) was injected into the HPLC column (Supercosil ODS-C_{18} column, 0.45 × 25 cm, 5 µm beads, Supelco, Bellefonte, PA, USA). The column was connected to a Beckman–Gold HPLC system and the amino acids were eluted with a gradient consisting of solution A (30 mM citrate phosphate buffer, pH 5.6, 5% acetonitrile) and solution B (90% acetonitrile in H_2O). The gradient was as follows: 0–15% B over 12 min; 15–100% B over 4 min, stayed at 100% B for 4 min and then returned to 100% A in 1 min. Amino acids were detected fluorometrically at an excitation wavelength of 330 nm and emission wavelength of 440 nm. D-Asp was eluted at 5.2 min, followed by L-Asp at 6.0 min and by L-Glu at 7.2 min. Following the sample analysis, the same procedure was carried out for the blank sample. The net area of D-Asp was then calculated by subtracting the peak area of D-Asp in the blank sample from that in the sample. Then, we carried out a standard amino acid HPLC curve consisting of 20 pmol of D-Asp and 100 pmol of each of the following amino acids: L-Asp, L-Glu, L-Asn, L-Gln, L-Ser, L-Thr, L-His, Gly, L-Ala, L-Arg, L-Met, L-Val, L-Tyr, L-Phe, L-Ile, L-Leu and L-Lys. Next, D-Asp concentration in the sample was

calculated with the following formula: Area of D-Asp from sample/area of D-Asp from the standard curve × 20 = pmol of D-Asp contained in 0.05 mg of original tissue. Analogously, L-Asp and L-Glu were calculated with the following formula: Area of L-Asp or L-Glu from sample/area of L-Asp or L-Glu from the standard curve × 100 = pmol of D-Asp contained in 0.05 mg of original tissue.

Data analysis

Statistical analyses were performed using Stat-Soft, 1997, 98th edition. Two-way ANOVA (control or treated rat per day) with repeated measurements was used to record the swim time to the platform. The correlation coefficient was determined according to Pearson's product × moment method. The *t* student $P < 0.05$ was considered statistically significant.

Results

Determination of D-Asp in rat brain by using the HPLC-D-AspO method

D-Asp in rat brain was determined by the HPLC method associated with D-AspO. This enzyme commonly enables researchers to determine with accuracy the actual amount of D-Asp in a sample. If a sample contains a molecule that interferes with D-Asp peak, HPLC determination of the sample before and after treatment with D-AspO assures the detection of the actual amount of the peak area reached by D-Asp. Panel a shown in Fig. 1 depicts a typical example of HPLC determination of D-Asp from a mixture of standard amino acids containing 20 pmol of D-Asp and 100 pmol of the other amino acids. Under HPLC conditions, whereas D-Asp, L-Asp**,** and L-Glu were well separated from each another, the other amino acids eluted after L-Glu were not. Panel B of the same figure shows a typical HPLC determination of the amino acids contained in 0.05 mg of rat hippocampus. Panel c, instead, reports the same analysis but after the sample was treated with D-AspO. We can observe that the peak which eluted at a retention time of 5.2 min completely disappeared, thus indicating that the entire peak area was identifiable with D-Asp.

Accumulation of D-Asp in rat brain treated with D-Asp

After 18 days of treatment with sodium D-aspartate (40 mM), the animals were killed to determine the amount of D-Asp accumulated in the brain, in comparison with the control group. We found that whereas the mean concentration of D-Asp in control rats was 30.6 ± 5.4 nmol D-Asp/g of brain, the concentration of D-Asp-treated rats was 82.5 ± 10.0 nmol/g. Thus, it increased by as much as 2.7-fold compared to the control (Fig. 2).

Effects of D-Asp on learning and memory in rats

This study indicates that D-Asp improves learning and memory ability in rats, as evidenced by their efficient performance in the Morris water maze acquisition test after oral treatment. In fact, rats receiving oral doses of D-Asp for 12 days required less time to reach the platform than did control rats receiving NaCl for the same number of days. In the last session of the first 3 days of the acquisition phase, when the platform was situated in the center of NW quadrant of the tank, rats treated with D-Asp reached the platform in much less time (5 ± 2 s) than did the control group (30 ± 5 s). Two-way ANOVA, with repeated measurements, revealed a significant effect ($F_{(2,\ 105)} = 57.29$; *P* value < 0.001). In addition, the same significant result was obtained when the platform was moved from the center of the NW quadrant to the center of the NE quadrant of the tank (reversal phase). As expected, in the last phase of this training session, the swim time of rats treated with D-Asp was significantly shorter than that of the control rats. Indeed, rats treated with D-Asp reached the platform in 5 ± 2 s, as opposed to the control group who spent 20 ± 3 s to accomplish the task, i.e, fourfold slower than the sample. Two-way ANOVA, with repeated measurements, revealed a significant effect ($F_{(2,\ 84)} = 27.624$; $P < 0.001$). A probe test was conducted at the end of both training phases (acquisition and reversal phases). The percentage of time (s) spent in each quadrant during the probe test was recorded. After the first training session (acquisition) rats treated with D-Asp remained for 42 ± 5% of the total time in the NW quadrant, for 15 ± 3% in the NE quadrant, for 22 ± 4% in the SW, and for 21 ± 3% in the SE quadrant. The control rats, instead, remained for 31 ± 4% in the NW quadrant, for 19 ± 4% in the NE quadrant, for 26 ± 5% in the SW quadrant, and for 24 ± 4% in the SE quadrant. Although both treated and untreated rats remained in the NW quadrant more than 25% of the total time, which is usually considered a time limit for learning and memory, the difference in the percentage of time spent by the treated rats in the NW quadrant was significantly higher than that of control ($P < 0.01$).

Relationship between the endogenous concentration of D-Asp in rat hippocampus and the ability of learning and memory

The second important experiment conducted in this study was to ascertain whether there existed a relationship between the endogenous concentration of D-Asp in rat hippocampus and the rat's ability to learn and memorize

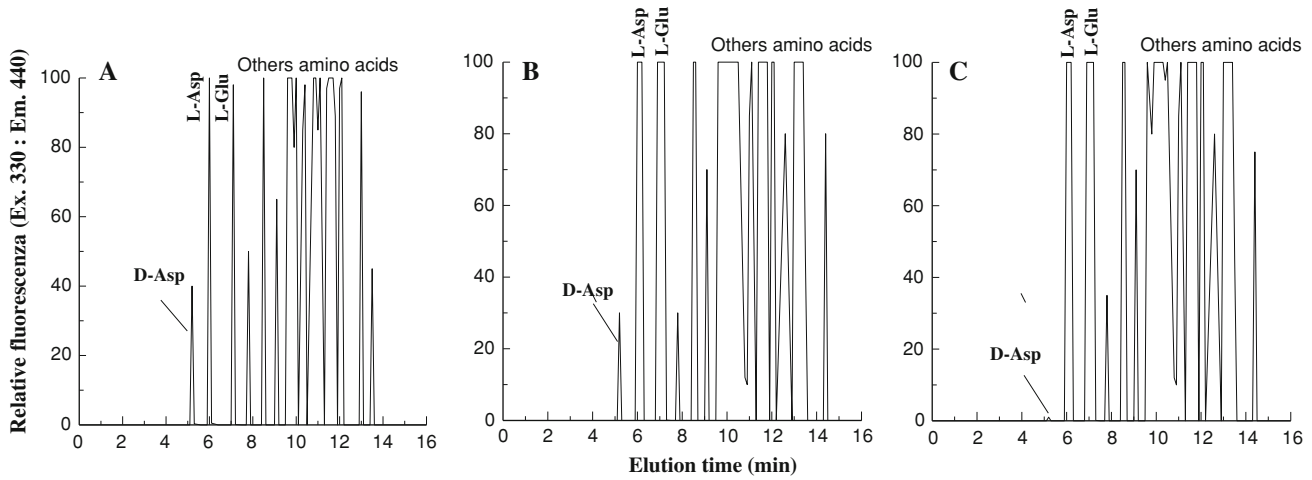

Fig. 1 Typical HPLC analysis for the determination of D-aspartic acid and others amino acids in rat hippocampus. Panel **a** HPLC profile of a standard mixture consisting of 20 pmol of D-Asp and 100 pmol of the following other amino acids: L-Asp, L-Glu, L-Asn, L-Gln, L-Ser, L-Thr, L-His, Gly, L-Ala, L-Arg, L-Met, L-Val, L-Tyr, L-Phe, L-Ile, L-Leu and L- Lys. D-Asp eluted at 5.2 min, L-Asp at 6.0 min, L-Glu at 7.2 min. The other amino acids were eluted after L-Glu but not separated owing to the rapid increase in the acetonitrile gradient. Panel **b** Typical example of HPLC analysis of amino acids from 0.05 mg of rat hippocampus analyzed by HPLC, as described in "Materials and methods". The peak of D-Asp is well visible at elution time of 5.2 min. Panel **c** The same sample was used previously, but after treatment with D-AspO. Before HPLC analysis, the sample was incubated with 2 µl (0.2 µg) of purified beef kidney D-AspO at 37°C for 20 min. Then the disappearance of the peak at retention time of 5.2 min indicated that the peak of D-Asp in panel **b** was all due to the occurrence of D-Asp

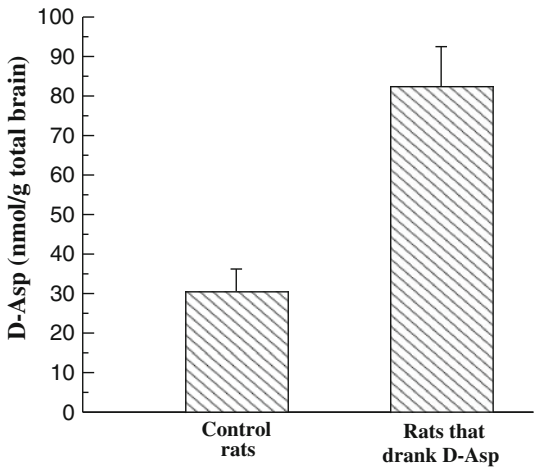

Fig. 2 Occurrence of D-aspartic acid in brain of control and D-Asp treated rats Fig. 2 reports the concentration of D-Asp in rat brain (nmoles/g tissue) of rats belonging to the control group (mean ± SD from 12 rats) and in the brains of rats treated with sodium D-aspartate (drank 40 mM for 16 days). *Left bar* indicates the concentration of D-Asp in total brain from the control rats (30.6 ± 5.4 nmol/g tissue) and *right bar* indicates the concentration of D-Asp in treated rat (82.5 ± 10 nmol/g tissue). Such differences were statistically significant for the Student *t* test $P < 0.0001$

the position of the platform in the Morris water maze system. To test this hypothesis, 20 new male rats aged 120 days were randomly selected from various families of rats and were trained in the Morris water maze system twice a day over three consecutive days. Following the last session, the time spent by each rat to reach the platform was recorded. Then, the concentration of D-aspartate in the hippocampus was determined following dissection. Next, the values of the swim time to reach the platform and the concentration levels of D-Asp in the hippocampus were fitted on an *XY* plot. Finally, the coefficient of correlation was calculated according to Pearson's method. In Table 1, we report the time spent by rats to reach the platform in relation to D-Asp levels found in their brains. The results obtained from this investigation demonstrated that there was indeed a good and significant correlation between the two parameters. The coefficient of correlation was $R = -0.916$ with a *df* of 18 and a *t* student of $P < 0.001$ (Fig. 4). Thus, in addition to the previous water maze experiment, this last one, based on the concentration ratio of D-Asp in the hippocampus in relation to the time needed to reach the platform, strongly indicates that D-Asp plays a pivotal role in improving learning and memory.

Discussion

In the present paper, we have proven that exogenous administration of D-Asp improves the learning capability of rats and enhances memory processes. Furthermore, we have also illustrated that learning performance in rats is significantly enhanced in the presence of higher concentrations of endogenous D-Asp in the hippocampus. Indeed, oral administration of D-Asp (40 mM) facilitated rats' performance in the Morris water maze system. Similarly,

Table 1 Relationship between the endogenous occurrence of D-Asp in rat hippocampus and the time it took rats to reach the platform in the Morris water maze system

	D-Asp in hippocampus (nmol/g tissue)	Time (s) for rat to reach the platform
1	13	25
2	13	27
3	14	25
4	18	19
5	21	20
6	21	15
7	23	18
8	25	19
9	29	17
10	30	22
11	31	15
12	31	15
13	32	14
14	35	16
15	45	10
16	48	7
17	50	8
18	52	8
19	55	6
20	62	5

Twenty adult rats (120 days old) were trained for learning and memory in the Morris water maze, as described in the text. The rats were dissected and D-Asp was determined in the hippocampus. Then the coefficient of correlation between concentration of D-Asp and the amount of time to reach the platform was determined. This correlation was calculated according Pearson's method which yielded an R of -0.916 with a df of 18 and a t student of $P < 0.001$

rats with higher endogenous concentrations of D-Asp in the hippocampus took much less time to reach the hidden platform than did rats with lower D-Asp levels. These results strongly support previous research suggesting the involvement of this amino acid not only in the endocrine system, but also in the nervous system, where it acts either as a neurotransmitter or as a neuromodulator in those brain regions strongly associated with learning and memory.

Recent investigations have shown that D-Asp plays an important physiological role in mammals. This amino acid occurs in the neuroendocrine tissues and is synthesized intracellularly by a D-aspartate racemase (Spinelli et al. 2006; Wolosker et al. 2000; Monteforte et al. 2008). In the pituitary gland it regulates the release and synthesis of LH, GH and prolactin), whereas in the testis it regulates the release and synthesis of testosterone (D'Aniello 2007; G. D'Aniello et al. 2000).

In the nervous system, D-Asp occurs at high concentrations in the synaptic vesicles (Spinelli et al. 2006) and displays many neuronal functions (see the "Introduction" section), thus indicating its role as a neurotransmitter or neuromodulator in the nervous system and its involvement in learning and memory processes. With regard to this last physiological function, we have previously reported that D-Asp is present in the human cortex and that its concentration levels are significantly reduced in Alzheimer's brain compared to the normal brains of aged control patients (Fisher et al. 1991). In addition, this phenomenon also occurs in other regions of the brain, e.g, in the frontal, parietal, occipital, cerebellum, amygdale, and hippocampus (D'Aniello et al. 1998b). Hence, given that Alzheimer's patients possess a reduced cognitive activity, we have deduced that D-Asp could also be involved in human learning and memory. Intriguingly, further research in this field revealed that in human ventricular cerebrospinal fluid obtained from the third ventricle (Fisher et al. 1994), and from the spinal cerebral fluid of Alzheimer's subjects (Fisher et al. 1998), the concentration of D-Asp is significantly higher than that found in the same tissue of healthy control subjects. A tentative explanation toward this phenomenon is that, for unknown reasons, in Alzheimer's disease, D-Asp is transferred from the brain to the ventricular and then to the spinal cerebral fluid.

Moreover, recent studies have demonstrated in mice that D-aspartate plays a direct role in regulating hippocampal synaptic plasticity. Indeed, 1 month of oral administration of sodium D-aspartate (20 mM) enhances NMDAR-dependent long-term potentiation (LTP) in mice (Errico et al. 2008a). The same phenomenon occurs in knock-out mice. Actually, in this genetically modified mouse, high values of endogenous D-Asp are present owing to the total or partial absence of D-AspO, a finding indicating that high levels of endogenous D-Asp do enhance LTP (Errico et al. 2008a). In addition, D-Asp is capable of boosting hippocampal *N*-methyl-D-aspartate receptor (NMDAR)-dependent memory, prevent cortical long-term depression (LTD), as well as induce an acute increase in cerebral cGMP levels (Errico et al. 2008b). However, although D-Asp increases LTP, no difference in spatial memory has been found in either mouse genetically modified for D-AspO or in mouse treated with D-Asp. The reasons for these conflicting results are still elusive. One tentative explanation is that genetic differences between rat and mice may be responsible for such discrepancies. An alternative possibility could be that, in the brain, D-Asp concentrations higher than baseline values are ineffective in improving learning and memory. In fact, the brain of the genetically modified mouse for D-AspO possesses endogenous high levels of D-Asp that can result 5–12 times higher than those of control (Errico et al. 2006). In addition, in the brains of experimental mice receiving 20 mM sodium D-Asp for 1 month, D-Asp levels are 5 times higher

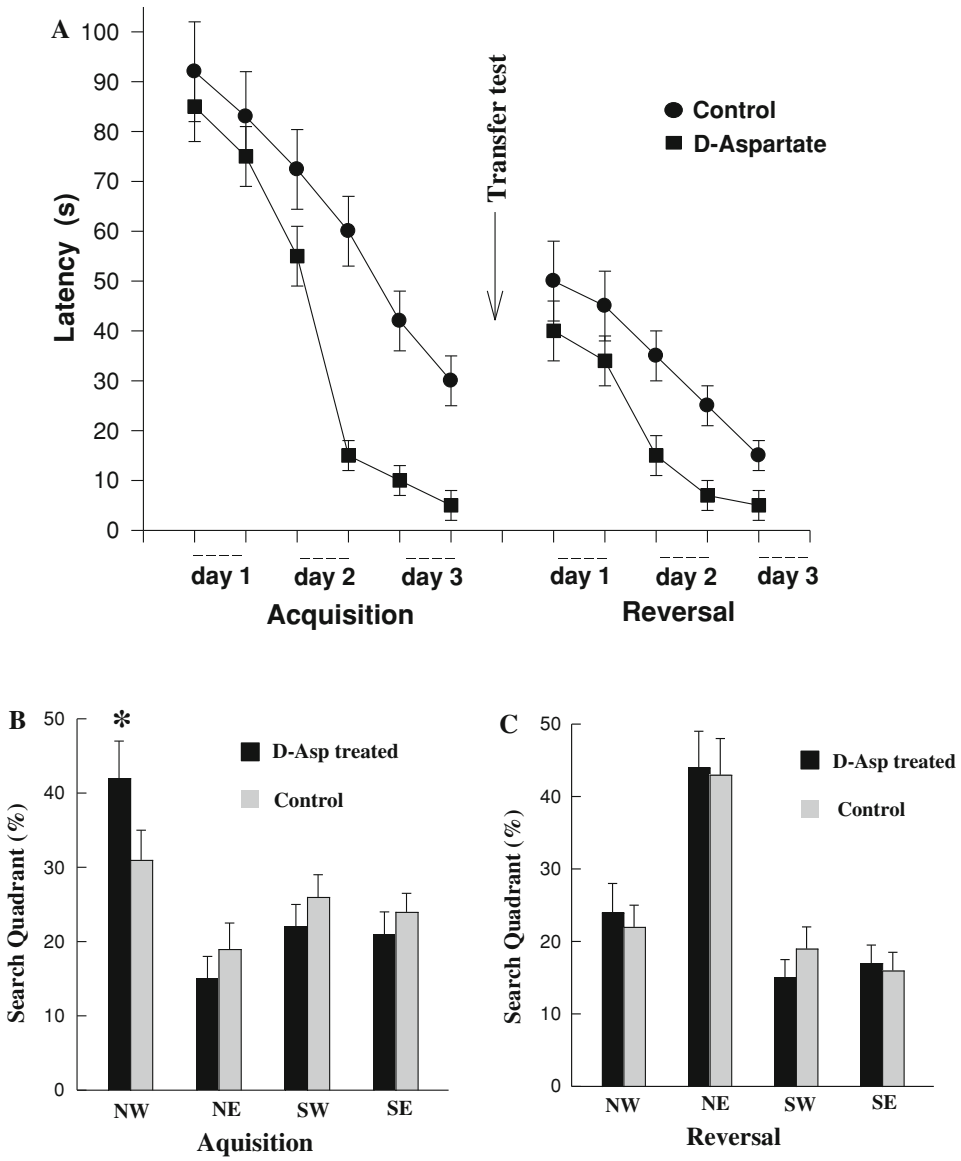

Fig. 3 Water maze training and memory acquisition of D-Asp-treated rats and controls **a** Mean escape latency of 12 rats treated with 40 mM sodium D-aspartate and of 12 control rats. The acquisition phase, carried out using the hidden platform in the center of the NW quadrant, consisted of two daily sessions carried out over a period of three consecutive days. Then, the platform was moved from the center of the NW quadrant to the center of NE quadrant and the rats continued their training phase twice a day for two more days and through the morning of the third day. Two-way ANOVA, with repeated measurement, revealed that both groups significantly improved their task performance during the acquisition phase [$(F_{2,\ 105}) = 57.29$; P value < 0.001], the reversal phase [$(F_{2,\ 84}) = 27.62$; P value < 0.001]. **b** A 60-s probe test was performed at the end of the acquisition phase. Whereas control rats did not show any preference for the target quadrant (*NW*), D-Asp-treated rats spent more time in the goal quadrant, whose significance is indicated by the *asterisk* (P value < 0.001). **c** A second probe test was performed at the end of the reversal phase. Both control and D-Asp-treated rats exhibited a preferential spatial search for the target quadrant (*NE*) (P value < 0.001). However, there was not significant difference between D-Asp-treated rats and controls (P value > 0.05)

than those of control rats (Errico et al. 2008a). In our experimental rats, instead, the levels of D-Asp in the brain increased 2.5 times compared to control. Thus, it is possible that moderate increases in D-Asp are more efficient than higher levels in improving learning and memory, a phenomenon that has not yet been fully clarified.

In this study, to delve deeper into the hypothesis that D-Asp is involved in the neuronal mechanisms underlying learning and memory processes, we conducted two basic experiments on rats. The first experiment consisted of administering oral solutions of sodium D-Asp to rats before subjecting them to the Morris water maze test. By doing so, we were able to verify whether D-Asp administration was able to increase the rats' spatial memory. The second experiment was aimed at assessing whether selectively randomized rats that possessed high endogenous

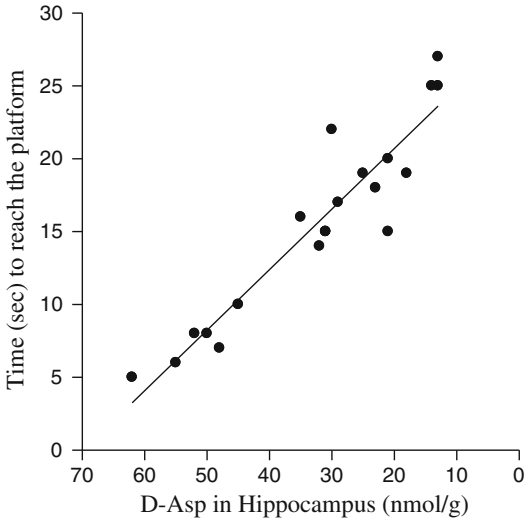

Fig. 4 Relationship between the endogenous occurrence of D-Asp in rat hippocampus and the time it took each rat to reach the platform in the Morris water maze system. Twenty adult rats (120 days old) were trained for learning and memory tasks in the Morris water maze system. Then, they were dissected and D-Asp was determined in the hippocampus. Finally, the coefficient of correlation between D-Asp concentration and the time to reach the platform was determined according to Pearson's method. The coefficient of correlation was calculated as $R = -0.916$ with a *df* of 18 and a *t* student of $P < 0.001$

concentrations of D-Asp in the hippocampus were faster than the ones containing lower concentrations at finding the hidden platform in the water maze. As expected, rats treated with sodium D-aspartate at a concentration of 40 mM for 12 days displayed a significant improvement in their spatial learning and memory ability compared with control rats (Fig. 3). In fact, as shown in Fig. 3, on the third day of the water maze experiments, the rats receiving sodium D-aspartate displayed enhanced spatial memory in comparison with control rats, indicating that exogenous assumption of D-Asp improved memory. In addition, the second and equally important experiment, which verified whether there existed a relationship between the endogenous occurrence of D-Asp in the brain and the ability to learn the position of the platform in a water maze system, also yielded strong indications in favor of D-Asp as a major player in memory enhancement. In fact, as is shown in Table 1 and Fig. 4, rats that possessed high concentrations of D-Asp in the hippocampus took less time to reach the platform, thereby indicating once again the direct involvement of D-Asp in learning and memory.

At present we do not know the exact mechanism by which D-aspartic acid induces an increase water maze memory. One plausible explanation could be that in the nervous system D-aspartic acid is localized mainly in synaptic vesicles and could be a novel neurotransmitter implicated in memory process. This speculation is in line with some of our recent results in which we have found that among all of the D-Asp present in rat brain the 80–90% is localized in synaptic vesicles (unpublished results).

In conclusion, the present work, by substantiating even further previously published data, conclusively demonstrates that, in the rat, D-Asp plays a major role in the neuronal mechanisms underlying learning and memory.

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