D-Aspartic acid is a novel endogenous neurotransmitter

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ABSTRACT **D-Aspartic acid (D-Asp) is present in invertebrate and vertebrate neuroendocrine tissues, where it carries out important physiological functions and is implicated in nervous system development. We show here that D-Asp is a novel endogenous neurotransmitter in two distantly related animals, a mammal (*Rattus norvegicus*) and a mollusk (*Loligo vulgaris*). Our main findings demonstrate that D-Asp is present in high concentrations in the synaptic vesicles of axon terminals; synthesis for this amino acid occurs in neurons by conversion of L-Asp to D-Asp *via* D-aspartate racemase; depolarization of nerve endings with K^+ ions evokes an immediate release of D-Asp in a Ca^{2+} dependent manner; specific receptors for D-Asp occur at the postsynaptic membrane, as demonstrated by binding assays and by the expansion of squid skin chromatophores; D-aspartate oxidase, the specific enzyme that oxidizes D-Asp, is present in the postsynaptic membranes; and stimulation of nerve endings with D-Asp triggers signal transduction by increasing the second messenger cAMP. Taken together, these data demonstrate that D-Asp fulfills all criteria necessary to be considered a novel endogenous neurotransmitter. Given its known role in neurogenesis, learning, and neuropathologies, our results have important implications for biomedical and clinical research.—D'Aniello, S., Somorjai, I., Garcia-Fernàndez, J., Topo, E., D'Aniello, A. D-Aspartic acid is a novel endogenous neurotransmitter. *FASEB J.* 25, 1014–1027 (2011). www.fasebj.org**

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D-AMINO ACIDS ARE PRESENT in small amounts in animal tissues, but possess important physiological functions (1–2). D-Serine (D-Ser) and D-aspartic acid (D-Asp) are the most abundant amino acids and have been extensively studied (3–4). D-Asp was first discovered in the brain of *Octopus vulgaris* (5). Subsequently, this amino acid was found in the nervous tissues of the mollusk opisthobranch *Aplysia californica* (6), in the amphioxus *Branchiostoma lanceolatum* (7), and the tunicate *Ciona intestinalis* (8), as well as in vertebrates, including chicken (9), rat (10–14), and human tissues (14–16). High levels of D-Asp have been found in the nervous system of rat and chicken embryos (9), in addition to human embryonic and adult brains (14–16), indicating that this amino acid could be involved in the development of the nervous system and in adult neurological activity. The presence of D-Asp at considerable levels, primarily in pituitary gland (12, 13, 17, 18), pineal gland (10), and testis (12, 13, 18–20), is also suggestive of its involvement in endocrine tissues. From a functional standpoint, studies on rat pituitary gland and testis have indicated that D-Asp is involved in the synthesis and release of luteinizing hormone (LH) and testosterone, respectively (17–18), as well as of α-melanocyte-stimulating hormone, GABA, and dopamine (21). In addition to the endocrine system, this amino acid plays well-defined roles in the nervous system of a variety of animals. For example, D-Asp is able to potentiate the effects of L-glutamate in the goldfish retina (22) and has a visual function in the retina of *Sepia officinalis* (23). Furthermore, it has been reported that K^+ ions induce the release of D-Asp from astrocytes (24) and from rat brain slices (25). Most recently, we found that D-Asp increases long-term potentiation in mice (26), prevents long-term depression, attenuates schizophrenia-like symptoms (27), and is able to rescue hippocampal age-related synaptic plasticity (28). In addition, D-Asp has been demonstrated to increase spatial memory in rats (29). Recently, it has been reported that D-Asp may function as a modulator of adult neurogenesis (30). In light of all these data, we formally propose that the aforementioned implication of D-Asp in neurological activity could be due to its function as a neurotransmitter. Therefore, the aim of this study was to test the hypothesis that D-Asp could be a novel endogenous neurotransmitter in the nervous system. We used two different models: a mammal, the rat (*Rattus norvegicus*); and a mollusk, the European squid (*Loligo vulgaris*). The results obtained from these investigations provide evidence that the principal criteria required to consider D-Asp an endogenous neurotransmitter have been satisfied. We thereby expand the functional repertoire of D-amino acids, providing important avenues for future medical research.

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MATERIALS AND METHODS

Materials

All amino acids, bovine serum albumin (BSA), *o*-phthaldialdehyde (OPA), *N*-acetyl-L-cysteine (NAC), Triton X-100, Tris (Tris hydroximethyl-aminomethane), in addition to all chemicals used for optic and electronic immunohystochemistry, were purchased from Sigma Chemical Co. (Milan, Italy). The solvents for high-performance liquid chromatography (HPLC) were reagent grade, and purchased from Merck (Milan, Italy) or C. Erba (Milan, Italy). Cation exchange resin (AG 50WX8; H^+ form, 100–200 mesh), and all chemicals for acrylamide gel electrophoresis were obtained from Bio-Rad Laboratories (Milan, Italy). All material for immunoblotting was from Amersham International (Dübendorf, Switzerland).

Animals

Wistar rats were purchased from Charles River Laboratory (Como, Italy) and housed 3/cage in a controlled environmental animal facility at 24°C on a 12-h light-dark cycle. The animals were fed standard laboratory food pellets and water *ad libitum*. Animal care was in accordance with local institutional guidelines. Rats were killed by decapitation. Specimens of the mollusk *L. vulgaris* were caught in the bay of Naples by local fishermen.

Preparation of synaptosomes, synaptic vesicles (SVs), and postsynaptic membranes (PSMs) from *R. norvegicus* and *L. vulgaris* brain

The preparation of rat synaptosomes was carried out according to the described methods (31) with a slight modification. Rat brain (5 g of 120 d-old Wistar rat) was homogenized in 100 ml of 0.32 M sucrose in 0.05 M Tris-HCl (pH 7.5), using a glass Potter homogenizer with a clearance of 0.20–0.25 mm (10–15 up-and-down strokes). The homogenate was centrifuged at 1000 *g* for 10 min. The supernatant was recentrifuged at 22,000 *g* for 20 min, and the resulting supernatant was stored for the determination of total brain amino acids. The precipitate was suspended in 25 ml of 0.32 M sucrose in 0.05 M Tris-HCl (pH 7.5), and each 5 ml of this suspension was layered on a discontinuous sucrose gradient consisting of 20 ml of 1.2 M sucrose (bottom) and 20 ml of 0.8 M sucrose (top) and centrifuged at 22,000 *g* for 2 h. The synaptosomal fraction, visible as a cream-colored band to the top level of the 1.2 M sucrose, was collected and diluted with an equal volume of physiological buffer (125 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 22 mM glucose, and 10 mM Na_2HCO_3, pH 7.4) and centrifuged for 90 min at 22,000 *g*. The precipitate of synaptosomes was suspended in 2 ml of 0.32 M sucrose in 0.05 M Tris-HCl (pH 7.5); stratified on a second sucrose gradient consisting of 14 ml each of 1.2, 0.95, and 0.75 M sucrose; and centrifuged at 22,000 *g* for 90 min. The top fraction containing synaptosomes was collected, diluted with an equal volume of physiological buffer, and centrifuged at 22,000 *g* for 10 min. The pellets (synaptosomes) were suspended in 5 ml of the same buffer and used for the following studies: electron microscopy, amino acid determination, release of D-Asp and L-glutamic acid (L-Glu) by KCl depolarization, preparation of PSMs, and/or isolation of the SVs. For the preparation of PSMs and SVs, 3 ml of synaptosome suspension was diluted in 60 ml of distilled water and homogenized with a glass Potter homogenizer with a clearance of 0.20–0.25 mm (10–15 up-and-down strokes). The synaptosomes were broken by osmotic lyses, left at 4–6°C for 30 min under slow agitation (the SVs were liberated and not lysed due to their small size), and centrifuged at 5000 *g* for 5 min. The supernatant contained the SVs and the precipitate the PSMs. The precipitate was suspended in 20 ml of the above buffer, mixed with 0.4 ml of 10% Triton X-100, and stirred at 18–20°C for 10 min, then centrifuged at 20,000 g for 60 min. The pellet consisting of PSMs was suspended in 3 ml of the same buffer and used for electronic microscopy and assessment of receptor binding activity and D-aspartate oxidase (D-AspO) activity. The supernatant containing the SVs was centrifuged at 15,000 *g* for 20 min; the precipitate was suspended in 1.0 ml of the above buffer and centrifuged for 60 min at 20,000 *g* on a sucrose gradient consisting of 14 ml each of 1.2 M sucrose (bottom), 0.9 M sucrose (middle), and 0.6 M sucrose (top). The SVs migrated at the interface between 0.9 and 1.2 M sucrose; they were collected and diluted with 10 ml of the above buffer and centrifuged at 20,000 *g* for 10 min. The precipitate was suspended in 3 ml of the same buffer and used for HPLC amino acid analysis, protein determination, and electron microscopy.

For *L. vulgaris*, the synaptosomes from optic lobes were prepared according to the method previously described (32), and the same procedures discussed above were used to prepare SVs and PSMs and determine postsynaptic density.

Amino acid and protein determination

D-Asp, L-Asp, L-Glu, and total amino acid levels were determined by HPLC according to the method described previously (23). Proteins were measured with bicinchoninic acid as described previously (33). Data were analyzed by Student's *t* test and expressed as means ± SD.

D-Aspartate racemase: synthesis of D-Asp in *R. norvegicus* and *L. vulgaris* brains

We next sought to determine whether endogenous D-Asp is synthesized *in vivo* from L-Asp by D-aspartate racemase. Aspartate racemase (EC 5.1.1.13), an enzyme that synthesizes D-Asp by conversion of L-Asp into D-Asp and *vice versa*, has been reported in the mollusk *Scapharca broughtonii* (34), the gastropod *A. californica* (6), the reptile *Podarcis s. sicula* (35), and the mammal *R. norvegicus* (25). In this study, we characterized D-aspartate racemase activity in the whole-brain homogenate and in synaptosomes and SVs obtained from *R. norvegicus* and *L. vulgaris* brains. The procedure was essentially the same as described previously, with few modifications (23). The general principle was based on the following reactions:

$$\text{L-Asp} \xrightarrow{\text{D-aspartate racemase}} \text{D-Asp}$$

$$\text{D-Asp} + O_2 + H_2O \xrightarrow{\text{D-AspO}} \alpha\text{-oxaloacetate} + H_2O_2 + NH_3$$

α-Oxaloacetate + 2,4-dinitrophenylhydrazine =
α-oxaloacetate-dinitrophenylhydrazine complex
(yellow color)

α-Oxaloacetate-dinitrophenylhydrazine complex + NaOH =
α-oxaloacetate-dinitrophenylhydrazone
complex (strong purple color)

In brief, tissues were homogenized (1:5) in 0.02 M potassium citrate-phosphate buffer (pH 7.5) containing 10 mM of EDTA and a protease inhibitor cocktail (1:100, Sigma) and

centrifuged at 30,000 *g* for 30 min at 2–4°C. The supernatant (500 μl) was mixed with 50 μl of 1 M sodium L-Asp and 100 μl of 1 M potassium citrate-phosphate buffer at pH 6.5 to 8.5 and incubated for 60 min at 37°C. After that, the assay mixture was brought to pH 8.2 with 1 M NaOH or HCl; 10 μl of purified D-AspO was added, and the mixture was incubated for 30 min at 37°C. The α-oxaloacetate generated for the oxidation of D-Asp by D-AspO was quantified as follows: 50 μl of 1 M perchloric acid was added to the sample, mixed, and centrifuged at 13,000 rpm for 5 min. The supernatant (500 μl) was mixed with 50 μl of 2,4-dinitrophenylhydrazine (5 mM in 5 M HCl) and left for 20 min at room temperature. Then, 100 μl of 4 M NaOH was added, mixed, and centrifuged at 13,000 rpm for 5 min. The supernatant was read at 445 nm against the respective blank, consisting of the sample treated with H_2O instead of L-Asp. The absorbance was used to calculate nanomoles of α-oxaloacetate, which also corresponded to nanomoles of D-Asp synthesized. A standard curve was determined by using 500 μl of 0.1 to 1 mM α-oxaloacetate instead of the sample supernatant and proceeding as above. One enzyme unit was defined as the amount of enzyme activity necessary to produce 1 nmol of D-Asp under the assay conditions. Specific activity was expressed as units per milligram of protein. The Michaelis-Menten constant K_m was determined using 0 to 100 mM L-Asp as substrate.

Effect of K^+ on the release of D-Asp from synaptosomes

One milliliter of purified synaptosomes (see above) from *R. norvegicus* or *L. vulgaris* brain was incubated with D-[^{3}H]Asp or with L-[^{3}H]Glu in a physiological buffer (125 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 22 mM glucose, and 10 mM Na_2HCO_3), and adjusted to pH 7.4 with a few drops of 1.0 M citric acid or 1 M NaOH (36–37). Next, the synaptosomes were washed extensively with the same buffer; 50 μl of 1.05 M KCl (final concentration 50 mM) was added, and the sample was incubated for 1 min at 20°C with shaking in the presence or absence of calcium ions (1.2 mM $CaCl_2$). Finally, the sample was centrifuged for 1 min at 5000 *g*, and the labeled amino acids thus released into the medium were determined. A further experiment was carried out to test the effects of K^+ ions on the release of endogenous D-Asp and L-Glu from the synaptosomes in the presence of 1.2 mM $CaCl_2$. For experiments in which labeled amino acids were used, the release of D-Asp and L-Glu was quantified by determining the radioactivity in the medium before and after KCl stimulation. HPLC was used to determine and measure the release of endogenous amino acids.

Binding assay for *R. norvegicus* and *L. vulgaris* PSMs

This was carried out according to the described method (38) and modified as follows: 100 μl of PSMs, obtained as described above (1 mg/ml of protein) and previously equilibrated in the physiological buffer, was mixed with 100 μl of D-[^{3}H]aspartate (100 nM) or 100 μl of L-[^{3}H]glutamate (50 nM) and 300 μl of physiological buffer. Then, 5.0 μl of each amino acid or amino acid analog (0 to 10 mM) was added to the assay mixture (final concentration between 0 to 100 μM), and incubated for 60 min at 20°C with shaking. Subsequently, the sample was centrifuged for 5 min at 14,000 rpm in a 2-ml Eppendorf microcentrifuge (Eppendorf, Hamburg, Germany). The supernatants were decanted, and the tubes were rapidly washed twice in cold distilled water. The pellet was dissolved in 400 μl of 0.1 M NaOH and mixed with 3 ml of scintillation fluid to determine the radioactivity (cpm). Calculation of specific binding was obtained by subtraction of the nonspecific binding component that persisted in the presence of 1 mM of unlabeled D-Asp or L-Glu. Data from binding experiments were analyzed using GraphPad Prism (GraphPad, San Diego, CA, USA), yielding the binding equilibrium, expressed as the dissociation constant K_D, which indicates the concentration of the amino acid displacer that was able to inhibit 50% (IC_{50}) of the binding sites for the ligands D-[^{3}H]aspartate or L-[^{3}H] [^{3}H]glutamate.

Effects of D-Asp and L-Glu on *L. vulgaris* skin chromatophores

L. vulgaris skin chromatophores are pigmented cells of 100–1000 μm diameter, and which belong to different families, each of which contains a variety of pigment types, including red, black, brown, yellow, and blue. Each chromatophore is linked to a set of striated radial muscles (39–40). When the skin of the animal is stressed, either by topical application of or immersion in neurotransmitter, the chromatophores are excited and immediately expand, thereby increasing their specific color. We have employed this unique system to test the effects of D-Asp and L-Glu on squid skin chromatophores. The experiment consisted of the immersion of a piece of fresh skin (2×3 cm) in seawater containing 1 mM of D-Asp or L-Glu (see **Fig. 5*A***). We determined the effects of these amino acids on the expansion of chromatophores of different colors, which are associated with specific receptors for D-Asp or L-Glu.

Determination of D-AspO in the cytosol and in PSMs from *R. norvegicus* and *L. vulgaris* brains

D-AspO (EC 1.4.3.1), an oxidative enzyme with a molecular mass of ~45 kDa, is a flavoprotein that contains 2 flavin adenine dinucleatide (FAD) residues/mol of enzyme and that specifically oxidizes D-Asp, D-Glu, and NMDA (41–42). The enzyme occurs primarily in the liver, kidney, and brain (41–44). In this study, we determined D-AspO activity in the cytosol and in the PSMs of *R. norvegicus* and *L. vulgaris* brain and in cytosol and plasma membranes of liver and kidney tissues. The activity measurement of this enzyme was based on the determination of α-oxaloacetate obtained from the following reaction:

$$\text{D-Asp} + O_2 + H_2O \xrightarrow{\text{D-AspO}} \alpha\text{-oxaloacetate} + H_2O_2 + NH_3$$

The enzymatic assay was as described previously (9), with modifications as described in the following paragraph.

The brain sample (1g) was homogenized with 10 ml of 0.1 M Tris-HCl (pH 8.2) and centrifuged at 30,000 rpm for 30 min. The supernatant fraction, consisting of the cytosol from cerebral tissues, was used for the enzymatic assay. The PSMs were prepared from purified synaptosomes as described above. For the preparation of cytosol and plasma membranes from liver and kidney, 1 g of each tissue was homogenized 1:10 in 0.1 M Tris-HCl and centrifuged as above. The supernatant was used as the cytosol fraction. The precipitate was homogenized in 10 ml of 0.1 M Tris-HCl containing 0.1% Triton X 100 and centrifuged as above; the resulting supernatant was used as the plasma membrane fraction. The enzymatic assay was carried out as follows: each sample (cytosol and PSMs obtained from brain, and cytosol and plasma membrane obtained from liver or kidney) was brought to 2 mg/ml; 500 μl of the fractions was mixed with 50 μl of 1 M sodium D-aspartate and incubated for 60 min at 37°C. The same assay mixture, but in which distilled H_2O replaced D-Asp, was used for the blank (control). After incubation, 50 μl of 5 M perchloric acid was added to the assay mixture, mixed, and centrifuged at 13,000 rpm for 5

min. The α-oxaloacetate that resulted from the oxidation of D-Asp was quantified with hydrazine as described above. Specifically, the supernatants of each sample and the blank were mixed with 50 μl of 2,4-dinitrophenylhydrazine (5 mM in HCl 5 M) and left for 20 min at room temperature. Then, 200 μl of 5 M NaOH was added, mixed, and centrifuged at 13,000 rpm for 5 min. The supernatants of each sample were read at 445 nm against the respective blank, and absorbance was used to calculate the enzymatic activity, expressed as milliunits per milligram protein. One unit was defined as the amount of the enzyme able to oxidize 1 nmol of D-Asp under assay conditions. The K_m was determined by plotting the concentration of the substrate *vs.* the enzymatic activity using D-aspartate as the substrate at a concentration ranging from 0 to 100 mM. Values are expressed as the means ± SD obtained from 5 experiments, each of which was carried out on 5 different preparations.

Antibody production against D-AspO

Beef kidney D-AspO was overexpressed in *Escherichia coli* and purified as described previously (23). The antibody was prepared as follows. A rabbit was immunized by the subcutaneous injection of an emulsion of the recombinant D-AspO (0.5 mg) mixed with 0.2 ml Freund's complete adjuvant. A booster immunization was repeated 3 times at 2-wk intervals using the D-AspO and Freund's incomplete adjuvant to produce a high titer of antiserum. A 10 ml aliquot of the resultant antiserum was applied to a protein A Sepharose column (2.5×0.8 cm, GE Healthcare, Little Chalfont, UK) previously equilibrated with 20 mM sodium phosphate (pH 7.0). After washing the column with the phosphate buffer, immunoglobulins were eluted with 0.1 M glycine buffer (pH 3.0). The eluate was immediately neutralized by the addition of 2 vol of 1 M Tris-HCl buffer (pH 7.5). An aliquot of the eluate (2 ml) was dialyzed against 1 L of 50 mM sodium phosphate (pH 7.5) containing 0.15 NaCl overnight at 4°C, and then applied to a D-AspO column preequilibrated with 20 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl. Anti-D-AspO immunoglobulins were eluted from the column with 1 ml of 0.2 M glycine buffer (pH 2.8). The eluate was dialyzed against 1 L of PBS overnight at 4°C. The dialyzed anti-D-AspO antibody (0.6 mg/ml) was portioned into aliquots and stored at −20°C until use.

Western blot analysis

Aliquots of the total tissue homogenate or purified D-AspO were incubated in the presence of 2% SDS and 5% 2-mercaptoethanol at ~80°C for 3 min, and then separated by SDS-PAGE using a 10% gel. The proteins were transferred to Clear Blot Membrane-P using a semidry blotter (Atto, Tokyo, Japan). The membranes were rinsed twice in 100 ml of PBS containing 0.1% Tween (buffer A) for 5 min, and then incubated with PBS containing 5% (w/v) skim milk and 0.1% Tween 20 for 1 h. The anti-D-AspO antibody (0.6 mg/ml stock) was diluted 500-fold with buffer A containing 1% BSA. The blocked membrane was incubated with the diluted anti-D-AspO antibody for 1 h, washed 3 times with buffer A, incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (1:20,000), diluted with buffer A (Amersham ECL Plus Western Blotting Reagent Pack; GE Healthcare) for 1 h, and finally rinsed 5 times with buffer A. The peroxidase was detected using Amersham ECL Western blotting detection reagent (GE Healthcare). To assess the specificity of the polyclonal anti-D-AspO antibody, 5 μl of 4 mg/ml D-AspO was incubated with 5 μl of D-AspO antibody at 2 mg/ml overnight at 4°C; this sample was used for electrophoresis and immunoblotting experiments (negative control).

Immunoelectronic microscopy of D-AspO at the PSMs

Immunoelectronic microscopy was conducted essentially as described previously (44). In brief, ultrathin sections of *R. norvegicus* and *L. vulgaris* synaptosomes were obtained by trimming appropriate areas from Unicril-embedded tissue. The sections were blocked for 1 h in drops of 4% BSA in 20 mM TBS (pH 7.4). Samples were then incubated for 10 min in 3% H_2O_2 to destroy endogenous peroxidase activity, washed with TBS, and finally incubated overnight with rabbit anti-D-AspO (0.1 mg/ml) protein diluted 1:100 in TBS buffer supplemented with 0.1% BSA. After several washes with TBS, the grids were incubated for 60 min at 4°C with a biotin-labeled secondary antibody at 1: 200 dilution (Vector Laboratories, Burlingame, CA, USA). Next, the slices were washed in TBS for 5 min, incubated with ABC (Vector Laboratories) for 30 min, and washed again in TBS for 5 min. The reaction was developed using diaminobenzydine (DAB)-hydrogen peroxide (prepared by dissolving 100 mg DAB-HCl in 200 ml TBS and 25 μl of 3% hydrogen peroxide). Samples were fixed with 1% glutaraldehyde, and the sample pellet was submitted to routine electron microscopy examination using a Leo 912 AB Omega (Leitz) transmission electron microscope (Leica Microsystems, Wetzlar, Germany).

Determination of cAMP concentrations

The concentration of cAMP in synaptosomes obtained from rat and squid brain was determined as previously reported (45). The experiment consisted of incubating synaptosomes (1 mg/ml) in a physiological buffer with D-Asp (1 mM final concentration) with shaking at 37°C for 30 min and then determining cAMP synthesis concentration.

Statistical analyses

Statistical analyses were performed using Statistica 1998 (StatSoft, Tulsa, OK, USA). Comparison of two groups was done by *t* test. Three or more group comparisons were conducted using ANOVA with Duncan's *post hoc* tests.

RESULTS

D-Asp in the whole brain, in synaptosomes, and in SVs

The first criterion that must be satisfied in order to consider D-Asp a neurotransmitter is that it be present at high levels in SVs. We first purified synaptosomes and SVs from *R. norvegicus* and *L. vulgaris* brains (**Fig. 1**) and then determined the concentration of D-Asp with respect to L-Glu and L-Asp, two well-known neurotransmitters (36–37). Rat and squid synaptosomes (0.8–1.2 μm diameter) are full of SVs (Fig. 1*A*, *B*, *D*, *E*). Figure 1*C*, *F* shows the purified SVs obtained from rat (40–60 nm in size) and squid synaptosomes (40–70 nm in size), respectively.

In rat whole-brain homogenate, the concentration of D-Asp is negligible (55.6±8.4 nmol/g brain) compared to L-Asp and L-Glu (5654±450 and 7610±840 nmol/g, respectively; **Fig. 2*A*** and **Table 1**). In *L. vulgaris,* it is of

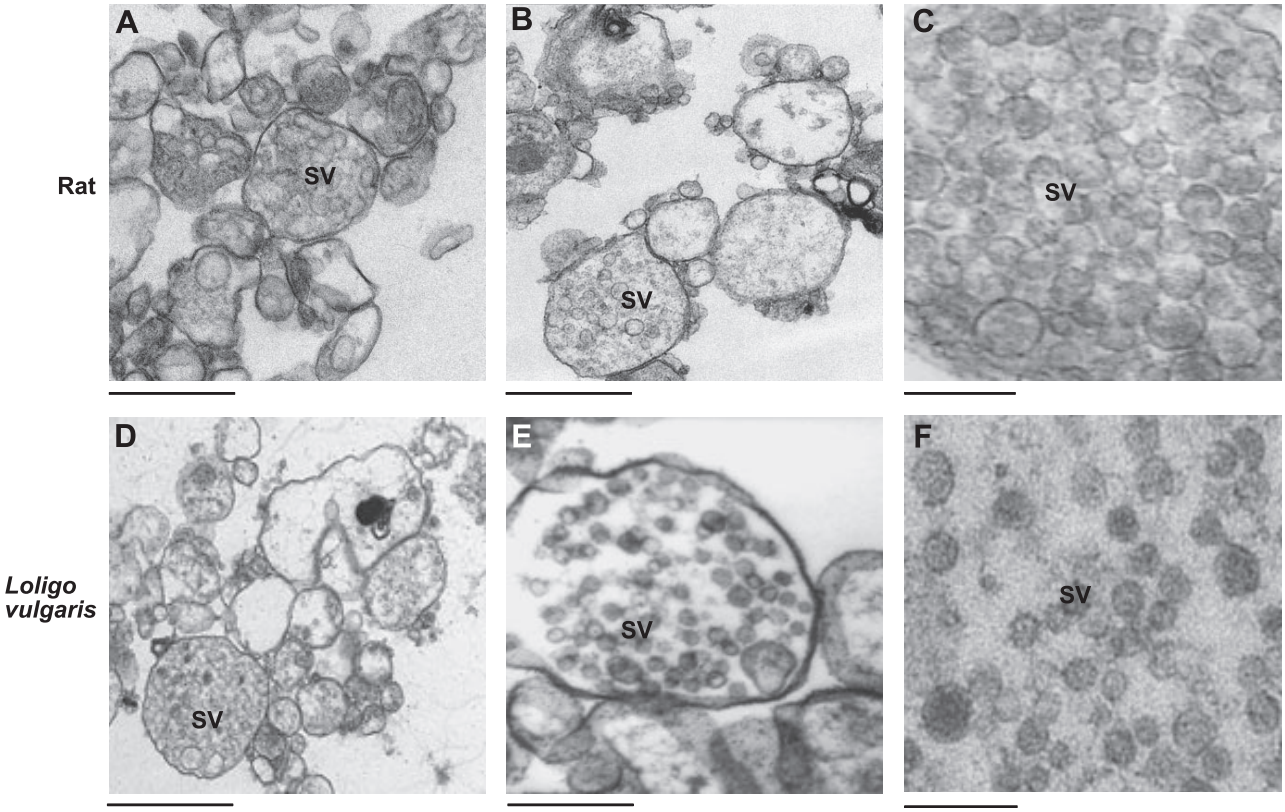

Figure 1. Electron microscopy of synaptosomes and SVs from rat and *L. vulgaris* brains. *A*, *B*, *D*, *E*) Purified synaptosomes (nerve endings) filled with SVs obtained from rat (*A*, *B*) and *L. vulgaris* brains (*D, E*); ×60,000. Synaptosomes from rat brains are 0.4–1.2 μm in size; those from *L. vulgaris*, 0.6–1.6 μm. *C*, *F*) Purified SVs obtained from lysis of rat (*C*) and *L. vulgaris* brain synaptosomes (*F*). SVs from rat brains average 40–60 nm in size; those from *L. vulgaris*, 50–80 nm. Scale bars = 1.0 μm (*A*, *B*, *D*, *E*); 0.1 μm (*C*, *F*).

the same order of magnitude as L-Asp and L-Glu (Fig. 2*D* and Table 1), as has previously been reported (5). Nevertheless, we found that D-Asp was highly concentrated in the synaptosomes of both species, corresponding to 1.9 and 10.3% of total amino acids, respectively (Fig. 2*B*, *E* and Table 1). In rat SVs, D-Asp reaches a concentration of 8.6% compared to total amino acids (Fig. 2*C* and Table 1), whereas in *L. vulgaris*, it reaches 18.4% (Fig. 2*F* and Table 1). This indicates that in both mammals and mollusks, D-Asp is present at high concentrations in SVs, as occurs for other well-known neurotransmitters. Figure 2 illustrates a typical example of D-Asp, L-Asp, and L-Glu determination by HPLC. The chromatograms clearly demonstrate that the peak at elution time 5.6 min, corresponding to D-Asp, disappears on treatment with D-AspO (compare Fig. 2*G* and *H*).

Synthesis of D-Asp: D-aspartate racemase

We also investigated the biochemical origin of D-Asp and whether machinery for its synthesis exists in nerve tissue. Conversion of L-Asp to D-Asp by aspartate racemase is the main source generating neuronal D-Asp (6, 23, 25). Our results demonstrate that D-aspartate racemase occurs in a consistent concentration in the whole-brain homogenate of *R. norvegicus* and *L. vulgaris,* corresponding to 510±70 and 715±80 mU/mg protein, respectively (**Fig. 3*A, B***). However, in synaptosomes and SVs, this enzyme was found at very low concentrations (Fig. 3*A*, *B*). The optimum pH for this enzyme is ~8.0 in both animals (Fig. 3*C*), and the K_m value was found to be 6.8 mM for rat and 7.5 mM for squid, as determined by Michaelis-Menten kinetics (Fig. 3*D*). The fact that neither the synaptosomes nor the SVs possess this enzyme led us to the conclusion that D-Asp is synthesized in the soma of the neuron, and that it is then transported along the axon to the nerve terminals. This particular result is consistent with that reported in *A. californica*, where D-Asp is synthesized in the pedal ganglion and transported along the axon (6).

Effects of K^+ ions on the release of D-Asp from *R. norvegicus* and *L. vulgaris* synaptosomes

Potassium ions play an important role in neurotransmission through their action in depolarization of the postsynaptic neuron's membrane. We investigated the effects of K^+-induced artificial depolarization on the release of D-Asp from synaptosomes using two different experimental approaches. In the first experiment, labeled D-[^{3}H]-Asp and L-[^{3}H]-Glu were allowed to incorporate into synaptosomes, after which the latter were treated with 50

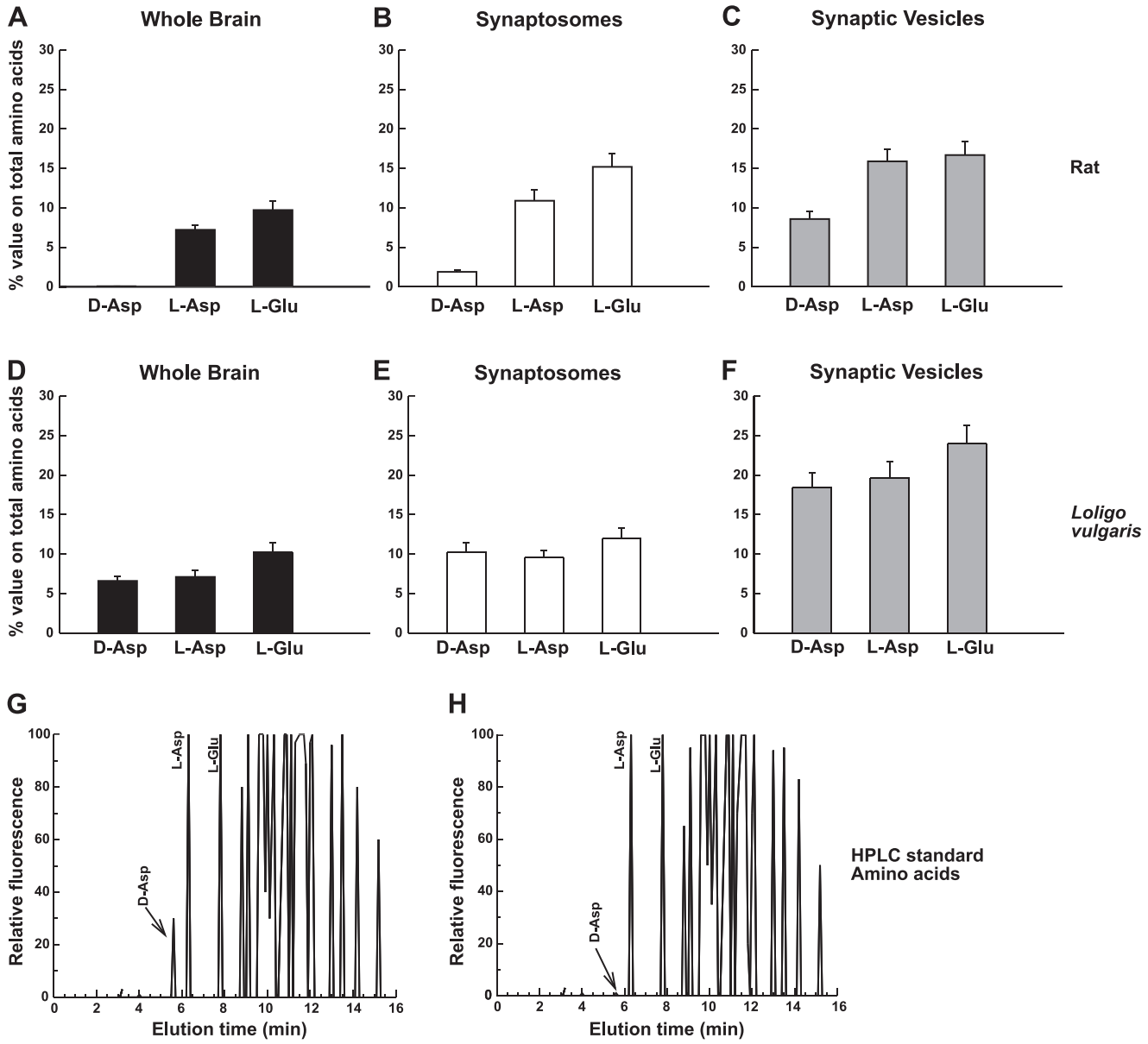

Figure 2. Levels of D-Asp, L-Asp, and L-Glu in whole brain and isolated synaptosomes and SVs from rat and *L. vulgaris*. *A*) Percentage values of D-Asp, L-Asp, and L-Glu relative to total amino acids in the whole rat brain homogenate. *B, C*) Percentage values of the same amino acids in the isolated synaptosomes (*B*) and SVs (*C*) compared to total amino acids. *D*) Percentage values of D-Asp, L-Asp, and L-Glu relative to total amino acids present in *L. vulgaris* whole brain homogenate. *E, F*) Percentage values of the same amino acids in the synaptosomes (*E*) and SVs (*F*). *G*) Typical HPLC analysis of a standard amino acid mixture consisting of 20 pmol D-Asp and 100 pmol of each of L-Asp, L-Glu, L-Ser, L-His, L-Thr, Gly, L-Arg, L-Ala, L-Thy, L-Val, L-Met, L-Ile, L-Leu, L-Phe, and L-Lys. *H*) Same HPLC analysis but after treatment with 5 ng of purified D-AspO (incubation 20 min, 37°C). Peak at elution time 5.6 min, corresponding to D-Asp, is completely absent (arrow).

mM KCl in the presence or absence of 1.2 mM $CaCl_2$. In the second experiment, we measured the endogenous levels of D-Asp and L-Glu in synaptosomes, and released by K^+. Our results show that when previously incubated synaptosomes were stimulated with K^+ in the presence of Ca^{2+}, almost 40–50% of total [^{3}H]D-Asp and [^{3}H]L-Glu incorporated into the synaptosomes was released into the medium within 60 s (**Fig. 4*A***). However, when calcium ions were omitted from the medium, the release of these amino acids was low, representing only ~5–10% of total incorporated [^{3}H]D-Asp and [^{3}H]L-Glu (Fig. 4*B*). These results indicate that D-Asp shows the same property as other neurotransmitters: it is depolarized by KCl in a calcium-dependent manner.

Receptors for D-aspartate

We proceeded to determine whether receptors for D-Asp occur in *R. norvegicus* and *L. vulgaris* brains, and whether these are specific to D-Asp or rather are common to L-glutamate receptors. Binding assays using radiolabeled D-[^{3}H]Asp on rat and squid PSMs demonstrated that the D-[^{3}H]Asp is able to displace ligand

TABLE 1. *Free amino acids in whole-brain homogenate, synaptosomes, and SVs of* R. norvegicus *and* L. vulgaris

Component	D-Asp	L-Asp	L-Glu	Total amino acids
R. norvegicus				
Whole-brain homogenate				
nmol/g brain	55.6 ± 8.4	5654.0 ± 450	7610.0 ± 840	78120 ± 5540
nmol/mg total protein[a]	0.55 ± 0.08	56.1 ± 4.5	75.5 ± 7.2	775 ± 55
% total amino acids[b]	0.07 ± 0.01	7.2 ± 0.6	9.7 ± 1.1	100
Synaptosomes				
nmol/mg total protein[a]	44.6 ± 4.2	255.0 ± 32	355 ± 41	2333 ± 280
% total amino acids[b]	1.9 ± 0.2	10.9 ± 1.4	15.2 ± 1.7	100
SVs				
nmol/mg total protein[a]	274 ± 35	504.0 ± 49	530 ± 55.4	3181 ± 310
% total amino acids[b]	8.6 ± 1.0	15.9 ± 1.5	16.7 ± 1.7	100
L. vulgaris				
Whole-brain homogenate				
nmol/g brain	7819 ± 820	8472 ± 950	12192 ± 1409	119680 ± 10800
nmol/mg total protein[a]	99.7 ± 11.5	105.9 ± 11.9	152.4 ± 17.6	1496 ± 155
% total amino acids[b]	6.6 ± 0.6	7.1 ± 0.8	10.2 ± 1.2	100
Synaptosomes				
nmol/mg total protein[a]	1125 ± 131	1052 ± 106	1312 ± 140	10935 ± 875
% total amino acids[b]	10.3 ± 1.2	9.6 ± 0.9	12.0 ± 1.3	100
SVs				
nmol/mg total protein[a]	2625 ± 266	2796 ± 300	3424 ± 350	14266 ± 13303
% total amino acids[b]	18.4 ± 1.9	19.6 ± 2.1	24.0 ± 2.3	100

Synaptosomes and SVs were isolated from *R. norvegicus* and *L. vulgaris* brains using a sucrose gradient. Values represent means ± SD from 5 different preparations, each starting with 5 g of brain tissue. [a]Sum of soluble (cytosolic) and insoluble (membrane) proteins. [b]Percentage value of each amino acid relative to total amino acids.

binding with high specificity. The concentration of cold D-Asp able to displace 50% (K_i) of D-[^{3}H]Asp was 0.1 μM (**Table 2**), whereas that of cold L-Glu was 2.5±0.4, which corresponds to 25 times reduction in displacement efficiency (Table 2). In contrast, when L-[^{3}H]Glu was used as the radioligand, cold L-Glu was the amino acid that displaced these binding sites at a higher rate, with a K_i of 0.09 (Table 2). In this case, cold D-Asp displaced the binding for L-[^{3}H]Glu at a concentration of 2.5±0.4 μM (27.7-fold concentration; Table 2). The other amino acids used as displacer compounds on D-[^{3}H]Asp and L-[^{3}H]Glu receptors,

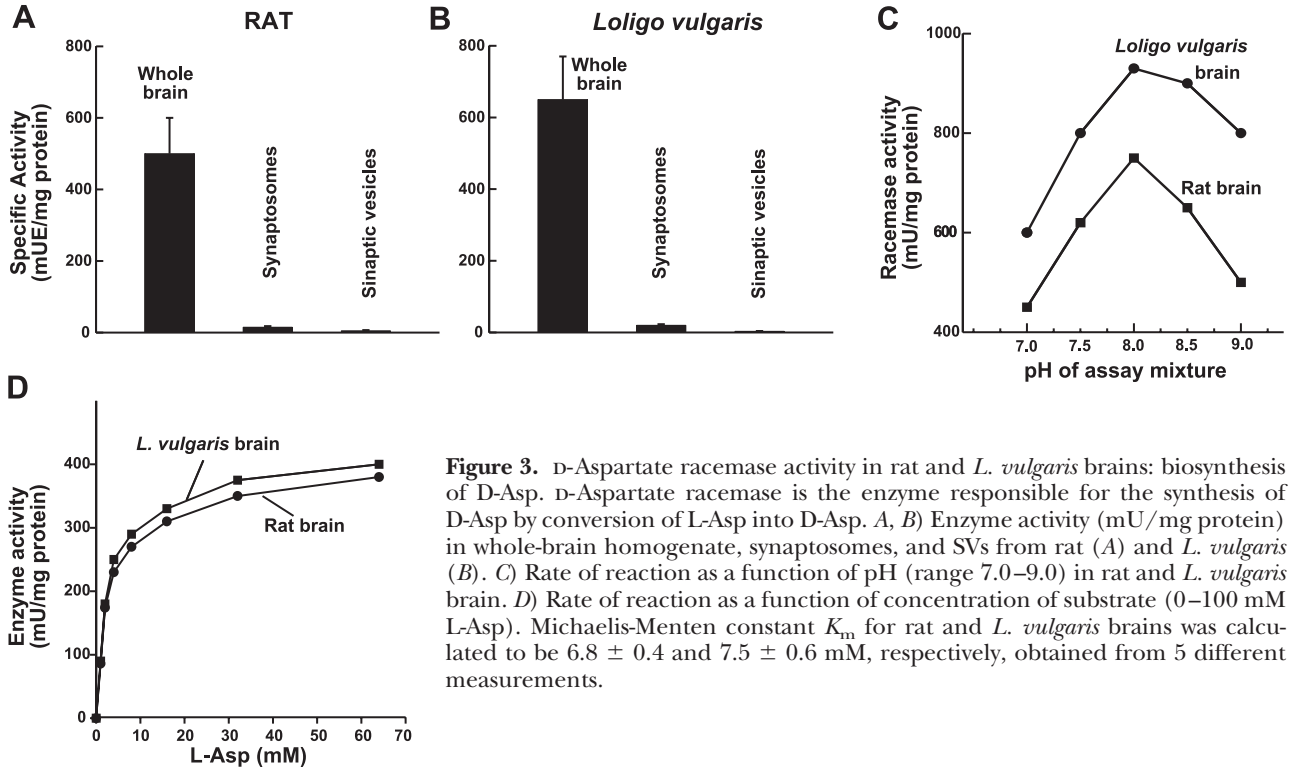

Figure 3. D-Aspartate racemase activity in rat and *L. vulgaris* brains: biosynthesis of D-Asp. D-Aspartate racemase is the enzyme responsible for the synthesis of D-Asp by conversion of L-Asp into D-Asp. *A*, *B*) Enzyme activity (mU/mg protein) in whole-brain homogenate, synaptosomes, and SVs from rat (*A*) and *L. vulgaris* (*B*). *C*) Rate of reaction as a function of pH (range 7.0–9.0) in rat and *L. vulgaris* brain. *D*) Rate of reaction as a function of concentration of substrate (0–100 mM L-Asp). Michaelis-Menten constant K_m for rat and *L. vulgaris* brains was calculated to be 6.8 ± 0.4 and 7.5 ± 0.6 mM, respectively, obtained from 5 different measurements.

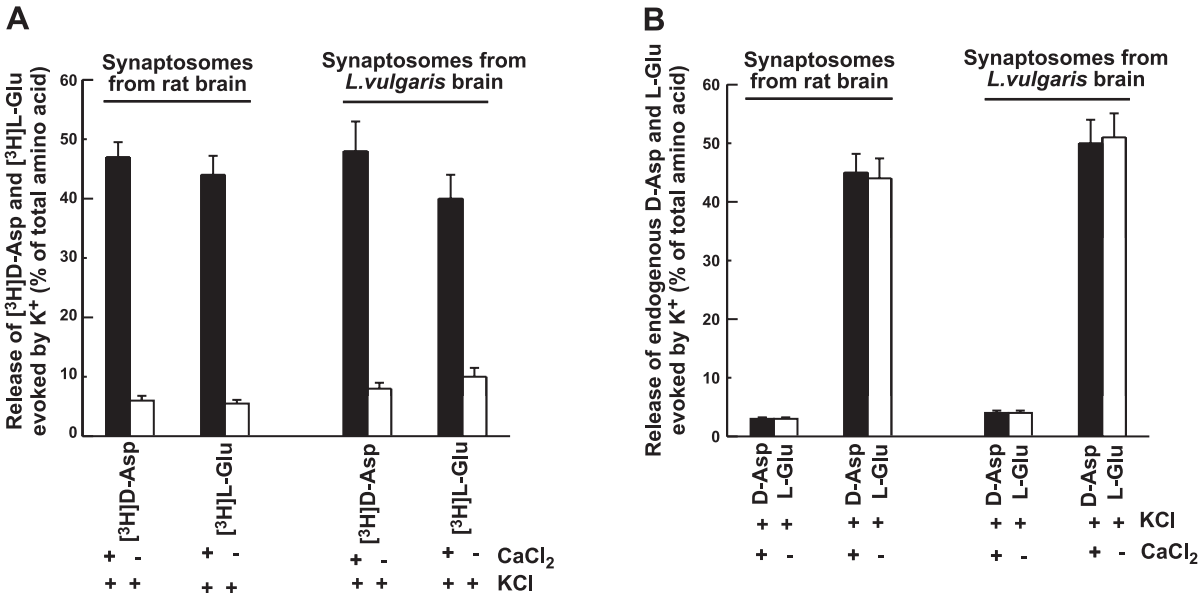

Figure 4. Effects of KCl and $CaCl_2$ on the release of D-Asp and L-Glu from synaptosomes isolated from rat and *L. vulgaris* brains. *A*) Effects of K^+ ions on the release of [^{3}H]D-Asp and [^{3}H]L-Glu from synaptosomes of rat and *L. vulgaris* brains, with or without Ca^{2+} ions. Synaptosomes were preloaded with [^{3}H]D-Asp or [^{3}H]L-Glu and then incubated for 1 min with 50 mM KCl in presence of 1.2 mM $CaCl_2$. Values are expressed as the percentage of [^{3}H]D-Asp and [^{3}H]L-Glu released over the respective total labeled amino acid incorporated in the synaptosomes. Data are means ± SD of 3 experiments. *B*) Effects caused by 50 mM KCl on the release of endogenous D-Asp and L-Glu from the synaptosomes purified from rat or *L. vulgaris* brains in the presence of 1.2 mM $CaCl_2$.

which included L-Asp, D-Glu, D-Asn, D-Gln, D-AP5, NMDA, quisqualate, kainate, and DL-homocisteate, did not show any appreciable binding. These results suggest that rat PSMs possesses a specific type of receptor for D-Asp, different from that which triggers L-Glu (Table 2).

Similar results were obtained on squid PSMs. In fact, when receptor binding assays using D-[^{3}H]Asp as radioligand were carried out on squid PSMs, cold D-Asp was able to displace 50% of the ligands at the lower concentration, with a K_i of 0.2±0.05 μM. In contrast, cold L-Glu was able to displace these ligands at a concentration 17 times higher (K_i 3.4±0.4 μM). In addition, as occurred with rat PSMs, when L-[^{3}H]Glu was used for the binding assay, L-Glu was the amino acid that inhibited 50% of the ligands at the lowest concentration (K_i 3.4±0.4 μM), and not D-Asp (Table 2). Thus, in *L. vulgaris* PSMs, D-Asp acts on specific receptors for D-Asp and not *via* L-glutamate receptors (Table 2).

TABLE 2. *Displacement of specific binding sites for D-[^{3}H]Asp and L-[^{3}H]Glu by various amino acids and amino acid analogues on the PSMs from* R. norvegicus *and* L. vulgaris *brains*

	K_i (μM)			
	R. norvegicus		*L. vulgaris*	
Ligand displacer	D-[^{3}H]Asp	L-[^{3}H]Glu	D-[^{3}H]Asp	L-[^{3}H]Glu
D-Aspartate	0.1 ± 0.01	2.5 ± 0.4	0.2 ± 0.05	3.4 ± 0.4
L-Glutamate	2.5 ± 0.3	0.09 ± 0.02	1.8 ± 0.2	0.1 ± 0.02
L-Aspartate	5.4 ± 0.6	4.4 ± 0.5	3.5 ± 0.25	3.5 ± 0.5
D-Glutamate	34 ± 4.5	28 ± 6.0	34 ± 4.6	22 ± 3.5
D-Asparagine	33 ± 4.5	45 ± 8.5	25 ± 5.5	34 ± 4.6
D-Glutamine	>100	45 ± 8.5	>100	35 ± 8.2
D-AP5	>100	1.1 ± 0.2	>100	28 ± 4.5
NMDA	>100	8.5 ± 2.0	>100	32 ± 6.0
Quisqualate	>100	25 ± 6.0	>100	4.5 ± 1.2
Kainiate	>100	>100	>100	26 ± 4.8
DL-Homocisteate	>100	30 ± 6.0	35 ± 7.5	22 ± 5.4

Displacers for binding sites of D-[^{3}H]Asp (100 nM) and L-[^{3}H]Glu (50 nM) on PSMs were tested in a concentration range of 0 to 100 μM. Inhibition constant K_i is the concentration of the displacer able to inhibit 50% of binding sites of D-[^{3}H]Asp or L-[^{3}H]Glu (IC_{50}). Values are mean ± SD concentrations (μM) obtained from 3 separate experiments. Values >100 indicate that the amino acid tested was completely ineffective in inhibiting the binding site.

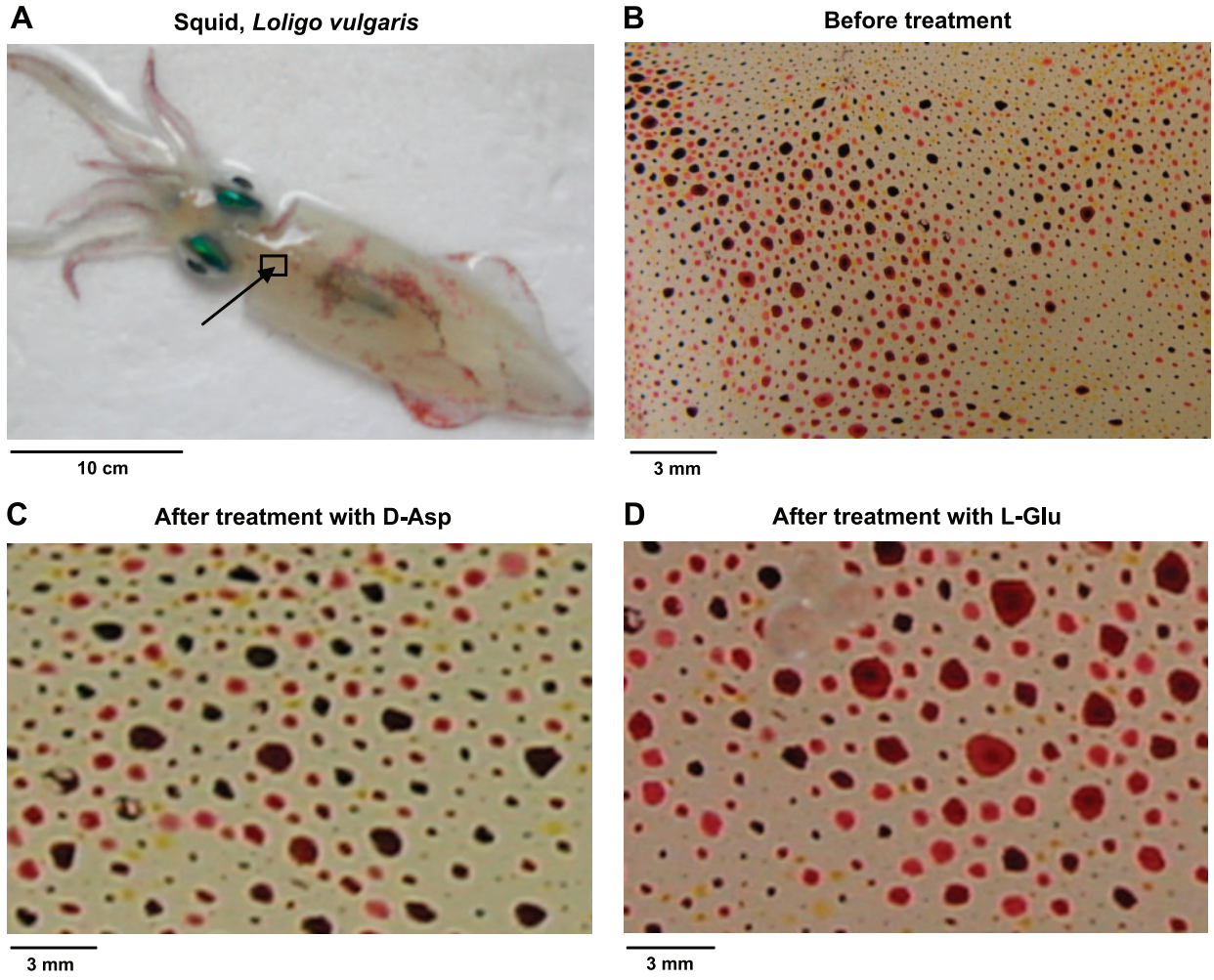

Figure 5. Effects of D-Asp and L-Glu on the expansion of *L. vulgaris* chromatophores. Chromatophores are skin cells (100–1000 μm diameter) controlled by a muscular system with nerves and glia. The chromatophores contain pigments of different colors, each of which represents a distinct family of chromatophores. When the *L. vulgaris* skin is stimulated tactically or by chemical stimulation (*e.g.*, by an amino acid), the chromatophores expand and retract with a strong emission of color, which is amino acid-dependent. *A*) Typical example of an adult squid, *L. vulgaris*. Arrow indicates the dorsal area of the mantle, from which a piece of skin (2×3 cm) was taken and used for amino acid treatment. *B*) Skin sample that has been immersed in seawater alone: chromatophores are quiescent and measure from 0.1–0.3 mm. *C*) Skin has been immersed for 1 min in seawater containing 1 mM sodium D-aspartate. In this case, chromatophores of a dark brown color have expanded to a size of 2–3 mm. *D*) Skin has been immersed for 1 min in 1 mM L-glutamate, causing red chromatophores to expand to a size of 2–3 mm.

To confirm the existence of specific receptors for D-Asp, we employed an innovative approach that consists in observing the behavioral response of *L. vulgaris* skin chromatophores to drug treatments. As previously mentioned, chromatophore cells are linked to striated muscle connected to a set of nerve cells (39–40). Therefore, when a piece of *L. vulgaris* skin is immersed in solution containing D-Asp or L-Glu, the receptors on the nerve cells are activated, causing contraction of radial muscular cells around the chromatophores and expansion of the pigment. Since each chromatophore is stimulated by a specific amino acid, the associated nerve must possess a specific receptor. In this study, we have examined the effects of D-Asp and L-Glu on squid skin chromatophores. When a piece of skin is immersed in seawater containing 1 mM D-Asp, only those chromatophores which contained black ink expanded (**Fig. 5*C***). However, when the skin was immersed in seawater containing 1 mM L-Glu, only those chromatophores containing red ink expanded (Fig. 5*D*). The data thus suggest that the nerve cells connected to the radial muscle are associated with chromatophores that contain specific receptors that are insensitive to L-Glu, as well as specific receptors for L-Glu that are insensitive to D-Asp.

D-AspO activity in the cytosol and in PSMs from *R. norvegicus* and *L. vulgaris* brains

An essential criterion for demonstrating that D-Asp may act as a neurotransmitter is the existence of an enzymatic system to destroy D-Asp at the PSMs, or to transport it to the prenerve terminal. We therefore undertook biochemical quantification and immunomicroscopic localization of D-AspO at the PSMs. Since this

TABLE 3. *D-AspO in the cytosol and in PSMs from* R. norvegicus *and* L. vulgaris *brains*

Component	*R. norvegicus*		*L. vulgaris*	
	Activity (mU/mg protein)	K_m (mM)	Activity (mU/mg protein)	K_m (mM)
Brain				
Cytosol	40.5 ± 5.2	75 ± 10	36.5 ± 4.5	86 ± 15
PSMs	240 ± 32	4.8 ± 0.5	220 ± 25	4.9 ± 0.4
Liver				
Cytosol	315 ± 45	360 ± 70	5.6 ± 0.4	5.2 ± 0.3
Plasma membranes	2.5 ± 0.6	3.1 ± 0.4	>100	>100
Kidney				
Cytosol	370 ± 60	210 ± 30	5.3 ± 0.3	5.5 ± 0.4
Plasma membranes	2.2 ± 0.6	2.9 ± 0.5	>100	>100

Enzyme activity is expressed as milliunits per milligram protein. One unit is defined as the amount of enzyme able to oxidize 1 nmol of D-Asp, generating 1 nmol of α-oxaloacetate under the assay conditions. *K_m was determined by plotting concentration of the substrate vs.* enzymatic activity, using D-aspartate as substrate at a concentration range of 0 to 100 mM. Values are means ± SD obtained from 5 experiments, each carried out on 5 different preparations.

enzyme is the specific protein that oxidizes D-Asp, we expected to find its presence on the membranes of the postnerve ending. For biochemical quantification, we determined the enzymatic activity of D-AspO in the cytosol and PSMs of *R. norvegicus* and *L. vulgaris* brains. For comparative purposes, the same experiment was conducted on liver and kidney cytosol and plasma membrane. The results demonstrate that, in both rat and squid brains, D-AspO occurs at higher concentrations in the PSMs than in the cytosol, indicating that this nervous system enzyme pays the principal role in the oxidation of D-Asp at the PSMs. Specifically, *R. norvegicus* PSMs contain 240±32 mU/mg protein and a K_m value of 4.8±0.5 mM (**Table 3**), whereas in the cytosol, the enzyme is ~6 times less active, with values of 40.5±5.2 mU/mg protein and a K_m of 75±10 mM (Table 3). The same phenomenon occurs in *L. vulgaris* brains, where D-AspO activity is concentrated in the PSMs and much lower in the cytosol (see Table 3 for numeric details). Interestingly, in liver and kidney of both species, the enzyme is mainly contained in the cytosol, and only very low amounts are present in the plasma membranes (Table 3).

In combination, these experiments demonstrate that D-AspO has a dual and well-differentiated function: it works to destroy or eliminate D-Asp after its action has been completed on the PSMs of neurons; in other tissues (*i.e.*, liver and kidney), it is present in a soluble form and possesses metabolic activity in the oxidation of endogenous and exogenous D-Asp for the production of ketoacids necessary for normal cell metabolism. Thus, the presence of D-AspO on the PSMs indicates that this enzyme plays a role in eliminating D-Asp from the postsynaptic neuron after it has triggered the postsynaptic membrane, an action similar to that of acetylcholinesterase at the neuromuscular junction (46).

D-AspO antibody production and confirmation of specificity by Western blot analysis

To localize D-AspO on the synaptosome PSMs, we have developed anti-D-AspO antiserum in rabbit, and purified it by affinity chromatography using purified D-AspO (23) fixed to the column. A Western blot analysis using the purified antibody revealed a single distinct band at a molecular mass of ~45 kDa (**Fig. 6*B***, lane 2). When the antibody was preincubated with the recombinant D-AspO (5 μl of 4 mg/ml D-AspO mixed with 5 μl of D-AspO antibody at 2 mg/ml and incubated overnight at 4°C), a strong reduction of the immunopositive D-AspO signal occurred, indicating the specificity of the reaction.

Immunoelectron microscopy of D-AspO at the PSM

Examination of purified synaptosomes obtained from *R. norvegicus* and *L. vulgaris* brains by immunoelectron

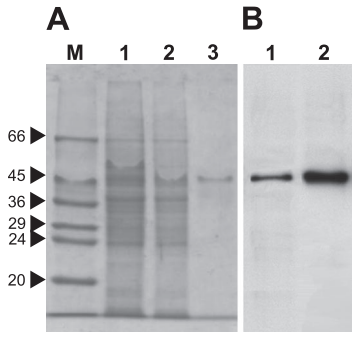

Figure 6. SDS-PAGE and immunoblot analysis to test the specificity of anti-D-AspO antibody for D-AspO in a rat brain homogenate. *A*) Ten percent SDS-gel electrophoresis stained with Coomassie blue. Lane M, molecular weight standard markers; lane 1, rat kidney crude homogenate fraction (30 μg); lane 2, rat kidney homogenate partially purified with ammonium sulfate at 50% saturation and dialyzed (10 μg); lane 3, purified D-AspO prepared from bovine renal cortex (0.6 μg). *B*) Immunoblot experiment. Blot was incubated with the anti-D-AspO antibody, and immunoreactive bands were visualized by the ECL technique. Lane 1, 0.1 μg of purified D-AspO preincubated with purified D-AspO antibody (5 μl of 4 mg/ml D-AspO plus 5 μl of 2 mg/ml D-AspO) and incubated overnight at 4°C; lane 2, 0.1 μg (10 μl of 2 mg/ml) of purified D-AspO from bovine kidney obtained by *E. coli* overexpression.

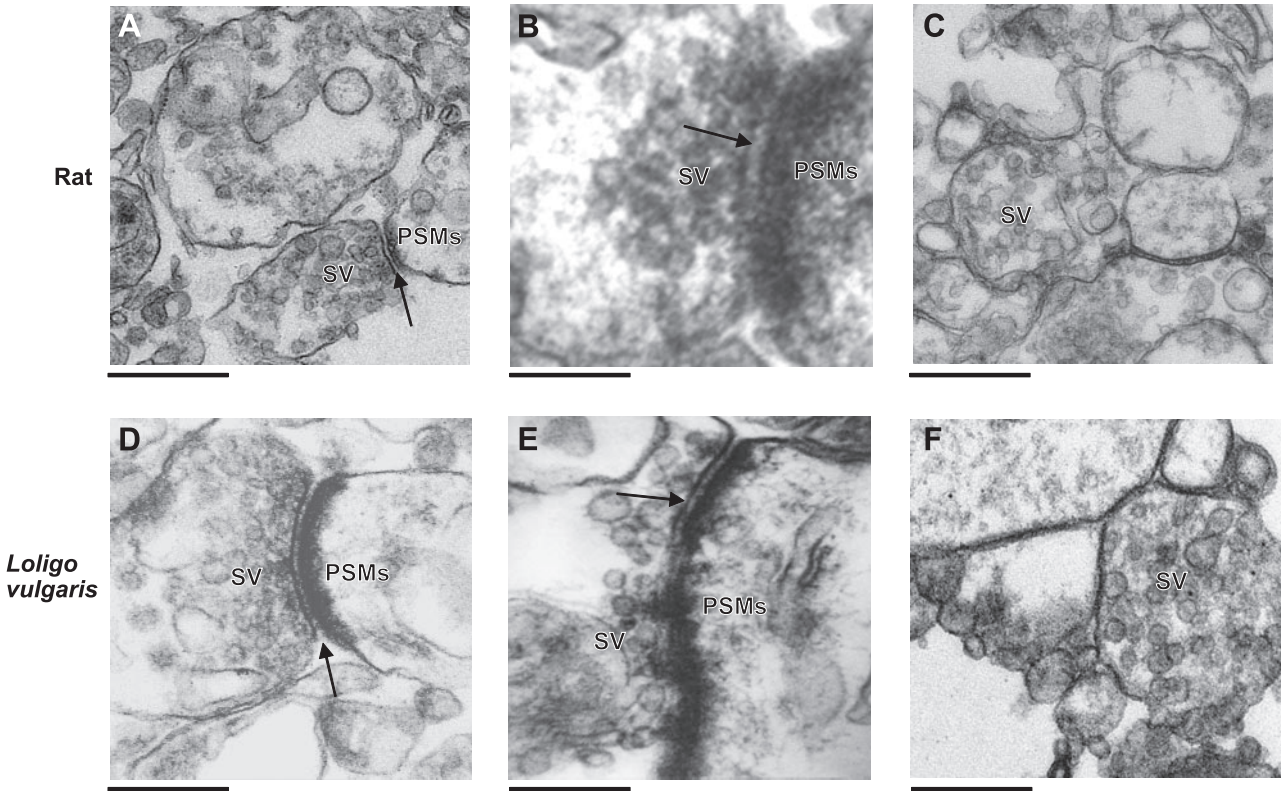

Figure 7. Immunoelectron localization of D-AspO at the PSMs of nerve endings from rat and *L. vulgaris* brains. *A*, *B*, *D*, *E*) Purified synaptosomes (nerve endings) filled with SVs obtained from rat (*A*, *B*) and *L. vulgaris* brains (*D*, *E*), and treated with the anti-D-AspO antibody raised in rabbit (×80,000). Secondary antibody was anti-rabbit conjugated with POD, and revealed using DAB. *C*, *F*) Control synaptosomes from rat (*C*) and *L. vulgaris* (*F*) in which the primary antibody was omitted. Pre- and postsynaptic nerve endings are separated by synaptic clefts of 15–20 nm (arrows). Immune positivity at the PSMs is clearly evident (*A*, *B*, *D*, *F*), indicating the presence of D-AspO at the postsynaptic axons. Scale bars = 0.5 μm (*A*, *C*, *D*, *F*); 0.1 μm (*B*, *E*).

microscopy using the anti-D-AspO antibody shows clear expression of D-AspO at the PSMs (**Fig. 7*A, B*** for rat and *D*, *E* for squid synaptosomes). The immunopositivity for D-AspO at the PSMs is more evident when pre- and postsynaptic membrane nerve endings are observed at a higher resolution (×130,000). Presynaptic synaptosomes obtained from both rat (Fig. 7*B*) and squid (Fig. 7*E*) brains are rich in SVs, and the PSMs are strongly immunopositive to D-AspO. Controls in which the primary antibody was omitted show no signal (Fig. 7*C*, *F*), demonstrating the specificity of the antibody. The pre- and postsynaptic nerve endings are clearly separated by a synaptic cleft of 15–20 nm (Fig. 7*A*, *B*, *D*, *E;* arrows).

D-Asp increases cAMP

To demonstrate that D-Asp induces a signaling event, we tested the effects of D-Asp in stimulating an increase of cAMP on synaptosomes obtained from rat and squid brains. The results demonstrate that the concentration of cAMP in rat synaptosomes treated with D-Asp increased four times compared to the control. A similar, though weaker, effect was observed when squid synaptosomes were used (**Fig. 8**). This change in cAMP levels was significant for both species, with a value of $P < 0.01$. The increase in levels of the second messenger cAMP, which activates specific phosphorylating enzymes and protein kinases, demonstrates that D-Asp is able to trigger the second messenger, thereby inducing physiological signaling from one neuron to another, similarly to other well-known neurotransmitters.

DISCUSSION

In neuroscience, the terms neurotransmitter and neuromodulator are generally adopted indifferently to define a targeted molecule that is implicated in neurotransmission. However, from a restrictive point of view, a neurotransmitter should be conceptualized as a molecule that responds to specific criteria; namely, it should be present in the presynaptic neuron and be released into the synaptic cleft; and be capable of raising the postsynaptic membrane depolarization through a specific receptor situated at the PSMs, or an ionic channel; then, it should be immediately destroyed or recaptured in the presynaptic axon through the glia. This is the case, for example, for the classic neurotransmitters, acetylcholine (46) and L-Glu (47). A neuromodulator, on the other hand, should be conceptualized as a neurotransmitter that is not reabsorbed by the presynaptic neuron or broken down into an inactive metabolite, and that is unable to induce a nerve impulse. Representatives

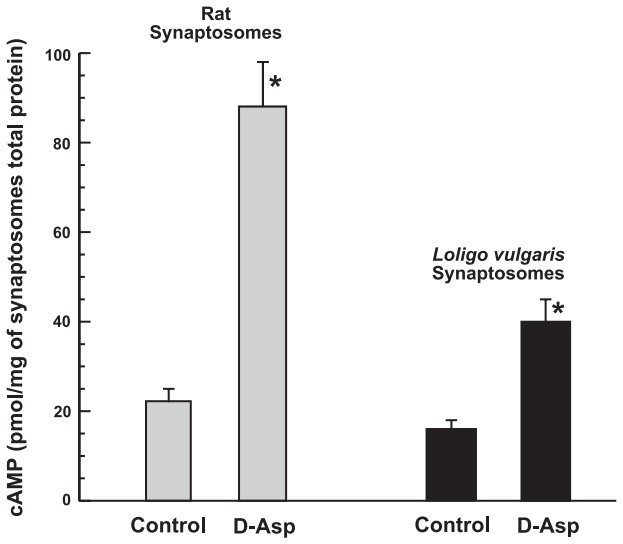

Figure 8. Effects of D-Asp on the synthesis of cAMP on isolated synaptosomes from rat and *L. vulgaris* brains. Concentration of cAMP (pmol/mg total protein) in isolated synaptosomes of rat and *L. vulgaris*, before treatment (control) and after treatment with 1 mM D-Asp (30 min at 37°C). Values represent means ± SD obtained from 5 experiments. Significant 4- and 2.3-fold increases in cAMP levels occurred in rat and *L. vulgaris* synaptosomes, respectively, after treatment with D-Asp. $*P < 0.01$ *vs.* control.

of this category include dopamine, serotonine, histamine, and peptides like enkephaline. Gases like nitric oxide (NO) and carbon monoxide (CO), as well as the amino acids glycine, GABA, and D-Ser, are also neuromodulators.

Several lines of evidence point to the fact that D-Asp may also fulfill the aforementioned functions of a neurotransmitter. In particular, in a previous study conducted on the squid *Aplysia limacina*, we demonstrated that D-Asp was present in SVs and that synthesis of D-Asp by D-aspartate racemase occurred in neurons (48). In another species, *A. californica,* it was additionally shown that D-Asp possesses the activity of a putative cell-cell signaling molecule (6). These data, taken together with the fact that D-Asp is present in the nervous system of various animal species (2), led us to formally test whether this amino acid fulfilled the important criteria necessary to be considered a novel endogenous neurotransmitter. To demonstrate the generality of the phenomenon, we performed experiments in two distantly related animal models, a mammal (the rat *R. norvegicus*) and a mollusk (the European squid *L. vulgaris).*

We first purified synaptosomes and SVs from rat and squid brains (Fig. 1) and determined the concentrations of D-Asp, as well as of L-Asp and L-Glu, two well-known amino acid neurotransmitters used here for comparative purposes. We found that D-Asp was highly concentrated in the SVs of both species (Table 1 and Fig. 2) at concentrations in an order of magnitude with those of L-Asp and L-Glu.

We then addressed the question of whether a biological system to synthesize D-Asp exists in neurons of both species. We demonstrate that in fact neurons possess the enzyme D-aspartate racemase, necessary for the synthesis of D-Asp by conversion from L-Asp, thereby ensuring the continuous presence of D-Asp in the neurons. The synthesis of D-Asp occurs in the soma and is then transported to the nerve periphery, where it is deposited in the SVs.

A third important test was to determine whether D-Asp is released efficiently from synaptosomes following stimulation with potassium ions, as has been demonstrated to occur for other well-known neurotransmitters. We found that when synaptosomes isolated from either rat or squid brains were incubated in 50 mM KCl, almost 50% of the total D-Asp or L-Glu in the synaptosomes was released within 60 s. This phenomenon can be demonstrated both by measuring the labeled amino acids previously incorporated into the synaptosomes, as well as the endogenous D-Asp and L-Glu present physiologically in the synaptosomes (Fig. 4*A*, *B*). These results provide additional and essential support for the hypothesis that D-Asp is an endogenous neurotransmitter.

Given that there exist specific receptors for other well-known neurotransmitters, such as L-Glu (38), we were interested in determining whether receptors for D-Asp are present in the nervous system. Binding studies conducted using D-[^{3}H]Asp on rat and squid PSMs in effect demonstrated the existence of binding sites, and therefore receptors, for D-Asp different from those for L-Glu (Table 2). This new and surprising finding was confirmed by a more innovative *in vivo* methodological approach consisting of observing the effects of D-Asp on the expansion of chromatophores in squid skin. The observation that D-Asp and L-Glu cause the expansion of different families of chromatophores of black and red color, respectively (Fig. 5), elegantly support the binding studies, indicating that D-Asp and L-Glu acts on different and specific classes. However, it is important to point out that it is currently impossible to exclude the possibility that D-Asp acts on a subunit of L-Glu receptors that has yet to be discovered. Nevertheless, it remains noteworthy that D-Asp triggers the postsynaptic neuron and induces a signal transduction event.

We also elucidate a system by which D-Asp can be eliminated from the postsynaptic neuron after its action, critical for defining a neurotransmitter. Both rat and squid PSMs possess the D-AspO that specifically oxidizes D-Asp (Table 3 and Fig. 7). However, one question arises concerning the mechanism by which this elimination occurs. Some researchers have reported that L-glutamate transporters are also able to efficiently transport D-Asp in the presynaptic axon through the glia (49), while other studies indicate that this is not the case (50). It is therefore possible that D-Asp is eliminated from the synaptic cleft either by L-glutamate transporters or *via* destruction by D-AspO. Both systems may work in combination simultaneously, thereby ensuring a more efficient elimination of D-Asp after its action has been completed.

Finally, we also report a significant increase of cAMP

in rat and squid synaptosomes when incubated with D-Asp. Since cAMP is a well-documented second messenger (45), this last result indicates that the neurotransmitter signaling of D-Asp is mediated by cAMP.

Our demonstration that D-Asp is a novel endogenous neurotransmitter in two distantly related species increases the neurotransmitter repertoire for D-amino acids. There is currently no other example in the literature of a D-amino acid implicated in neurotransmission, with the exception of D-Ser. This amino acid was propelled to the forefront by a pioneering study conducted by Snyder and colleagues (51), who demonstrated that D-Ser is a synaptic modulator acting as an endogenous ligand (coagonist) for the glycine site of the NMDA receptor (3). D-Ser has been implicated in schizophrenia since levels of D-Ser are lower in both the serum and cerebrospinal fluid (CSF) of patients with schizophrenia (52). Moreover, increasing NMDA receptors by administration of D-Ser to patients with schizophrenia improves their cognitive symptoms (53).

While the presence of D-Asp in the nervous system and its role as a chemical neurotransmitter are now well defined, it is important to qualify its physiological role in the brain. Multiple studies have indicated that D-Asp is implicated in different neuronal activities, including the physiology of vision (23), neurogenesis (26–28), and learning and memory processes (29). In rats and chickens, high levels of D-Asp occur during the last stages of embryonic life, implying that D-Asp could play an important role in the development of the nervous system (9, 14), as it does in human embryos (16). With respect to its role in learning and memory, we found that in brain tissue and ventricular cerebrospinal fluid of patients with Alzheimer's disease, D-aspartate is present at significantly reduced levels compared to healthy controls (15, 54–56). More recently, we also showed that D-Asp increases long-term potentiation in mice (26), prevents long-term depression, attenuates schizophrenia-like symptoms (27), and rescues hippocampal age-related synaptic plasticity (28). Furthermore, D-Asp increases spatial memory in rats (29), while depletion of aspartate racemase in newborn neurons of the adult mouse hippocampus causes a reduction of D-Asp in the neurons as well as defects in dendritic development, implying that D-aspartate is a modulator of adult neurogenesis (30).

In summary, we have presented extensive data to support our hypothesis that D-Asp is a novel endogenous neurotransmitter in the nervous system. Notably, D-Asp fulfills a restrictive set of criteria necessary and sufficient to place it in the same category as other classic neurotransmitter amino acids like L-Glu. Our results, taken in conjunction with data implicating D-Asp in neuronal development, behavior, and neurological disease prevention, highlight the importance of this amino acid in future biomedical and neuroscience research. FJ

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