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Aminoacyl-histidine dipeptides in the glial cells of the adult rabbit forebrain ^,[1]

Paolo Peretto[a], Federico Luzzati[a], Luca Bonfanti[b], Silvia De Marchis[a], Aldo Fasolo[a,*]

[a]Department of Human and Animal Biology, University of Turin, via Accademia Albertina 13, 10153 Turin, Italy
[b]Department of Veterinary Morphophysiology, University of Turin, via Nizza 52, 10126 Turin, Italy

Abstract
The mammalian nervous system contains high amounts of the aminoacyl-histidine dipeptides carnosine and homocarnosine. In the brain, they prevalently occur mainly in glial and ependymal cells, their role(s) still remaining obscure. In vitro studies indicate that these molecules exert diverse protective effects, and in vivo they are frequently associated with extracellular fluid compartments. Recently, carnosine-like immunoreactivity has been found in the subependymal layer (SEL) of adult rodents, a region endowed with persistent cell proliferation and migration. Unlike rodents, the SEL of the rabbit has a persistent olfactory ventricle. We show here that the morphologic organization of the SEL is different in these species, with particular reference to the glial/non glial cell compartments. The distribution of carnosine-like immunoreactivity in the rabbit displays some differences only within the SEL, which could be linked to its arrangement and compartmentalization.

Keywords: Carnosine; Subependymal layer; Neurogenesis; Immunohistochemistry

1. Introduction

The role of small peptides such as aminoacyl-histidine ipeptides in the cell biology of the nervous tissue is at the present unclear and frequently relies on in vitro assays or neurochemical localization cues. Carnosine (b -alanyl-Lhistidine) and other related dipeptides, such as homocarnosine and anserine, have been described to occur in the nervous system of many vertebrates, particularly in sensory organs (for review see Ref. [7,9]). In mammals, including humans [17,30], carnosine is abundant in olfactory receptor neurons of the olfactory mucosa and their central projections [3,22], whereas homocarnosine is prevalent in the brain [17,8]. The lack of monospecific antibodies unables the immunocytochemical detection of each single peptide [3,29], which might be referred to as carnosine-like immunoreactivity (carnosine-LI), in the mammalian brain corre corresponding to either carnosine or homocarnosine (reviewed in Ref. [7]).

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* Corresponding author. Tel.: 139-0118123224; fax: 139-0118124824.
E-mail address: fasolo@dba.unito.it (A. Fasolo).
[1]In memory of Vittorio Erspamer who gave me through the years invaluable expertise on amphibian skin peptides and carnosine, but far more an outstanding example in life and science. (A.F.).

The functional role of aminoacyl-histidine dipeptides in the nervous system is still a matter of debate. An involvement in neurotransmission within the olfactory system has been proposed in the past [23]. Nevertheless, the distribution of carnosine-LI in the central nervous system (CNS), prevalently associated to glial/ependymal cells [3,7,26] along with the identification of several effects displayed by these molecules in vitro [4,5,13,18,24], strongly suggest that they could subserve other functions [7].
In the mammalian brain, carnosine has been detected almost exclusively in astrocytes, oligodendrocytes, ependymal cells and tanycytes [3,10,26; for review see Ref. [7]), although at present it is not clear if all these cell types are capable of synthesizing the dipeptide in vivo, or simply to uptake it, as demonstrated for astrocytes in cell cultures [14]. Recently, a striking amount of carnosine-LI has been described in the subependymal layer (SEL) of the rat and mouse forebrain [26]. This region, which spans from the lateral ventricle to the olfactory bulb, is characterized by persisting cell proliferation and migration accounting for adult olfactory bulb neurogenesis [20], and is particularly enriched in astrocytes. The SEL is composed of two distinct cell compartments: chains of migrating neuroblasts enwrapped by astrocytic glial tubes [15,21,25; for review see Ref. [27]). Unlike most antigens detected within this region, which usually are segregated to one of the compartments [27], carnosine-LI appears to fill both of them. Moreover, since migrating neuroblasts are immunoreactive for carnosine exclusively when they form chains in the SEL, but not when they migrate isolately through the olfactory bulb, the presence of the dipeptide does not seem to be related to cell proliferation/migration itself [7,26]. Such an hypothesis, is supported by the absence of carnosine-LI in the SEL during the early postnatal period, when striking neurogenesis and tangential migration already occur, and by its later appearance in coincidence with the assembling glial tubes [26].
It is well known that the anterior part of the SEL, or rostral extension, originates in rat and mouse after the occlusion of the primitive olfactory ventricle [1,31], thus losing a continuous, monostratified ependymal layer, as well as any direct contact with the cerebroventricular fluid. In other species such as rabbit, this occlusion never occurs, the olfactory ventricle persisting throughout adulthood [19]. Thus, we have studied here the organization of the subependymal layer in the forebrain of adult rabbits, with particular reference to the distribution of glial cells and carnosine-like immunoreactivity.

2. Methods

2.1. Tissue preparation

Brains were obtained from 4 adult New Zealand White rabbits (four to five months old; Charles River, Milan, Italy) and 2 adult Wistar rats (three to six months old; Charles River). All experiments were performed in accordance with D.L. 116/92 from the Italian Government and received the approval of the ethical committee of the University of Turin (Italy). After deep anesthesia by intraperitoneal (i.p.) injection of a solution of Rompun-xilazina (Bayer AG, Leverkusen, Germany, 0.25 mg/Kg) and Ketavet 100 (Gellini, Aprilia –LT-, Italy, 0.2 ml/Kg), animals were perfused intracardially with an heparinized saline solution (25 UI/ml in 0.9% NaCl, 3–5 min) followed by a freshly prepared solution of 4% paraformaldehyde and 2% picric acid in 0.1M sodium phoshate

buffer (PBS), pH 7.4. After dissection, brains were postfixed overnight in the same solution. The tissues were subsequently rinsed in PBS, dehydrated and embedded in paraffin wax, or cryoprotected in increasing sucrose solutions (7.5, 15, 30%) and frozen in liquid nitrogen-cooled isopentane at –80°C. Serial coronal and sagittal sections were cut on a microtome (paraffin, 10 μm) or cryostat sectioned (10–20 μm), collected onto gelatinecoated slides, and then treated for conventional histology and immunohistochemistry.

2.2. Conventional histology

The organization of the subependymal layer was examined in serial coronal and parasagittal sections (10–20 m m) cut through the rostral lateral ventricle and the olfactory bulb and stained with hemalum-eosin.

2.3. Antibodies

The following primary antisera and antibodies were used: i) anti-carnosine, a polyclonal rabbit IgG (F. Margolis, Baltimore, U.S.A.), diluted 1/1000; ii) anti-anserine, a polyclonal rabbit IgG (F. Margolis) diluted 1/400; iii) anti-glial fibrillary acidic protein (GFAP), a monoclonal IgG (Boehringer, Mannheim, Germany), diluted 1/100; iv) anti-29 39 - cyclic nucleotide 39 -phosphodiesterase (CNP), a monoclonal IgG (Boehringer), diluted 1/200; v) anti-PSA-NCAM, a monoclonal IgM raised against the capsular polysaccharides of meningococcus group B that share -2,8-PSA residues with polysialyc acid on the neural cell adhesion molecule (NCAM) (G. Rougon, Marseille, France), diluted 1/1000. All the antibodies were diluted in a solution of 0.01M PBS, pH 7.4, containing 0.1% Triton X-100.
The specificity of anti-carnosine and anti-PSA-NCAM is described respectively in [3] and [28].

2.4. Immunohistochemistry

Immunohistochemical reactions were carried out by using single and double immunofluorescence methods. Cryostat sections were washed for 10 min in 0.01M PBS, then incubated overnight at 4°C with a single primary antibody or a combination of primary antibodies (double labellings) and 1% normal serum of the same donor species of the secondary antiserum.
Affinity purified goat anti-mouse IgG (Fab-specific, Sigma Aldrich SRL, Milan, Italy, 1/50) immunoglobulin conjugated to fluorescein isothiocyanate (FITC), swine antirabbit (Dako SPA, Milan, Italy, 1/50) coupled to rhodamine, biotinjlated goat anti-rabbit (Vector, U.K:, 1/200), and Texas-red avidin (Vector, Burlingame, USA 1/500), served as immunolabels.
Sections were mounted in a solution of PBS/glycerol (9/1) and observed with a conventional epifluorescence microscope, using appropriate filters. For double staining a combination of two indirect immunofluorescence procedures using FITC + rodhamine or FITC + Texas-redconjugated antibodies were used.
Immunocytochemical controls included: (i) incubation of sections in carnosine antiserum preadsorbed with 10 m M of bovine serum albumin (BSA)e (BSA)-carnosine conjugated peptide; (ii) omission of primary antibodies; and (iii) incubation of sections in primary antibodies followed by inappropriate immunolabels.

3. Results

3.1. Organization of the subependymal layer (SEL) in adult rabbits

As previously described [19], our morphologic analysis of the adult rabbit anterior forebrain revealed the occurrence of an olfactory ventricle, extending through the olfactory tubercle and connecting the lateral ventricle with the center of the olfactory bulb.
In hemalum-eosine stained specimens all the ventricular cavities were lined by a continuous monolayer of large globose ependymal cells. Beneath the ependyma, an area corresponding to the SEL and characterized by cells with large, darkly stained nuclei, concentrated in a higher density than in the surrounding nervous tissue, was observed both at the lateral and olfactory (Fig. 1A) ventricular levels. However, in comparison with the corresponding levels of the rat forebrain (Fig. 1B), in the rabbit, cells appeared far less numerous and densely packed, thus preventing a clearly morphologic delimitation between the SEL and the mature brain parenchyma. Such a difference appeared even more evident in the rostral portions, namely around the olfactory ventricle (Fig. 1A,B).
At the level of the lateral ventricle, the ill-defined SEL of the rabbit was detectable on almost the entire ventricular perimeter (including the medial region of the septum), in contrast with the prevalent expansion on the lateral ventricular wall, characteristic of rodents (see Ref. [27]). Moreover, isolated clusters of tightly packed cells were frequently visible at different locations within the striatum. In the most posterior and ventral aspects, the SEL did not appear in the form of a continuos layer, but it was formed by discontinuous bulks of undifferentiated cells

3.2. PSA-NCAM immunoreactivity

By using the anti-PSA-NCAM antibody, a wide mass of immunoreactive cells was detectable in the tissue surrounding the ventricles (Fig. 1C). This mass of cells roughly overlaps the ill-defined area of the SEL, at both the lateral and olfactory ventricular levels. Although frequently tightly packed, the PSA-NCAM-positive cells did not appear associated each other to form regularly arranged, continuous chains.

3.3. Glial fibrillary acidic protein (GFAP) immunoreactivity

Immunocytochemistry for GFAP, a marker of mature astrocytes which can be used to visualize the meshwork of glial cells forming the glial tubes [27], revealed a particular density of astrocytic cells all along the SEL, from the lateral ventricle to the olfactory bulb (Fig. 3B,D). Along the olfactory ventricle, the area enriched in GFAP-positive cells was consistently far larger at the dorso-lateral aspect of the ventricular perimeter (Fig. 3D).
The analysis of coronal and sagittal sections revealed that, in spite of the abundance of astroglial cells in the rabbit SEL, they do not form clearly distinct glial tubes, as previously described in rat and mouse (see Ref. [27]). In particular, in the coronal view, the GFAP-positive cells did not delineate well defined, immunonegative fields.

Ependymal cells were GFAP-immunonegative, nevertheless many singular thick GFAP-positive processes, originating from the subjacent astroglial meshwork, can be seen at higher magnification among cells of the ependymal monolayer (not shown). These processes appeared frequently directed toward the ventricular lumen, especially in correspondence of the olfactory ventricle.

3.4. Carnosine-like immunoreactivity (-LI)

A wide population of cells showed a carnosine-LI in the adult rabbit forebrain. After preabsorbtion of the anti-carnosine serum on BSA-conjugated peptide, immunoreactivity was completely abolished within the brain areas examined. Similarly, no immunoreactivity was observed by using an anti-anserine antiserum.

As expected, strong carnosine-LI was observed in the external part of the olfactory bulb, corresponding to the olfactory nerve layer containing the axons of the olfactory receptor neurons, which terminate into the olfactory glomeruli (not shown). In the remaining tissue of the rostral forebrain, carnosine-LI was associated with three main cell populations: stellate-shaped, process-bearing cells reminiscent of the astrocytic morphology (Fig. 2A), round-shaped cell bodies preferentially localized in white matter bundles (Fig. 2C), and ependymal cells (Fig. 3 A,C). Double labelings with anti-carnosine and anti-GFAP identified the process-bearing cells as astrocytes (Fig. 2 A,B), leaving unstained the round-shaped cells. On the other hand, double carnosine/CNP immunostainings demonstrated that these GFAP-negative cells are indeed mature oligodendrocytes (Fig. 2 C,D). In the SEL, a dense carnosine-LI was consistently detected around the lateral ventricle (Fig. 3A), and to a lesser extent along the olfactory ventricle (Fig. 3C). This distribution was largely overlapping with the area occupied by the densely packed astrocytic cells, and carnosine/GFAP double labelings indeed confirmed that the dipeptide is prevalently associated to these glial cells of the SEL (Fig. 3). In the rostral extension (olfactory ventricle), although carnosine-LI was rather faint, this association was still detectable in the dorso-lateral part of the SEL, particularly enriched in glial cells (Fig. 3 C,D). By contrast, in carnosine/PSA-NCAM double labellings a consistent coexistence of the two antigens in the same groups of cells was not observed (not shown).

As to carnosine-LI in ependymal cells, it was not uniformly detected along the entire monolayer, rather it was associated to groups of ependymal cells lining the side of greater SEL thickness and higher glial cell density, in the olfactory ventricle corresponding to the dorso-lateral part (Fig. 3 C,D).

4. Discussion

The distribution of aminoacyl-histidine dipeptides in the mammalian nervous system is quite heterogeneous [7]. Both carnosine and homocarnosine are present, the former abundant in olfactory receptor neurons of the nasal cavities, the latter also detectable in the brain, where these substances are prevalently associated with cells of the glial lineage [3,7,10]. In a recent study, the localization of carnosine-LI has been described in the SEL of rodents, associated with both astrocytes of the glial tubes and migrating neuroblasts [26]. This region share the feature of continuous neurogenesis during adulthood with the olfactory epithelium, another area which has been

previously found to highly express carnosine-LI (see Introduction). However, a role of the dipeptides in the context of cell renewal is difficult to explain for several reasons (discussed in Ref. [7]), and does not account for the presence of these molecules in widespread glial cell populations.

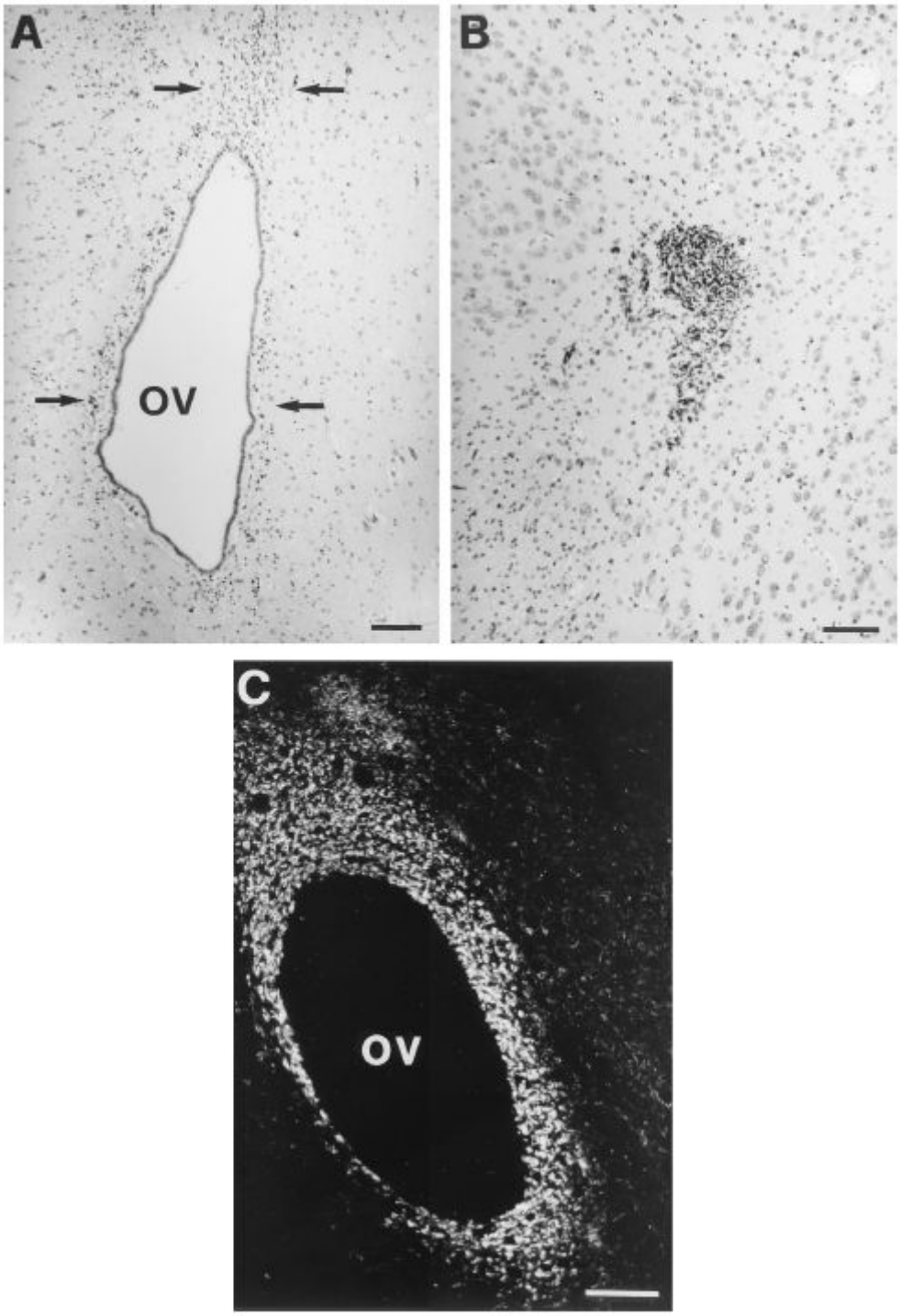

Fig. 1. A,B, Coronal sections cut at the level of the SEL rostral extension of the adult rabbit (A) and rat (B), stained with hemalum-eosin. Note the densely packed cells clearly delineating the SEL in the rat but not in the rabbit (arrows indicate the ill-defined border between the SEL and the brain parenchyma). C, Immunoreactivity for PSA-NCAM around the olfactory ventricle of the adult rabbit forebrain. ov, olfactory ventricle. Top, dorsal; left, lateral. Scale bars = 80 μm.

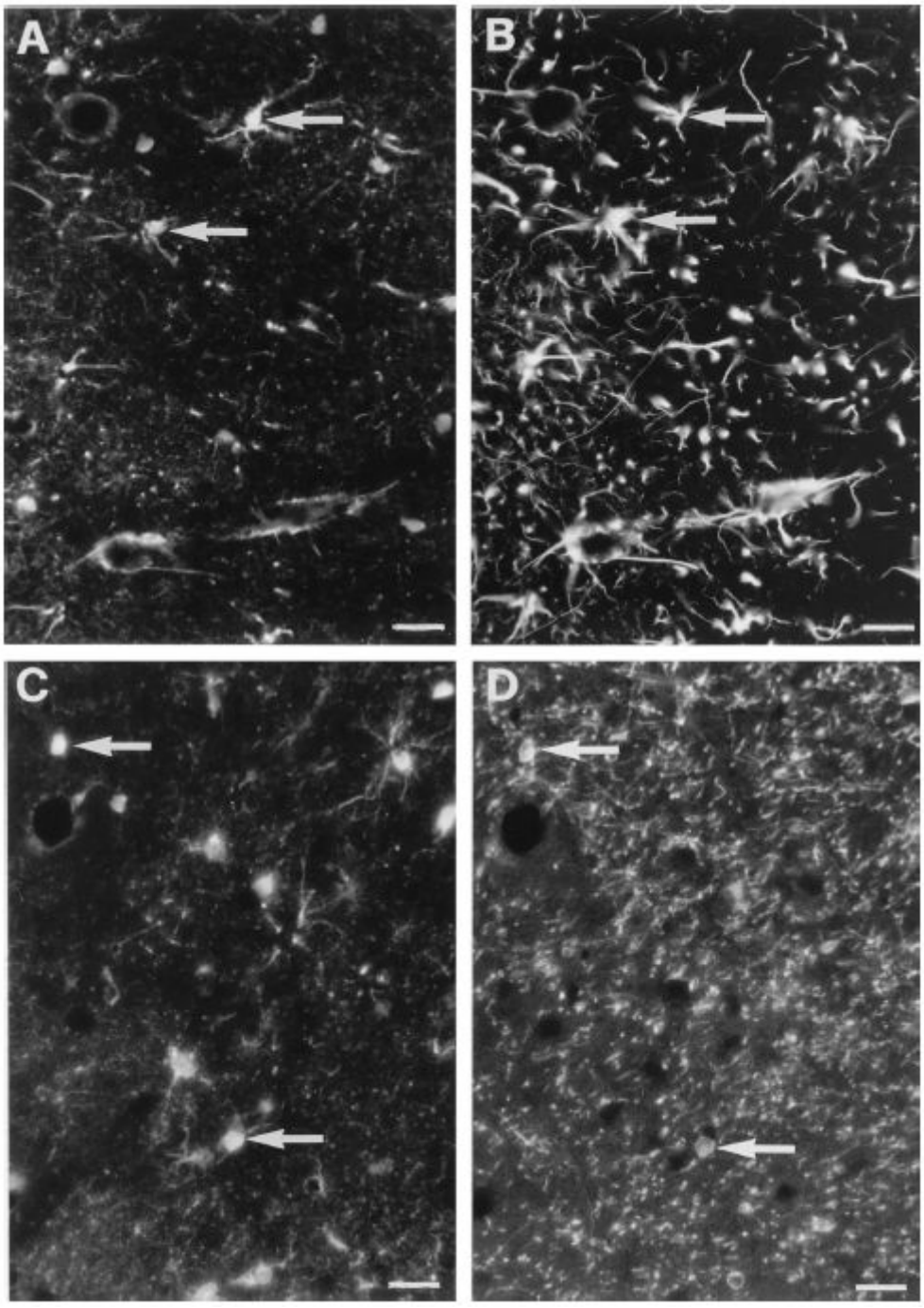

Fig. 2. Carnosine-LI (A,C) in glial cells of the adult rabbit forebrain. A,B Simultaneous localization of carnosine (A) and GFAP (B) in the olfactory tubercle. Carnosine-LI is present in a subpopulation of astrocytes (arrows) and in round-shaped cells which are GFAP-immunonegative. C,D Double labeling for carnosine (C) and CNP (D) in the olfactory tubercle. Mature CNP-immunoreactive oligodendrocytes (arrows) show a carnosine-LI. Scale bars = 40 μm.

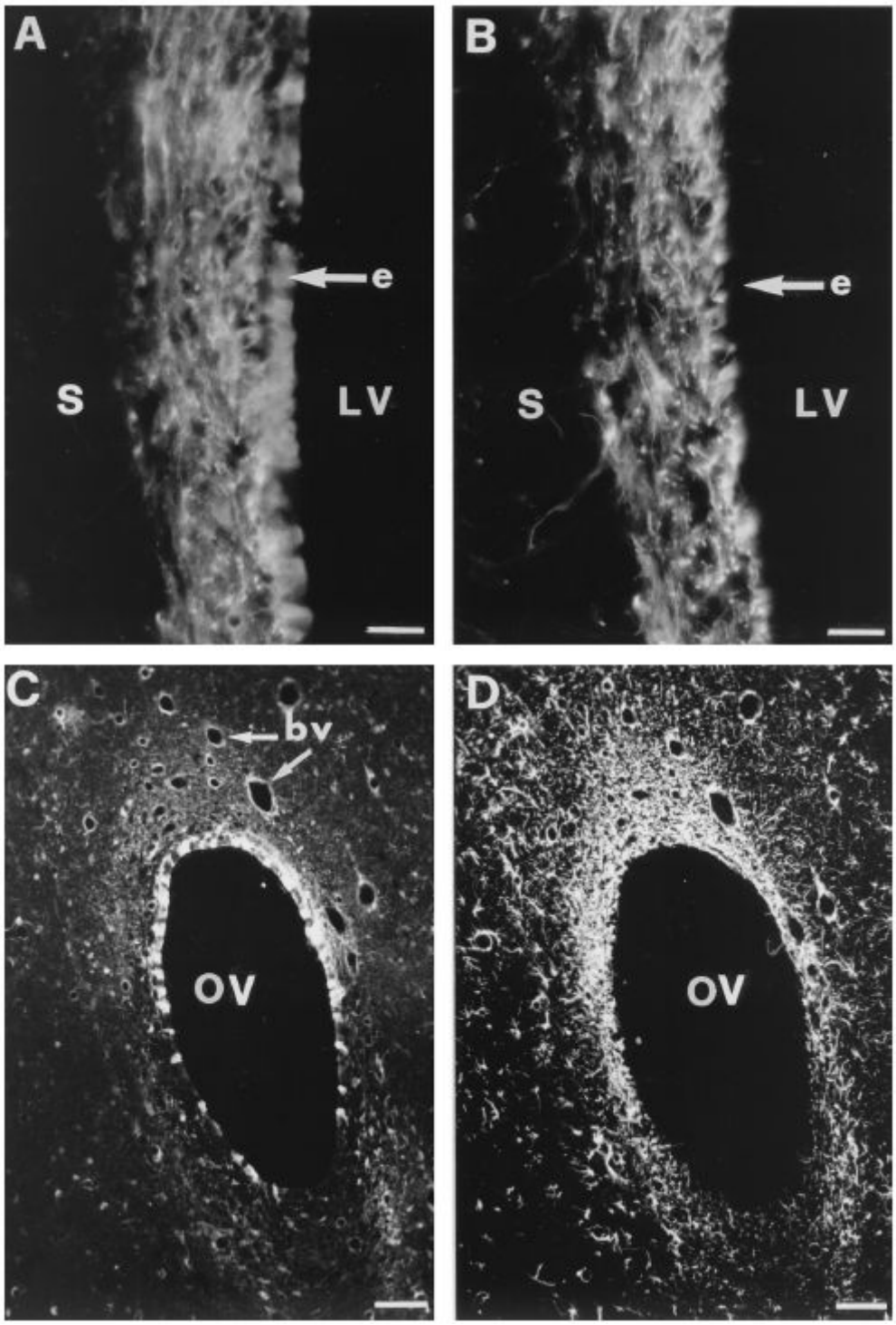

Fig. 3. Immunoreactivities for carnosine (A,C) and GFAP (B,D) in the SEL surrounding the lateral (A,B) and olfactory (C,D) ventricles (coronal sections). Numerous, densely packed astrocytes immunoreactive for both the antigens form a meshwork along the SEL. At the olfactory ventricle level (C,D) astrocytes are particularly abundant in the dorso-lateral part (D, top left). Note that, although densely packed, the glial cells do not compartmentalize the SEL in well distinct 'glial tubes'. Carnosine-LI in the more rostral levels (C) is less intense in comparison with the lateral ventricle (A), but it is prevalently associated with glial cells. This association is also visible in astrocytic processes surrounding blood vessels (bv). Among the two antigens, only carnosine is present in the ependymal monolayer (e). In the olfactory ventricle (C), note that the vast majority of carnosine-LI ependymal cells line the part of the SEL corresponding to a greater thickness and glial cell density. Scale bars: A,B = 15 μm; C,D = 60 μm.

In the present study we have described the distribution of carnosine-LI in the forebrain of adult rabbits, which broadly overlaps that previously observed in rodents, being abundant in olfactory axon terminals within the olfactory bulb, and in cells of the glial lineage, such as astrocytes, oligodendrocytes and ependymal cells. Nevertheless, some differences were noticed in the SEL of the olfactory ventricle, which itself morphologically differs from the corresponding 'rostral extension' described in other species (see Ref. [27]), being not obliterated after birth [19]. Here we show that the SEL of the rabbit is a rather ill-defined tissue, very different from that described in rat and mouse which is characterized by many tightly packed layers of cells sharply separated from the brain parenchyma. Moreover, SEL astrocytes in the rabbit do not compartmentalize the SEL forming glial tubes, rather they are uniformly intermingled with PSANCAM-immunoreactive cells, which represent the newly generated/migrating elements in rodents [6]. It is at present unknown if cell migration and neurogenesis in the olfactory bulb do occur in rabbits. However, by using proliferating cell nuclear antigen (Bonfanti and Merighi, unpublished data) and bromo-deoxyuridine administration at different survival times (Peretto and Bonfanti, ongoing work) reveal the presence of a certain amount of cell proliferation along the SEL of the adult rabbit, apparently with a lower rate of cell migration in comparison with rodents.

In the rabbit, where aminoacyl-histidine dipeptides have been biochemically detected in high concentrations [16], carnosine-LI in the SEL was not very abundant, and progressively decreased in intensity toward the more rostral levels. Unlike laboratory rodents, it was prevalently associated with astrocytes, being virtually absent in the mass of PSA-NCAM-positive cells. Thus, the only relevant difference in the distribution of carnosine-LI between rabbit and laboratory rodents mainly concerns the non-glial compartment of the SEL, and it seems to be related to a different morphologic organization of the SEL itself (lack of clear compartmentalization in glial tubes and cell chains), likely reflecting different rates and/or modalities of cell migration. Moreover, the distribution of astrocytic cells and PSANCAM-positive cells in the SEL of adult rabbits appears rather similar to that described in the postnatal rats before the assembling of glial tubes [26,27], a stage in which carnosine-LI is absent in the SEL [26].

The role(s) of aminoacyl-histidine dipeptides in the CNS still remains obscure, although several hypotheses have been advanced since the first description of carnosine in meat extracts, in 1900 [12]. Most of the current knowledge concerning the function(s) of these molecules comes from in vitro studies, suggesting that they are able to act in different but correlative ways to exert a protective effect in cells, as antioxidants, metal chelators, free radical scavangers, and inhibitors of protein glycosylation (reviewed in Ref. [7]). It has been suggested [7] that a possible explanation for the presence of aminoacyl-histidine dipeptides in the brain could be a protective/regulatory role in cells which directly contact extracellular fluid compartments, either outside the brain parenchyma (e.g. the olfactory mucosa and the ventricular cavities) or at certain locations of the brain, such as the SEL, where fully mature and embryonic tissue coexist, and widened or rapidly changing extracellular spaces are retained. In agreement with this hypothesis, aminoacyl-histidine dipeptides have been recently recognized as a group of osmolytes which could participate in the intercompartmental cotransport of water in cells of the nervous system

[2].
In conclusion, the existence of some differences in the distribution of carnosine-LI in different mammals, linked to a different morphologic organization of the SEL, supports the hypothesis that aminoacyl-histidine dipeptides could be somehow implicated in some aspect of structural plasticity occurring therein. Further studies are required to ascertain if such differences actually reflect alternative forms of neurogenesis or alternative modalities of cell migration across mammalian species (see for example Ref. [11]), and if aminoacyl-histidine dipeptides could play a direct or indirect role in such phenomena.

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