NO-tryptophan: a new small molecule located in the rat brain

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Abstract

A highly specific monoclonal antibody directed against nitric oxide-tryptophan (NO-W) with good affinity (10^{-9} M) and specificity was developed. In the rat brain, using an indirect immunoperoxidase technique, cell bodies containing NO-W were exclusively found in the intermediate and dorsal parts of the lateral septal nucleus. No immunoreactive fibres were found in the rat brain. This work reports the first visualization and the morphological characteristics of cell bodies containing NO-W in the mammalian brain. The restricted distribution of NO-W in the rat brain suggests that this molecule could be involved in specific physiological mechanisms.

Introduction

Nitric oxide (NO) plays an important role in inflammatory and neurodegenerative diseases[1-3] and, in this sense, the possible involvement of this molecule in the pathogenesis of multiple sclerosis has been suggested.[4-6] It is known that NO modifies amino acids (*e.g.*, tryptophan, cysteine, histidine) originating NO-amino acid species which generate an immune response.[7,8] Thus, after an immunoscreening, the presence of NO-tryptophan (NO-W) by ELISA techniques has been reported in the sera of patients suffering from multiple sclerosis and in experimental animal models (*e.g.*, experimental allergic encephalomyelitis) by the identification of circulating antibodies directed against NO-W.[8,9] The detection of these antibodies show indirectly the presence of NO-W.[8,9] However, to date, no study has been carried out regarding the distribution of fibres and cell bodies containing NO-W in the mammalian central nervous system. The role of this NO molecule remains to be studied in-depth and hence it is important to know its neuroanatomical distribution. In light of the above, the first aim of this study was to raise a highly specific monoclonal antibody directed against NO-W and the second to know the distribution of immunoreactive structures containing NO-W in the rat brain by using an immunohistochemical method.

Materials and Methods

The experimental design, protocols, and procedures of this work were performed under the guidelines of the ethics and legal recommendations of Spanish, French and European law. This work was also approved by the experimental research commission of the University of Salamanca (Spain).

A primary antiserum was developed in BALB/c mice (Janvier Labs, Le Genest-Saint-Isle, France) after immunization with NO-W-bovine serum albumin (BSA) immunogen linked *via* glutaraldehyde (G) [NO-W-G-BSA (NO-W conjugate)]. G complexes were prepared as previously described;[10] thus, 10 mL of W were dissolved in 1 mL of 1.5 M acetate buffer (pH 8.0) and a second solution was prepared containing 20 mg of BSA dissolved in 1 mL of the same buffer. In order to conjugate W with BSA, 100 µL of a 2.5 M G-water solution were added to the solution containing the W dissolved; at this step, the first and the second solutions were mixed at room temperature, reduced and dialyzed as previously described.[10] The final dialyzed solution was nitrosylated by acidifying the antigen solution with 2 N HCl (75 µL per mg of a 1 mg/mL W-G-BSA solution), mixed with 100 µL of an aqueous sodium nitrite solution (100 µL per mg of W-G-BSA) and incubated for 2 h shaking at 37°C. At the end, the nitrosylated conjugate was purified in phosphate buffered saline (PBS) using dialyze membranes with cut-off limits between 12 and 16 KDa. The purification was carried out in a 1 litre bucket at 4°C for 2 h and the nitrosylation was controlled by ultraviolet spectroscopy, scanning the conjugate solution between 250 to 500 nm. The characteristic peak at 336 nm confirmed the correct nitrosylation of the W-G-BSA conjugate. Moreover, an indirect ELISA test, using a rabbit polyclonal antibody directed against NO-W-G-BSA (AP071; Gemacbio, Saint-Jean-d'Illac, France), was carried out and confirmed that the antigen was specifically recognized by this polyclonal antibody.

After the synthesis of NO-W-G-BSA, mice were immediately immunized by one injection every 2 or 3 weeks with the immunogen (containing NO-W-G-BSA). Each immunization (subcutaneous injection) was carried out by administering 50 µL of an immunogenic NaCl solution and 50 µL of complete (only used in the first immunization) or incomplete Freund adjuvant (Sigma Aldrich, St. Louis, MO, USA). As previously described,[10,11] after the second immunization serum samples were collected and the antisera were pre-purified by immunoabsorption and tested by ELISA. Once a highly specific polyclonal antibody was obtained, the fusion of SP2/O/Ag myeloma cells and mice splenocytes was carried out. Then, the screening and the selection of specific clones were conducted. The screening was carried out in order to select the best well-plate containing the cell that produces a specific monoclonal antibody. Once the highly specific monoclonal antibody directed against the NO-W conjugate was obtained, cells were expanded in plastic flasks. Supernatant was recuperated every week, centrifuged and pre-purified with a saturated $(NH_4)_2SO_4$ solution, dialyzed in PBS and finally purified in an HiTrap protein G HP column (17-0404-01, GE Healthcare. Little Chalfont, UK). Later, an Isotyping kit (26179, ThermoScientific, Waltham, MA, USA) was used to determinate the type of immunoglobulin and chain. Thus, the antibody used here was characterized as an isotype Ig G_{2b} with a kappa chain.

Five adult male Wistar rats (weight 300-350 g) obtained from commercial sources

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(Charles River, Écully, France) were used in this study. Animals were kept under standardized conditions, anaesthetized, heparinized and perfused *via* the ascending aorta with cold physiologic saline and with a solution containing 4% paraformaldehyde and 2% of glutaraldehyde.[10,12] As previously reported,[10,12] brains were dissected out, post-fixed in 4% paraformaldehyde for 12-16 h and cryoprotected for histological studies. Using a freezing microtome, 40-50 µ-thick brain sections were obtained, kept in PBS solution (0.1 M, pH 7.2) and processed free-floating for immunohistochemistry.[10,12] Thus, in order to avoid possible interference by endogenous peroxidase, sections were treated with methanol and H_2O_2 (2:1) for 30 min, washed in PBS and pre-incubated in PBS containing 0.3% of Triton X-100 and 1% of normal horse serum (mix solution) for 30 min. Sections were incubated overnight at 4°C in the mix solution containing the monoclonal anti-NO-W antiserum (diluted 1/1,000), washed in PBS µincubated with biotinylated anti-mouse immunogammaglobulin (BA-9200, Vector Labs, Burlingame, CA, USA), diluted 1/200 in the mix solution (60 min). After a rinse in PBS, sections were incubated with the avidin-biotin-peroxidase complex (Vectastain PK-6100, Vector, USA) (1/100) for 60 min. Sections were washed in PBS (30 min) and in Tris-HCl buffer (10 min) and then the tissue-bound peroxidase was developed with H_2O_2, using 3, 3' diaminobenzidine as chromogen. Finally, the sections were washed in PBS and coverslipped with PBS/Glycerol (1:3). Histological controls were carried out to confirm the specificity of the immunoreactivity: 1) omission of the primary and/or secondary antisera; and 2) pre-absorption of the anti-NO-W antibody with an excess (100 g/mL) of NO-W. No residual immunoreactivity was found in either case. The stereotaxic atlas of Paxinos and Watson[13] was used for mapping. The size and density of cell bodies were considered as previously described.[14] The density was considered high (more than 20 perikarya/nucleus/section were visualized), moderate (10-20 perikarya/nucleus/section) or low (less than 10 perikarya/nucleus/section). Cell bodies were small (showing a diameter below 15 µm), medium-sized (diameter between 15-25 µm) or large (diameter above 25 µm). Photomicrographs were obtained with an Olympus DP50 digital camera attached to a Kyowa Unilux-12 microscope. To improve the visualization of results, only the brightness and contrast of the images were adjusted, without any further manipulation of the photographs. Adobe Photoshop CS software was used to view the images and modify their brightness and contrast.

Results

Using ELISA tests, the monoclonal antibody obtained was fully characterized (Table 1). In these assays, the parameters studied were titration, avidity, and specificity *versus* other structural analogues (Table 1; Figure 1) according to protocols previously described.[10,12] ELISA tests were carried out with the dilution of 1/75,000, corresponding to an optical density of 1, at 492 nm.[10,12,15] Accordingly, competition experiments were performed with different competitors (Table 1). All competition experiments (dilution: 1/75,000) were carried out with the same amount of antibody but using different concentrations of the conjugated antigen. The monoclonal antibody used here recognized the specific target at very low concentrations and this means that its avidity is very high for the target.[10-12] In this assay, we tested the available closest analogues of NO-W and, in general, we observed a very low cross-reactivity between the target molecule and the closest compounds (Table 1).

After the application of the immunohistochemical technique, no immunoreactive fibres containing NO-W were detected in the rat brain, whereas cell bodies containing this small molecule were only found in the intermediate and dorsal parts of the lateral septal nucleus (Figure 2). According to the stereotaxic atlas of Paxinos and Watson,[13] these parts extend from Bregma 1.7 mm to Bregma -0.8

Table 1. Affinity and specificity of antibodies directed against conjugated NO-tryptophan.

Compound	Cross reactivityat half-displacement (IC50)
NO-Tryptophan-Gc	1
L-Tryptophan-Gc	1/100
NO-Tyrosine-Gc	1/50
NO-Cysteine-Gc	1/50,000
NO-Histidine-Gc	1/50,000

Using competition ELISA assays, cross-reactivity was calculated from the displacement curves at half-displacement (see Figure 1): the best recognized conjugate was NO-Tryptophan-Gc, whose concentration was divided by the concentration of each of the other conjugates. Gc, glutaraldehyde protein carrier.

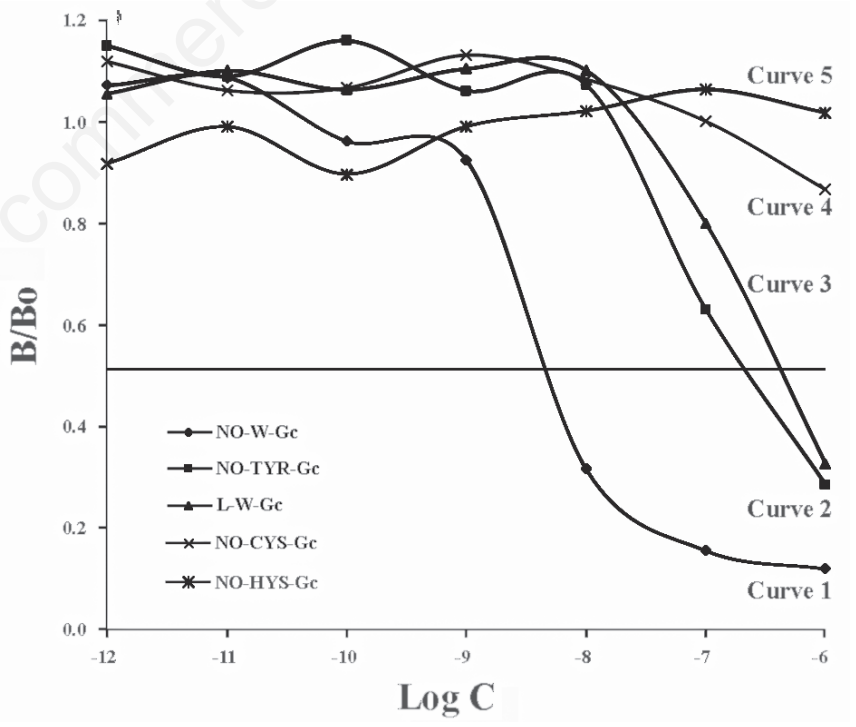

Figure 1. Antibody affinity and specificity resulting of competition experiments in ELISA test. B, optical density with competitor; B0, optical density without competitor. Curve 1, competition with NO-W-Gc; curve 2, competition with NO-Tyrosine (TYR)-Gc; curve 3, competition with L. W-Gc; curve 4, competition with NO-Cysteine (CYS)-Gc; curve 5, competition with NO-Histidine (HYS)-Gc; Gc, conjugated *via* glutaraldehyde to the protein carrier; Log C, logarithm of concentration.

mm. A high density of NO-W-immunoreactive perikarya was observed by the whole rostro-caudal extension of the intermediate part of the lateral septal nucleus (Figure 2 C-F) and a low density by the whole rostro-caudal extension of the dorsal part of the lateral septal nucleus (Figure 2C). No immunoreactive cell body was observed in the ventral part of the lateral septum. In general, immunoreactive cell bodies were round or piriform and small in size. One-two dendrites were also observed (Figure 2 C-F). The immunoreactivity was not observed when the anti-NO-W antibody was pre-absorbed with NO-W (Figure 2B).

Discussion

The ELISA tests, applied here to check the NO-W antiserum, showed that the monoclonal antibody used here had a rather high affinity (the estimated IC_{50} was 10^{-9} M for NO-W) and fairly good specificity. Moreover, the histological controls carried out in this study confirmed that the immunoreactivity observed was specifically due to NO-W. This is in agreement with the restricted distribution of the immunoreactivity observed (exclusively found in one nucleus of the rat brain). Moreover, this distribution and the degree of the immunoreactivity were similar in all the animals studied.

Regarding the development of antibodies directed against NO-W, it is important to remark that before the fusion of the splenocyte with the myeloma cell, a polyclonal antibody (directed against NO-W) showing a very high affinity and specificity was developed. Moreover, the purification of the antibody is a crucial step when developing new monoclonal antibodies. Here, the monoclonal antibody used was pre-purified and purified using protein G HP columns. After using an Isotype kit, the finally characterized antibody was determined: isotype Ig G_{2b} with a kappa chain. Then, after the fusion, a screening was conducted in order to select the best cell producing a specific monoclonal antibody. This highly specific antibody was characterized by competition experiments and allowed to discriminate NO-W from the closest molecules. We found that NO-W was, at least 100 times, better recognized than the conjugated W. This highly specific target discrimination is similar to that previously shown for antibodies discriminating L and D isomers of the same aminoacid,[10] since the only difference between NO-W and W is due to the NO group. Moreover, it is important to note that during the nitrosylation of W there are two possible nitrosylation positions: N and C_6. N position is preferentially nitrosylated when comparing with C_6 position. This means that the NO-W recognized by the monoclonal antibody used here was placed in the position N of the NO-W. NO is a very sensible species which is very quickly oxidized to NO_2; this must be taken into account during the different phases of the production of antibodies (polyclonal and monoclonal) and when testing samples and conducting the screening. Thus, as reported above, in each step of the process a new antigen was produced and immediately controlled. In this sense, the pre-absorption of the monoclonal antibody used in this work was carried out using a new and recently synthesized NO-W conjugate.

This work describes for the first time the presence and the morphological characterization of NO-W-immunoreactive cell bodies in the mammalian brain. By using the Golgi method, different morphological types of neurons have been found in the three parts of the rat lateral septal nucleus.[16] Our findings are in agreement with this previous study,[16] since in some cases, the morphological characteristics of the NO-W-immunoreactive neurons (round or piriform cell bodies showing two dendrites) are similar to those neurons reported in the dorsal and intermediate parts of the lateral

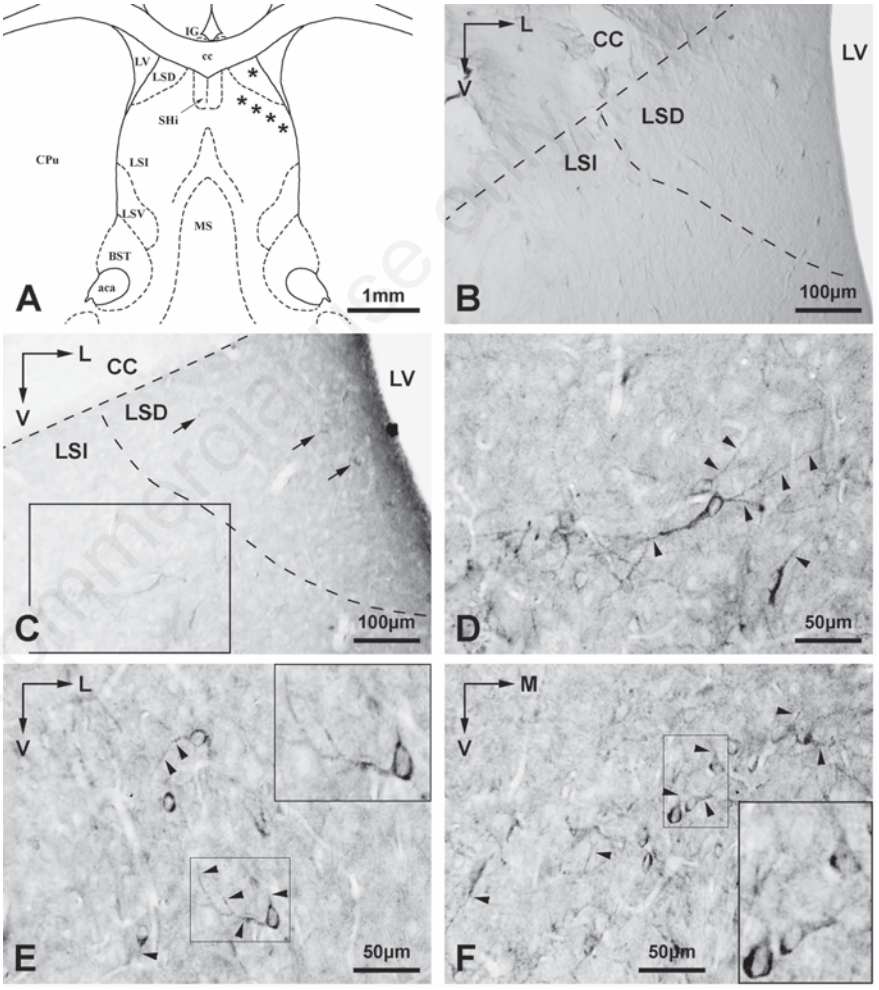

Figure 2. A) Frontal section (Bregma 0.2 mm) of the rat telencephalon. NO-W-immunoreactive cell bodies (asterisks) in the dorsal (LSD) and intermediate (LSI) parts of the lateral septal nucleus. B) Note the absence of immunoreactivity in both the intermediate (LSI) and dorsal (LSD) parts of the lateral septal nucleus after the pre-absorption of the first antibody; compare this with panel C. C) Cell bodies (arrows) containing NO-W located in the intermediate (LSI) and dorsal (LSD) parts of the lateral septal nucleus. D) Higher-power magnification of the region delimited by the rectangle shown in panel C. E,F) NO-W-immunoreactive perikarya located in the intermediate part of the lateral septal nucleus; arrowheads indicate dendrites; inset: higher-power magnification of the region delimited by the rectangle. Aca, anterior commissure, anterior part; BST, bed nucleus of the stria terminalis; cc, corpus callosum; CPu, caudate-putamen; D, dorsal; IG, induseum griseum; L, lateral; LSD, lateral septal nucleus, dorsal part; LSI, lateral septal nucleus, intermediate part; LSV, lateral septal nucleus, ventral part; LV, lateral ventricle; MS, medial septal nucleus; Shi, septohippocampal nucleus; V, ventral; VDBD, nucleus of the vertical limb of the diagonal band, dorsal part.

septal nucleus after using the Golgi method.[16] It is known that the different parts of the lateral septum (ventral, intermediate, dorsal) are interconnected.[17] However, according to our morphological observations, no immunoreactive fibre has been observed in the rat brain. The absence of NO-W in fibres could be due to the intraneuronal transport mechanisms of this molecule (stored exclusively in perikarya and dendrites), as it has been reported for some neuropeptides and D-amino acids (*e.g.*, somatostatin, D-glutamate).[10,18] It is also possible that in fibres the level of NO-W is very low and then the immunocytochemical technique is not sensitive enough to detect it.

Currently, the physiological actions of NO-W located in the mammalian brain are unknown. However, the presence of NO-W in the lateral septal nucleus suggests that this molecule could be involved in affective and motivational processes.[17] The lateral septum receives inputs from the hippocampus, hypothalamus, midbrain periaqueductal gray, ventral tegmental area, locus ceruleus, amygdala and the entorhinal cortex, whereas it sends projections to regions of the central nervous system (*e.g.*, diencephalon, limbic system) that control affective and motivational processes.[17,19-21] It is known that the lateral septum integrates cognitive and affective information and then transmits it to regions of the central nervous system that control the behavioural response.[17]

The data reported here are important because for the first time the presence of NO-W is reported in normal animals. It is known that NO is produced during the course of several pathologies (*e.g.*, multiple sclerosis) and that this molecule is involved in the modification of specific and different targets (*e.g.*, NO-W, NO-cysteine, NO-tyrosine).[8,9] NO is an unstable molecule hard to quantify *in vivo* and currently the local production of NO in tissues remains technically difficult to detect. However, the characterization of circulating antibodies directed against NO-modified self-antigens (*e.g.*, anti-NO-W) provides indirect evidence for the involvement of NO in pathogenic processes.[8,9] Moreover, it has been suggested that NO is involved in the blood-brain barrier breakdown and that this molecule participates in inflammatory processes.[22]

In conclusion, we demonstrate here for the first time the presence of immunoreactive perikarya containing NO-W in the mammalian central nervous system after using a highly specific monoclonal antibody against NO-W. The distribution of these cell bodies is quite restricted. Our study will serve in the future to compare the results reported here in normal animals with those reported in experimental animal models showing different pathologies (*e.g.*, multiple sclerosis, ischemia, depression). For example, in multiple sclerosis the involvement of NO in the pathogenesis of the disease has been suggested[4-6] and hence it should be important to know whether the distribution of NO-W-immunoreactive structures is widespread or restricted in the human central nervous system. Moreover, it is known that the mammalian lateral septum is involved in mood, social behaviour, fear, depression and motivation.[17] In the future, our neuroanatomical study will serve for a better understanding of the physiological roles played by the NO-W in the mammalian lateral septum.

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