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IMMUNOCYTOCHEMICAL VISUALIZATION OF D-GLUTAMATE IN THE RAT BRAIN

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Abstract—Using highly specific antisera directed against conjugated D-amino acids, the distribution of D-glutamate-, D-tryptophan-, D-cysteine-, D-tyrosine- and D-methionine-immunoreactive structures in the rat brain was studied. Cell bodies containing D-glutamate, but not D-glutamate-immunoreactive fibers, were found. Perikarya containing this D-amino acid were only found in the mesencephalon and thalamus of the rat CNS. Thus, the highest density of cell bodies containing D-glutamate was observed in the dorsal raphe nucleus, the ventral part of the mesencephalic central gray, the superior colliculus, above the posterior commissure, and in the subparafascicular thalamic nucleus. A moderate density of immunoreactive cell bodies was observed in the dorsal part of the mesencephalic central gray, above the rostral linear nucleus of the raphe, the nucleus of Darkschewitsch, and in the medial habenular nucleus, whereas a low density was found below the medial forebrain bundle and in the posterior thalamic nuclear group. Moreover, no immunoreactive fibers or cell bodies were visualized containing D-tryptophan, D-cysteine, D-tyrosine or D-methionine in the rat brain. The distribution of D-glutamate-immunoreactive cell bodies in the rat brain suggests that this D-amino acid could be involved in several physiological mechanisms. This work reports the first visualization and the morphological characteristics of conjugated D-glutamate-immunoreactive cell bodies in the rat CNS using an indirect immunoperoxidase technique. Our results suggest that the immunoreactive neurons observed have an uptake mechanism for D-glutamate. **© 2006 IBRO. Published by Elsevier Ltd. All rights reserved.**

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Several years ago, it was assumed that in eukaryotes only the L-series of amino acids could be found. However, D-amino acids have been located in mammalian tissues using high-performance liquid chromatography, gas chromatography, and immunocytochemical techniques (Man et al., 1983; Dunlop et al., 1986; Hashimoto et al., 1992a,b, 1993; Nagata et al., 1994; Kera et al., 1995). In this sense, the presence of D-aspartic acid and D-serine in the human and rat brains has been reported (Man et al., 1983; Dunlop et al., 1986; Hashimoto et al., 1992a,b, 1993; Nagata et al., 1994; Kera et al., 1995; Yasuda et al., 2001), but to our knowledge no information is available in the literature about the presence and localization of other D-amino acids in the mammalian CNS such as D-tryptophan, D-cysteine, D-tyrosine or D-methionine.

In addition, only partial data can be found about the distribution of D-glutamate in the rat CNS, since free D-glutamate has been assayed using high-performance liquid chromatography in the rat liver, kidney, and brain (Kera et al., 1995). No previous information appears to be available in the literature concerning the presence of fibers and cell bodies containing D-glutamate in the mammalian brain. Accordingly, it would appear to be very important to study the distribution of immunoreactive fibers and cell bodies containing D-amino acids, in general, in the mammalian CNS. The distribution in the mammalian brain of D-glutamate-immunoreactive structures seems to be an important focus of study, considering that in the mammalian CNS L-glutamate plays crucial roles (e.g. it is the most widespread excitatory amino acid neurotransmitter in the mammalian CNS; Storm-Mathisen et al., 1983; Ottersen and Storm-Mathisen, 1984, 1985; Hepler et al., 1988; Chagnaud et al., 1989).

In light of the above and in order to study the localization of several D-amino acids in the rat brain, the first aim of our study was to obtain specific antisera directed against D-glutamate, D-tryptophan, D-cysteine, D-tyrosine and D-methionine. Using immunocytochemical techniques, the second issue addressed was the visualization of fibers and cell bodies containing these D-amino acids in the rat CNS. The present report first describes the presence of D-glutamate-immunoreactive cell bodies in the mammalian brain.

EXPERIMENTAL PROCEDURES

Animals

Ten adult Sprague–Dawley male rats (weight 300–500 g) obtained from commercial sources (Charles River, Châtillon-sur-Chalarone, France; and CERJ Janvier, Le Gemest/Isle, France) were used to study the distribution of immunoreactive structures containing D-glutamate. The animals were kept under standardized light and temperature conditions and had free access to food and water. The animals remained for at least 10 days in their cages before the

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunoassay; G, glutaraldehyde; PB, phosphate-buffer; PBS, phosphate-buffered saline; RT, room temperature.

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experiments. The experimental design, protocols, and procedures of this work were performed under the guidelines of the ethics and legal recommendations of Spanish and European legislation. This work was also approved by the research commission of the University of Salamanca (Spain). Moreover, during all the experimental procedures, every effort was made to minimize the number of animals used and their suffering.

Tissue preparation

The animals were deeply anesthetized with urethane (1–1.5 g/kg, i.p.), heparinized, and perfused via the ascending aorta, using a peristaltic pump, with 50–100 ml of cold physiologic saline (0.9% NaCl) and with 900 ml of cold 4% paraformaldehyde and 2% glutaraldehyde (G) in 0.1 M phosphate-buffer (PB), pH 7.2 (flow rate of the fixative: 150 ml/min for 2 min and 60 ml/min for 10 min).

The brains were then dissected out and placed in 4% paraformaldehyde in 0.1 M PB at 4 °C for 12–16 h and were finally immersed in increasing concentrations of sucrose (5–30%) until they sank. Fifty-micrometer-thick serial brain sections were obtained using a freezing microtome and kept at 4 °C in phosphate-buffered saline (PBS) (0.1 M, pH 7.2) until they were processed for immunostaining. In general, six out of seven sections were used for immunocytochemistry, and the remaining section was stained for Nissl substance with Cresyl Violet.

Immunocytochemistry

Two different protocols for immunocytochemistry were followed. In the first, sections were reduced with a solution of 10^{-2} M sodium borohydride (Chagnaud et al., 1989) and rinsed with PBS (two times), whereas in the second protocol the reduction procedure was not carried out.

In order to avoid possible interference with endogenous peroxidase, free-floating, reduced and unreduced sections were treated with a mixed solution containing methanol and H_2O_2 (2/1) for 30 min. Then, the sections were washed for 30 min (at room temperature, RT) in 0.15 M PBS (pH 7.2) and pre-incubated for 30 min at RT in PBS containing 10% of normal horse serum and 0.3% of Triton X-100. The sections were then incubated overnight at 4 °C in the same buffer containing the antisera to conjugated D-amino acid diluted at 1/20,000 for D-glutamate and at 1/10,000 for D-tryptophan, D-cysteine, D-tyrosine and D-methionine. The sections were then washed in PBS (30 min) (RT) and incubated for 60 min at RT, with biotinylated anti-rabbit immunogammaglobulin (Vector, Burlingame, CA, USA) diluted 1/200 in PBS. After a 30 min wash with PBS at RT, the sections were incubated for 60 min at RT with 1/100 diluted avidin–biotin–peroxidase complex (Vector). Finally, after washing the sections in PBS for 30 min at RT and Tris–HCl buffer, pH 7.6, for 10 min, at RT, the tissue-bound peroxidase was developed with H_2O_2, using 3,3′-diaminobenzidine as chromogen. The sections were rinsed with PBS and coverslipped with PBS/glycerol (1/1).

Preparation of G complexes

Ten milligrams of each small molecule was dissolved in 1 ml of 1.5 M acetate buffer (pH 8.0). Indolyl and tyrosyl amino acids were previously diluted in 200 μl dimethylsulfoxide. A second solution was prepared containing 10 mg of bovine serum albumin (BSA) dissolved in 1 ml of acetate buffer. To conjugate the small molecule with the BSA, we added 100 μl of a 2.5 M G–water solution to the first solution (containing the small molecule dissolved); at this stage, the first and the second solutions were mixed at RT. Thus, the coupling reaction was finished when the solution became yellow and confirmed by a stable pH. In order to saturate the double bonds, 200 μl of a water solution containing 10 mM sodium borohydride was added. When the mixture became translucent, the saturation reaction was over. This final solution was dialyzed against distilled water for 24 h at 4 °C.

Antisera

Polyclonal primary antisera were obtained from commercial sources (Gemacbio SA, Cenon, France) and were raised in rabbits with their respective immunogens (D-amino acid–reduced-G–BSA). The animals were immunized with one injection every two or three weeks over two months. Each s.c. administration was a mixture of 250 μg of a fresh immunogenic conjugate in NaCl solution and 500 μl of complete or incomplete Freund adjuvant (Sigma, Saint Quentin Fallavier, France) depending on whether it was the first immunization or consecutive immunizations. This mixture was injected at both sides of the rachis, the antibody titers being elevated after four immunizations.

Antisera (1 ml) were preabsorbed with 10 mg of reduced and lyophilized BSA–G. Preabsorption was accomplished by contact, mixing the antisera with BSA–G (previously reduced and lyophilized), the antisera remaining in a three-dimensional plate for two hours at 37 °C. After centrifugation, the prepurified antisera were purified depending on the request of the researchers and their application [$(NH_4)_2SO_4$ precipitation, PBS dialysis and, when necessary, high trap columns]. Antisera were previously absorbed with the carrier protein used to induce the immunogenic response (BSA–G reduced) in order to neutralize possible spurious signals in late applications of these antisera such as those carried out here in the enzyme-linked immunoassay (ELISA) and immunocytochemical techniques.

ELISA

For a good characterization of antibodies present in antisera it is very important to have titration values prior to performing competition experiments. Thus, the best choice was to have an optical density of 1 at 492 nm after 10 minutes of dark development with the different dilutions of antisera for a known concentration of the antigen. This means that after 10 minutes, in the case of D-glutamate for example, one obtained different optical densities (dilution 1/5000, optical density measured 3; dilution 1/10,000, optical density measured 2; dilution 1/20,000, optical density measured 1; dilution 1/40,000, optical density measured 0.5) for different concentrations of antibody. For application of these values, it was estimated that the best concentration of antibodies was the one that shows an optical density of 1. This value was very important because the IC_{50} is considered to be the point at which there is enough competitor to reach an optical density of 0.5. Thus, the concentration of the antigen present in one aliquot, which makes that optical density

Table 1. Affinity and specificity of antibodies directed against conjugated D-glutamate

Compound	Cross-reactivity at half-displacement (IC_{50})
D-Glutamate–G–BSA	1
L-Glutamate–G–BSA	1/50,000
D-Glutamine–G–BSA	1/50,000
L-Glutamine–G–BSA	1/50,000
L-Aspartate–G–BSA	1/50,000
D-Aspartate–G–BSA	1/50,000
D-Cysteine–G–BSA	1/50,000
Taurine–G–BSA	1/50,000
D-Methionine–G–BSA	1/50,000
GABA–G–BSA	1/50,000
D-Tyrosine–G–BSA	1/50,000
D-Tryptophan–G–BSA	1/50,000

Using competition ELISA assays, cross-reactivity was calculated from the displacement curves at half-displacement: the best recognized conjugate was D-glutamate–G–BSA, whose concentration was divided by the concentration of each of the other conjugates.

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