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Detection of pantothenic acid-immunoreactive neurons in the rat lateral septal nucleus by a newly developed antibody

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Abstract

Introduction. The available immunohistochemical techniques have documented restricted distribution of vitamins in the mammalian brain. The aim of the study was to develop a highly specific antiserum directed against pantothenic acid to explore the presence of this vitamin in the mammalian brain.

Material and methods. According to ELISA tests, the anti-pantothenic acid antiserum used showed a good affinity (10^{-8} M) and specificity. The antiserum was raised in rabbits. Using an indirect immunoperoxidase technique, the mapping of pantothenic acid-immunoreactive structures was carried out in the rat brain.

Results. Pantothenic acid-immunoreactive perikarya were exclusively found in the intermediate part of the lateral septal nucleus. These cells were generally small, round, fusiform or pyramidal and showed 2–3 long (50–100 μm) immunoreactive dendrites. Any immunoreactive axons containing pantothenic acid were detected.

Conclusions. The very restricted anatomical distribution of the pantothenic acid suggests that this vitamin could be involved in some specific neurophysiological mechanisms. *(Folia Histochemica et Cytobiologica 2016, Vol. 54, No. 4, 186–192)*

Key words: vitamin B5; pantothenic acid; new antibody; rat; brain; lateral septal nucleus; IHC

Introduction

Vitamins acting as coenzymes or cofactors play important metabolic roles in many well-known biochemical pathways. Many studies, using chromatography, vitamin-binding protein assays, enzymatic immunoassays and radioimmunoassays, have reported the presence of vitamins in body fluid and in several organs. However, using immunohistochemical (IHC) techniques, the anatomical distribution of these substances in the mammalian brain is less known. Since 2004 and using highly specific antibodies, the distribution of axons and/or perikarya containing several vitamins has been reported in the mammalian brain [1–7]. Thick and smooth immunoreactive axons containing thiamine (vitamin B1), riboflavin (vitamin B2), pyridoxal (vitamin B6) or folic acid (vitamin B9) have been observed in the mesencephalon, thalamus, anterior commissure and/or cerebral cortex of the monkey (*Macaca fascicularis*) [1–5], whereas cell bodies containing pyridoxal or vitamin C have been respectively visualized in the

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hypothalamus and in the somatosensorial cortex (precentral gyrus) of the same species [4, 5]. Moreover, the distribution of immunoreactive structures containing vitamin C has been recently reported in the brainstem of children [8] and direct visualization of retinoic acid, in perikarya located in the rat hypothalamus, was also reported [6]. Most of vitamins studied using IHC techniques belong to the B series (B1, B2, B6 and B9) [1–5]. The studies reported above have shown that the distribution of vitamins in the mammalian brain is restricted or very restricted and this suggests that vitamins may be involved in some very specific unknown mechanisms different from their known metabolic roles reported to date [1–6]. In order to increase our knowledge on the neuroanatomical distribution of B vitamin series, we have studied for the first time by the IHC technique the distribution of immunoreactive structures containing pantothenic acid (vitamin B5) in the mammalian brain. To carry out this research, a highly specific antiserum directed against D-pantothenic acid (PA) was developed.

Material and methods

Development of a new and highly specific antiserum directed against D-pantothenic acid. D-Pantothenic acid of 99.3% chemical purity was purchased from ICN Biomedicals (Aurora, OH, USA).

PA was dissolved in methanol (solution 1) and bovine serum albumin (BSA) in water (solution 2), both solutions contained triethylamine. A third solution was prepared containing dimethylformamide and ethylchloroformiate (ECF). Then, solution 3 was poured into solution 1 (activation process) and, after 10 min, the resulting solution was transferred drip by drip into solution 2. The final solution, containing the synthesized PA-BSA, was purified by dialysis in dialyze membranes with cut-off limits between 12 and 16 KDa. The purification was performed in one liter bucket at 4°C during 36 h, changing the bath every 2–3 h.

Animals. The animal studies were carried out according to the Spanish, French and European law and they were also approved by the research ethical committee of the University of Salamanca (Spain). Male rabbits (New Zealand white, body mass 2 kg, age 8 months) were immunized with one injection every two or three weeks over two months. Each subcutaneous administration (1 mL) was a mixture of PA-BSA (250 μg) and of complete or incomplete Freund adjuvant [9]. Two weeks after the second immunization, serum samples were obtained. Antisera were preabsorbed with lyophilized BSA-ECF, centrifuged and the floating phase was purified as described previously [9]. Antisera were tested before and after the purification process [9].

Immunohistochemistry. Five male adult Wistar rats (300–350 g) were used. Rats were perfused *via* the ascending aorta with physiological saline and paraformaldehyde (4%) [1–4, 6]. Brains were dissected out, post-fixed in the same fixative for 12–14 h and cryoprotected. Using a freezing microtome, 40–50 μm-thick sections were obtained, kept in phosphate-buffered saline (PBS) and processed for immunostaining as previously reported [1–4, 6]. In brief, free-floating sections were treated with H_2O_2 and methanol, pre-incubated in PBS containing 0.3% Triton X-100 and 1% normal horse serum (mix solution) and then incubated overnight in the mix solution containing the PA antiserum (diluted 1:1,000) (denoted at Gemacbio, Saint Jean d'Illac, France, as AP090). Sections were washed in PBS and incubated with biotinylated anti-rabbit immunogammaglobulin (BA-1,000, Vector, Burlington, CA, USA), diluted 1:200 in the mix solution. Then, sections were incubated with a 1:100-diluted avidin-biotin-peroxidase complex (Vectastain PK-6,100, Vector) and washed in PBS and Tris-HCl buffer. Finally, tissue-bound peroxidase was developed with H_2O_2, using 3,3'-diaminobenzidine as chromogen.

For mapping and nomenclature, the stereotaxic atlas of Paxinos and Watson [10] was used. An Olympus DP50 digital camera attached to a Unilux-12 microscope (Kyowa, Tokyo, Japan) was used to obtain photomicrographs. Using an Adobe Photoshop CS software (Adobe Systems Inc., San José, CA, USA), only the contrast and brightness of the images were adjusted.

Results

Specificity of the anti-pantothenic acid antiserum

As previously described, antibodies were assessed and characterized by ELISA tests [9]. After titration and for competition experiments, the optimal dilution of the anti-PA antiserum was observed at 1:60,000–1:70,000 (at 492 nm, the optical density was 1) [9]. This procedure was carried out for each structure considered as competitor; the choice of the competitor was made based on the similarity of its chemical structure to that of the target molecule (see Table 1).

The estimated antibody avidity (IC_{50}) was fairly high (10^{-8} M for anti-PA-BSA antibodies, *i.e.* anti-PA-antibodies) (Table 1). The specificity of these antibodies was very high, since the antiserum discriminated analogous structures very well: pyridoxine, choline, retinoic acid, alpha-tocopherol, vitamin C and D-biotin (Table 1, Figure 1). No cross-reactivity was observed with any of the molecules tested. Moreover, the part of the antigen corresponding to the protein carrier was not detected when ELISA tests or the IHC technique were carried out, since in both cases no cross-linking was

Table 1. Affinity and specificity of antibodies directed against D-pantothenic acid

Compound	Cross-reactivity at half-displacement (IC_{50})
D-Pantothenic acid-BSA	1
Pyridoxine-BSA	> 1/1,000
Choline-BSA	> 1/50,000
Retinoic acid-BSA	> 1/50,000
α-tocopherol-BSA	> 1/50,000
Vitamin C-BSA	> 1/50,000
Biotin-BSA	> 1/50,000

Using competition ELISA assays, cross-reactivity was calculated from the displacement curves at half-displacement: the best recognized was D-pantothenic acid-BSA, whose concentration was divided by the concentration of each of the other compounds. Abbreviations: BSA — bovine serum albumin.

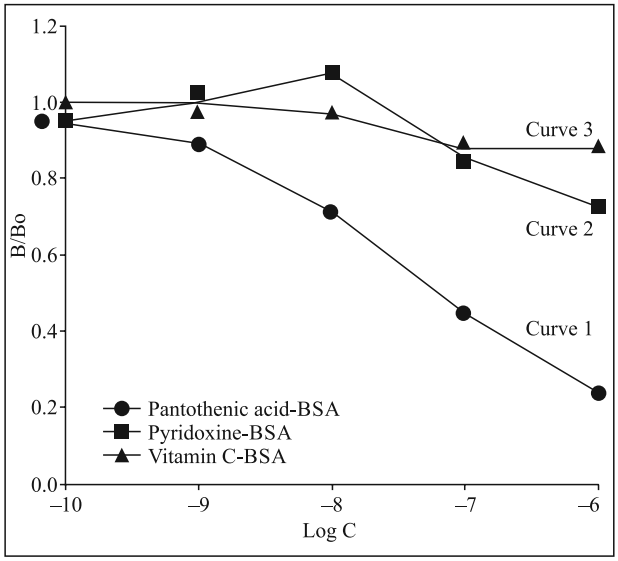

Figure 1. Antibody affinity and specificity resulting from competition ELISA tests of pantothenic acid. Curve 1: competition with D-pantothenic acid-BSA. Curve 2: competition with pyridoxine-BSA. Curve 3: competition with vitamin C-BSA; this curve is also representative of the competition with choline-BSA, retinoic acid-BSA, α-tocopherol-BSA or biotin-BSA. For a better visualization of Figure 1, the curves of the latter four compounds have not been included. Abbreviations: B — optical density with competitor; Bo — optical density without competitor; BSA — bovine serum albumin; Log C — logarithm of concentration.

observed. Thus, it may be concluded that the antiserum is specifically directed against PA due to the absence of cross-reactivity.

Specificity of the immunoreactivity

The anti-PA antiserum was absorbed with the protein carrier (BSA) and the lyophilized coupling agent (ECF) (both used to synthesize the antigen). In order to avoid spurious signals, the absorption with ECF--BSA was performed before the IHC technique was carried out. Moreover, the immunoreactivity observed in the intermediate part of the lateral septal nucleus disappeared when the anti-PA antiserum was pre-absorbed with an excess of PA (Figure 2B) and when the first and/or the second antibodies were omitted.

Neuroanatomical observations

We observed a very restricted distribution of PA-immunoreactive structures. Cell bodies containing this vitamin were exclusively found in the intermediate part of the lateral septal nucleus (Figures 2, 3). No immunoreactive structures were visualized in the ventral and dorsal parts of the same nucleus. Moreover, no PA-immunoreactive axons were observed in the rat brain. In the intermediate part of the lateral septal nucleus, we found a high density of immunoreactive perikarya (more than 20 cell bodies/nucleus/section) throughout the whole rostro-caudal extension of the intermediate part (Figures 2, 3). In all animals studied, we observed the same density and distribution of immunoreactive cell bodies. In general, these cell bodies were small (with a diameter below 15 μm), round, fusiform or pyramidal and showed two-three long (50–100 μm) immunoreactive dendrites (Figures 2, 3).

Discussion

Specificity of the developed anti-pantothenic acid antiserum

Due to the development by us at Gemacbio S.A. (Saint Jean d'Illac, France) of a new and highly specific antiserum directed against PA, it has been possible to map the immunoreactive structures containing PA in the rat brain. This vitamin is a small molecule (a compound with a molecular weight of 238.27 Da). Small molecules are not immunogenic and, for this reason, they are usually linked through a coupling agent (*e.g.*, aldehydes, carbodiimides, picric acid) to a carrier protein [9]. The immune response is triggered by molecules weighing 1 to 2 kDa, which is the minimum size for stimulating antibody production. Animals receiving the immunogen (PA-coupling agent-carrier protein) will generate different types of polyclonal antibodies, some of them will recognize PA, while other populations will recognize the carrier protein or the coupling agent (cross-linking). Antibodies directed against the carrier protein or the coupling agent could give a spurious signal that must be blocked, reduced

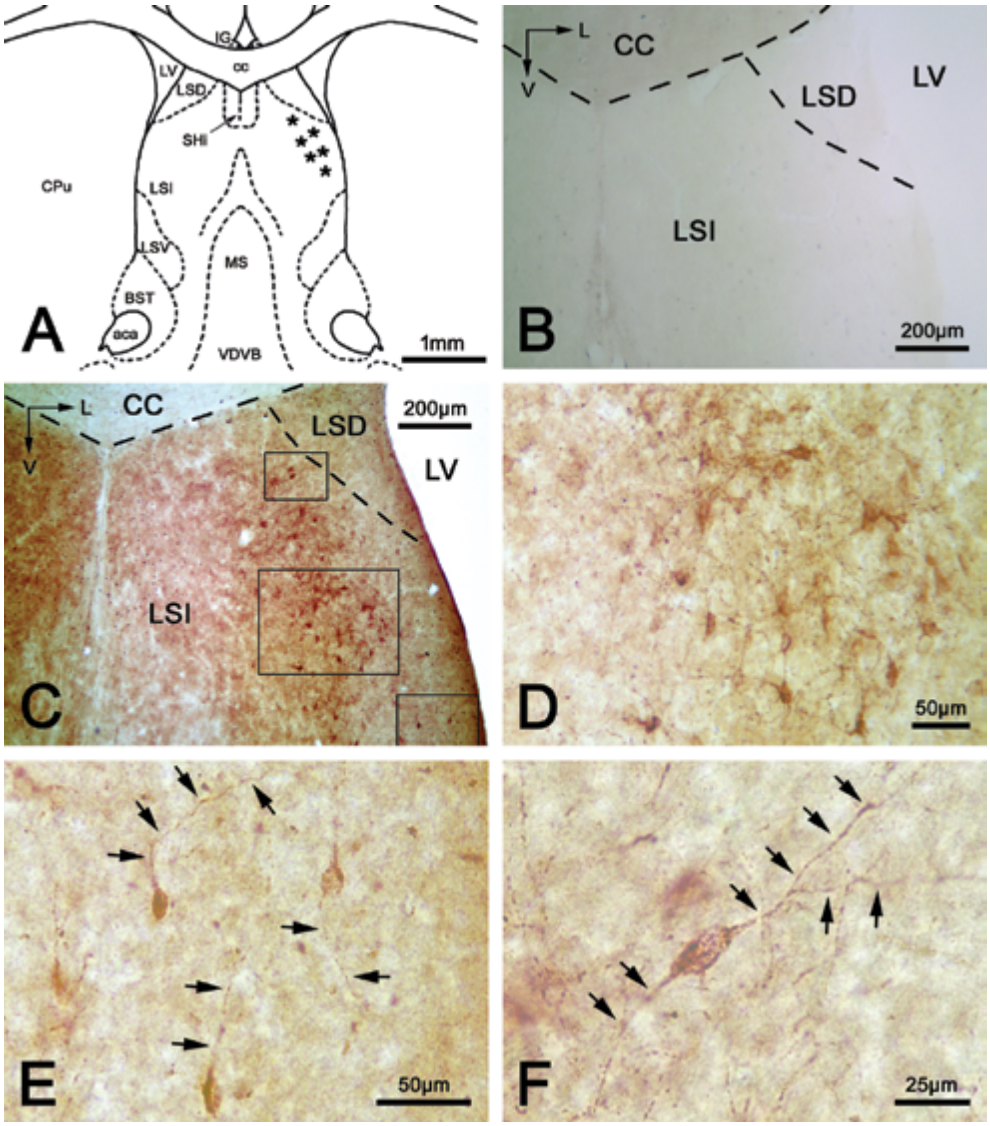

Figure 2. The distribution of pantothenic acid (PA) in lateral septal nucleus of rat brain. **A.** Frontal section (Bregma 0.2 mm) of the rat telencephalon. PA-immunoreactive cell bodies (asterisks) are present in the intermediate (LSI) part of the lateral septal nucleus; **B.** After the pre-absorption of the first antibody, note the absence of immunoreactivity in the intermediate (LSI) part of the lateral septal nucleus. Compare this figure with Figure 2C; **C.** Cell bodies containing PA are located in the intermediate (LSI) part of the lateral septal nucleus; **D.** Higher-power magnification of the region delimited by the rectangle (middle) shown in Figure 2C; **E, F.** Higher-power magnification of the regions delimited by the lower and upper rectangles, respectively, shown in Figure 2C. Arrows indicate dendrites. Abbreviations: 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.

or suppressed by later purification and checked after application of the respective controls.

According to the controls carried out in this study, it seems that the first antiserum (anti-PA) used here is very specific. Thus, the anti-PA antiserum was pre-absorbed with the carrier protein and the coupling agent in order to discard spurious signal due to antibodies directed against these parts of the antigen (cross-linking signal). Moreover, the pre-absorption of the first antiserum with D-PA-BSA and the omission of the first and/or second antisera showed the specificity of the immunoreactivity observed. ELISA tests revealed that D-PA was much better recognized than other competitors. Thus, the data demonstrate that the antibody directed against

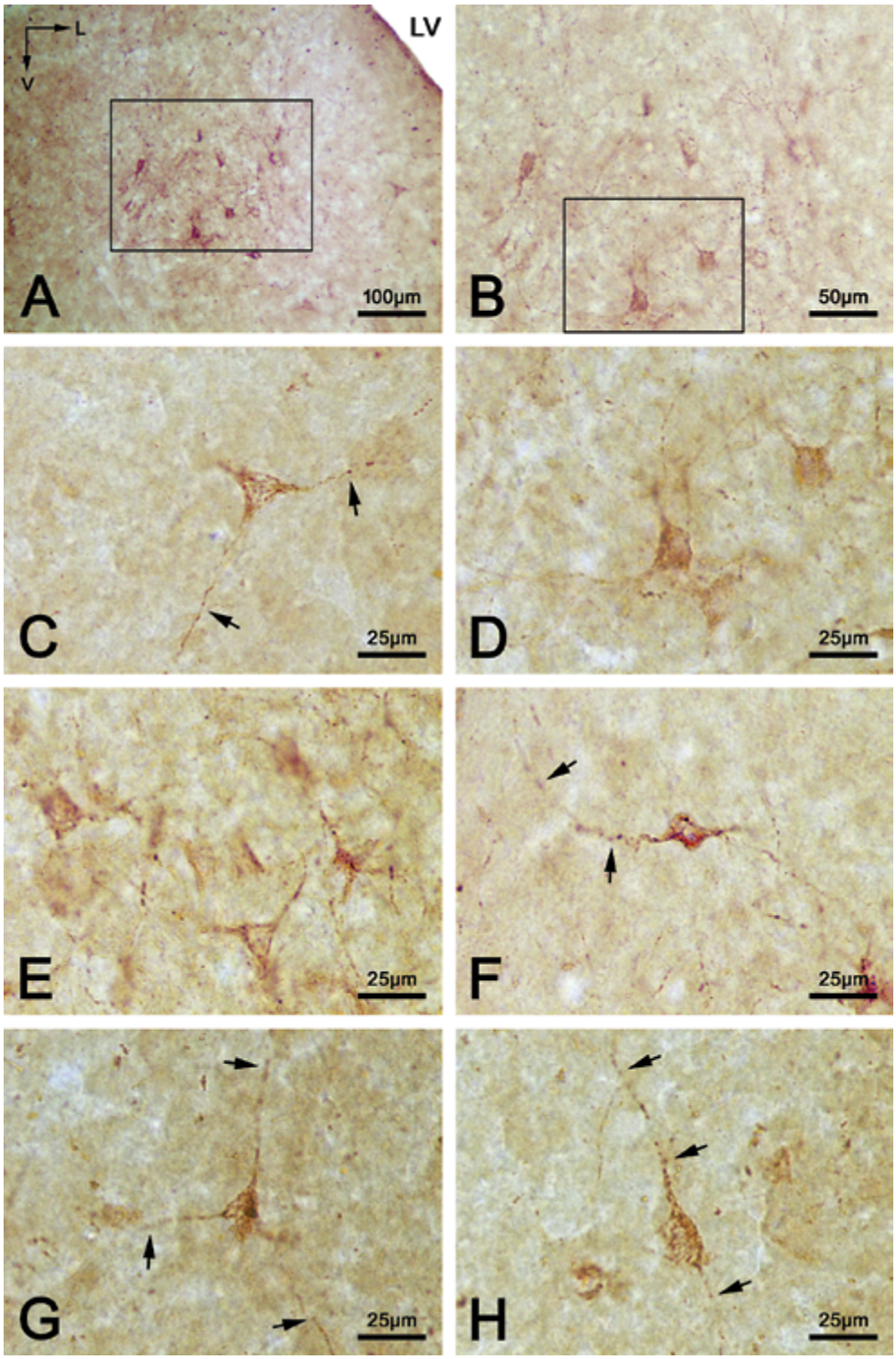

Figure 3. Immunoreactive neurons containing pantothenic acid (PA) in the rat lateral septal nucleus. **A.** Cell bodies containing PA; **B.** Higher-power magnification of the region delimited in the rectangle shown in Figure 3A; **C.** A PA-immunoreactive cell body; **D.** Higher-power magnification of the region delimited in the rectangle shown in Figure 3B; **E–H.** Cell bodies containing PA. Arrows indicate dendrites. Abbreviations: L — lateral; LV — lateral ventricle; V — ventral.

D-PA is highly specific. To sum up, we have applied two different techniques (IHC, ELISA) and both confirmed the specificity of the immunostaining visualized in the rat lateral septal nucleus.

The presence of pantothenic acid in the lateral septal nucleus

After applying the Golgi staining method, the morphological characteristics (*e.g.* long dendrites, fusiform or

pyramidal cell bodies) of the neurons located in the rat lateral septum have been previously reported [11]. Our results are in agreement with this previous work [11]. Thus, comparing the morphological characteristics of the rat septal neurons (shape of the cell body, dendrites) with those reported here for the PA-immunoreactive cell bodies located in the intermediate part of the lateral septal nucleus, it seems that some neurons show the same morphological characteristics. Thus, for example, fusiform cell bodies with two long dendrites or pyramidal cells with three long dendrites can be observed when the Golgi method [11] or the IHC technique were carried out. It is known that the intermediate, dorsal and ventral parts of the lateral septal nucleus are interconnected [12]; however, no immunoreactive axons have been observed in the rat brain. The lack of immunoreactivity in axons could be due to the intraneuronal transport mechanisms of PA (stored exclusively in dendrites and perikarya). This has been previously published for some D-amino acids (*e.g.* D-glutamate) and neuropeptides (*e.g.* somatostatin) [9, 13]. Moreover, it is also possible that the IHC technique applied here is not sensitive enough to detect PA because the level of PA stored in the axons is very low.

Our previous works on the neuroanatomical distribution of vitamins in the mammalian brain [1–6] suggested the involvement of these substances in stress, somatosensorial, motor, food intake, visual, neuroendocrine and auditory mechanisms. To date, the role played by PA in the rat brain is unknown, but the presence of this vitamin in the intermediate part of the lateral septal nucleus suggests that PA could be involved in motivational and affective processes [12]. It is known that the lateral septal nucleus send projections to the limbic system, which controls affective and motivational processes [12, 14], and receives afferents from the hypothalamus, amygdala, entorhinal cortex and hippocampus. Through these connections, the lateral septum integrates affective and cognitive information and sends this information to the brain regions involved in the control of the behavioral response [12].

Vitamins in the mammalian brain

Our results are in agreement with previous reports showing a restricted or a very restricted distribution of the immunoreactive structures containing vitamins in the mammalian brain [1–7]. Using IHC techniques, vitamins have been exclusively observed in axons (folic acid, riboflavin), cell bodies and dendrites (vitamin C, retinoic acid) or dendrites, axons and cell bodies (thiamine, pyridoxal) [1–7]. To date, the immunoreactive axons containing vitamins (folic acid, thiamine, riboflavin, pyridoxal) showed the same morphological characteristics: thick, smooth and unbranched [1–5]. These axons are very different from immunoreactive axons containing neuropeptides, since in general they show varicosities and are thin [15]. Here, we visualized cell bodies and dendrites containing PA, as it has been previously reported for vitamin C and retinoic acid [4, 6]. To date, immunoreactive structures containing vitamins have been found in the mesencephalon, thalamus, hypothalamus, cerebellum, anterior commissure and cerebral cortex [1–7]. Thus, we reported here for the first time the presence of immunoreactive structures containing a vitamin (PA) in the mammalian lateral septal nucleus. Moreover, our results confirm that in general vitamins show various anatomical distributions in the mammalian brain, although there are some brain nuclei (*e.g.,* medial geniculate, pulvinar) in which two or three vitamins have been located (folic acid, thiamine and/ /or riboflavin) [1–3, 5]. Comparing the distribution of the vitamins studied in the mammalian brain, it is important to note that folic acid showed the most widespread distribution in the mammalian brain (immunoreactive axons were observed in 13 nuclei/ /regions located in the mesencephalon and thalamus), followed by thiamine (9 nuclei/regions located in the mesencephalon, thalamus and cerebral cortex), riboflavin (7 nuclei/regions located in the mesencephalon and thalamus), pyridoxal (4 nuclei/regions located in the hypothalamus), retinoic acid (2 nuclei/regions located in the hypothalamus) and vitamin C (1 region located in the cerebral cortex) [1–7]. Thus, to date, the distributions of PA and vitamin C are the most restricted in the mammalian brain. The restricted distribution of PA suggests that the anti-PA antiserum used here is very specific against PA, because if the immunoreactivity observed was due to non-specific staining, this staining might have been distributed throughout the brain and not in a specific part of the lateral septal nucleus. Finally, in monkey, an IHC study has been carried out on the distribution of nicotinamide (the amide of nicotinic acid or vitamin B_3) and pyridoxine [4]. This study failed to demonstrate immunoreactive structures containing both vitamins.

The anatomical distribution of vitamins in the mammalian central nervous system by using highly specific antibodies directed against these substances is a new line of research that merits to be developed in the future. Vitamins show a specific neuroanatomical distribution in the mammalian brain and this suggests that these compounds may be involved in unsuspected physiological roles. Neuroanatomical studies are necessary in order to gain insight into the role played by vitamins in the brain regions in which vitamins are

found. The presence of PA-immunoreactive perikarya in the intermediate part of the lateral septal nucleus suggests that this vitamin could be involved in affective and motivational processes (*e.g.*, depression, social behavior, mood, fear). Complementary physiological studies should be carried out to confirm the involvement of PA in these processes.

In summary, using a highly specific anti-PA antiserum, we have reported for the first time in the mammalian brain the presence and the morphological characteristics of the cell bodies containing PA.

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