UNIVERSITÀ DEGLI STUDI DI TORINO

CARNOSINE-LIKE IMMUNOREACTIVITY IN ASTROCYTES OF THE GLIAL TUBES AND IN NEWLY-GENERATED CELLS WITHIN THE TANGENTIAL PART OF THE ROSTRAL MIGRATORY STREAM OF RODENTS

P. PERETTO,* L. BONFANTI,*‡ A. MERIGHI* and A. FASOLO†
*Dipartimento di Morfofisiologia Veterinaria, Via Nizza 52, I-10126 Torino, Italy
†Dipartimento di Biologia Animale, Via Accademia Albertina 17, I-10123 Torino, Italy

Abstract- In the nervous system, the aminoacylhistidine dipeptide carnosine (-alanyl-ξ-histidine) has been shown to be expressed in the olfactory receptor neurons and in brain astrocytes. 5 Using immunocytochemical techniques, we report here a dense carnosine-like immunoreactivity in the subependymal layer of the rodent forebrain. Since the subependymal layer involves two distinct compartments (astrocytic cells forming glial tubes and newly-generated cells of the rostral migratory stream, here organized to form chains contained within the glial tubes [Brannon Thomas L. et al. (1996) Glia 17, 1–14; Jancovski A. and Sotelo C. (1996) J. comp. Neurol. 258, 112–124; Lois C. et al. (1996) Science 271, 978–981; Peretto P. et al. (1997) Brain Res. Bull. 42, 9–21]), we investigated in detail the cellular distribution of carnosine-like immunoreactivity in this area. By using double labelling techniques with antisera raised against carnosine and specific markers of glial tubes or chains of migrating cells, we show that carnosine-like immunoreactivity is associated with both the compartments. On the other hand, unlike markers of the rostral migratory stream, carnosine-like immunoreactivity was not observed in isolated, migrating cells located outside the subependymal layer, which spread through the olfactory bulb in a radially-oriented manner. This suggests that carnosine is transiently expressed by cells of the rostral migratory stream when moving in the tangentially-oriented part of the migration route. Moreover, we investigated the distribution of carnosine-like immunoreactivity in the postnatal rat forebrain and found that it is detectable in the subependymal layer only starting from the third postnatal week, although it is well known that the dipeptide is present in the olfactory receptor neurons since the embryonic day 16 [Biffo S. et al. (1992) J. chem. Neuroanat. 5, 51–62].
Taken together, these results show that carnosine, other than abundantly present in astrocytes of the glial tubes, is associated to the tangential part of the rostral migratory stream.

Key words: aminoacylhistidine dipeptides, plasticity, cell proliferation, cell migration, subependymal layer, brain.

Carnosine (β-alanyl-L-histidine) is an aminoacylhistidine dipeptide present in muscle and in discrete cell populations of the nervous system of many vertebrates. [17] It is synthesized by carnosine synthetase from its component amino acids and degraded by carnosinase. Although many theories have been proposed about its biological function(s) [24,43] in the nervous system or elsewhere, none of them have yet been substantiated (for review see Ref. 38).

‡To whom correspondence should be addressed.
Abbreviations: BrdU, bromodeoxyuridine; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; LI, like immunoreactive/ immunoreactivity; NCAM, neural cell adhesion molecule; P, postnatal day; PBS, phosphate-buffered saline; PSA, polysialic acid; PSA-NCAM, polysialylated isoform of NCAM; RMS, rostral migratory stream; SEL, subependymal layer.

In the nervous system of adult mammals, carnosine has been found both in neurons and glial cells, [5] with a singular distribution. Its neuronal localization is restricted to a cell population which is located outside the brain: the olfactory receptor neurons. These receptors undergo a striking plasticity during adulthood, including an intense cell renewal and growth of their axonal processes directed to the olfactory bulb (for review see Ref. 15). Indeed, within the brain, the only neuronal structures containing carnosine are the primary olfactory neuron projections in the glomerular layer of the olfactory bulb. On the other hand, in the brain, carnosine is associated with a large population of glial-like cells, scattered within both the white and gray matter, and to ependymal cells which line the ventricular cavities. [5] Previous studies indicated that most of the carnosine-like immunoreactive (LI) cells in the brain are astrocytes, including a particular type of astrocytic cells in the cerebellum, known as Bergmann glia, which represent a remnant of the embryonic radial glia subserving the postnatal migration of cerebellar granule cells. [5]

An example of specialized glia, associated with persisting cell proliferation and migration, has been recently demonstrated to occur in the forebrain of the adult mouse [14,22,26] and rat. [36] It consists of a meshwork of astrocytic cells and processes organized to form channels within a strip of tissue originating from the primitive subventricular zone and referred to as the subependymal layer (SEL, [13] see Fig. 1), which surrounds the anterior horn of the lateral ventricle and extends toward the olfactory bulb (SEL rostral extension). These channels are particularly evident in the rat, wherein they have been indicated as ‘‘glial tubes’’, 36 serving as a glial guidance for cells generated in the SEL and directed to the olfactory bulb, [22,26,36] most of which are thought to be neuronal precursors. 25–27 Since the fate of these cells, at different levels of the SEL, is still a matter of debate, we will generally refer to them as ‘‘subependymal cells’’. [36] Ultrastructural studies confirmed that the cellular composition of the SEL involves at least two distinct compartments: the glial cells of the tubes and the subependymal cells [14,22,26,36] (Fig. 1). The entire mass of migrating cells is referred to

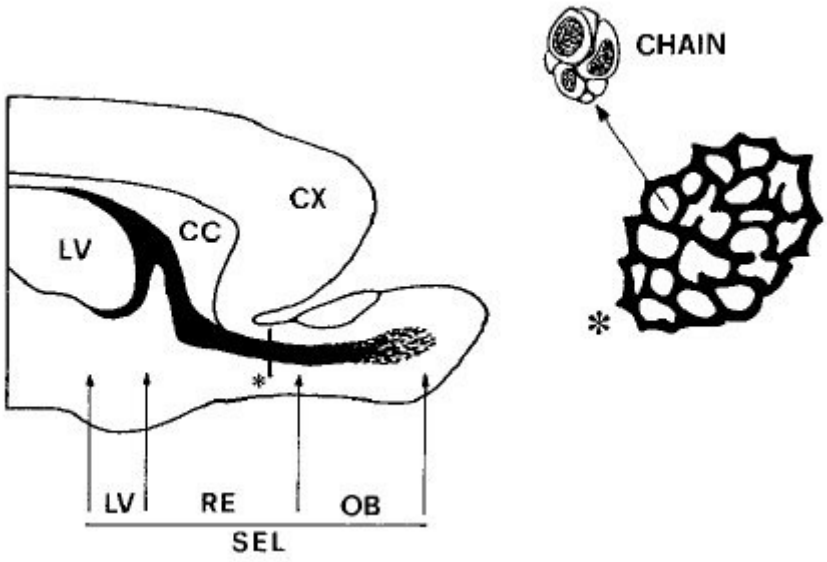

Fig. 1. Schematic representation of the subependymal layer (SEL) in the adult rodent brain. In parasagittal view (left), the SEL (black area) can be divided into: a portion lining the anterior part of the ventricular wall (LV) and the SEL rostral extension (RE). The anterior part of this latter, located in the centre of the olfactory bulb (OB), appears larger and ill-defined. The SEL tissue is characterized by two cell compartments, which are easily recognizable in coronal sections of the rostral extension [22,26,36] (asterisk and right): astrocytes forming glial tubes (in black) and chains of migrating neuronal precursors (subependymal cells). CC, corpus callosus; CX, cerebral cortex; LV, lateral ventricle

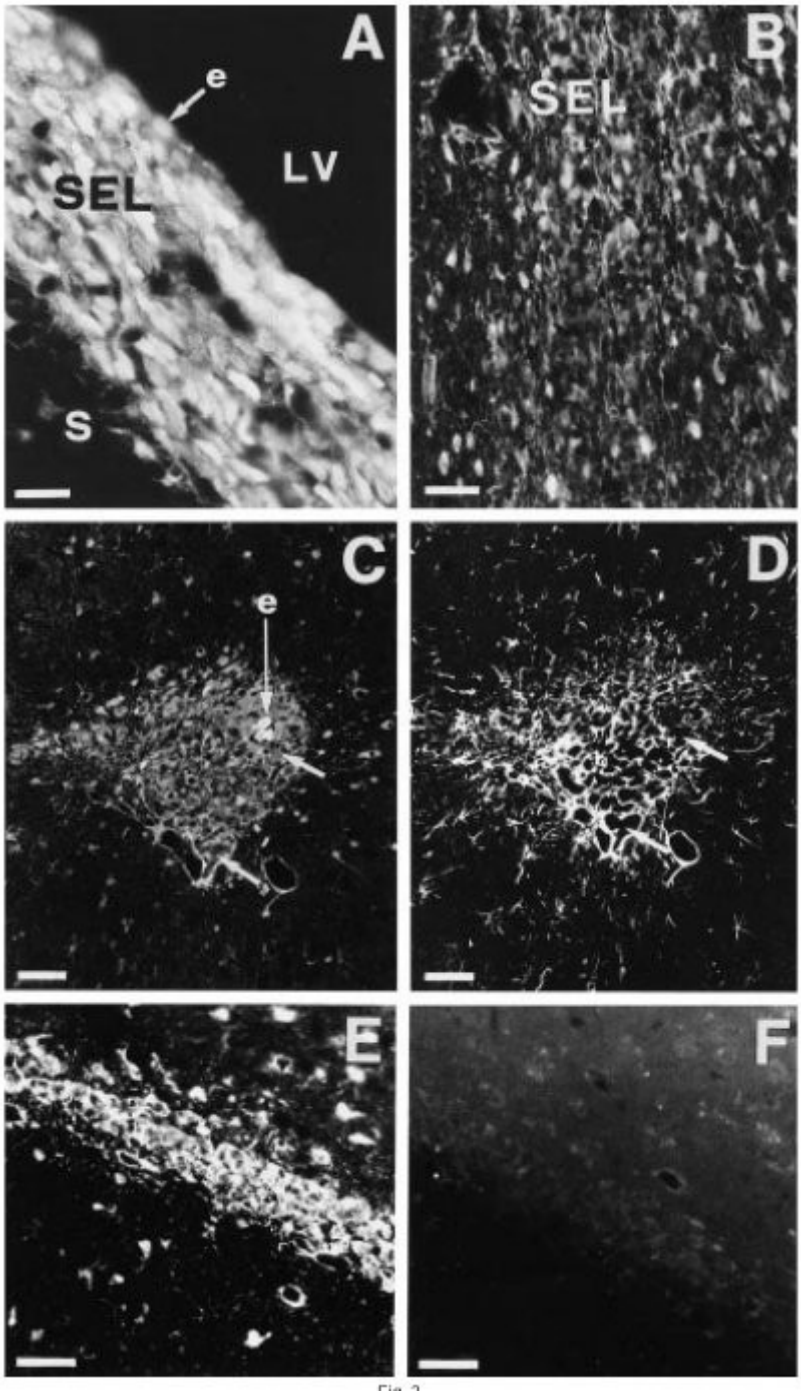

Fig. 2. Distribution of carnosine-LI in the SEL of the adult rat. (A) Parasagittal section cut in the anterior part of the lateral ventricle (LV); virtually all cells in the SEL are highly immunoreactive, in contrast with the adjacent striatum (S). Most of the immunoreactive cells are elongated, with a prevalent longitudinal arrangement. Also the ependyma (e) lining the ventricle appears strongly immunopositive. (B) Coronal section in the center and ventral portion of the olfactory bulb: the carnosine-LI cells are less tightly-packed than in (A) and prevalently correspond to astrocytic-like cells localized both within and outside the SEL (very ill-defined at this level). (C,D) Simultaneous localization of carnosine (C) and GFAP (D) in a coronal section of the SEL rostral extension (horizontal arm). Carnosine-LI is associated to a great number of cells localized in the SEL area, most of which filling the glial tubes (see arrows for example), and to scattered glial cells in the surrounding tissue. A cluster of three highly-immunoreactive (GFAP-negative), residual ependymal cells (e) is also visible. (E,F) Adjacent sections of the SEL rostral extension, treated with the anti-carnosine serum (E) or after absorption with BSA-conjugated peptide (F). Scale bars: A=15 μm; C,D=50 μm; B,E,F=30 μm.

as the rostral migratory stream (RMS, see Refs 1 and 25). In the SEL, the cells of the RMS form tangentially- oriented chains moving inside the glial tubes. By contrast, these cells undergo two striking changes as they reach the olfactory bulb: (i) they leave the SEL and the glial tubes (which became ill-defined at this level, see Ref. 36), spreading in a fan-shaped manner through the granule and periglomerular layers; (ii) they cease to be organized in tangentially-oriented chains, moving as isolated, radially-oriented neuroblasts. [12,25,27]

In the present study, by using immunocytochemical techniques, we describe the occurrence of a high concentration of carnosine-like immunoreactivity in the SEL of adult rodents. We investigated

in detail its distribution in this area of the brain, since it is characterized by the co-existence of a population of newly-generated cells and a specialized type of glia. This study was carried out in the rat, in which the two SEL compartments are clearly distinguishable [36] and in the adult mouse, wherein such type of cell migration has been well characterized. [14,22,23,25,26]

With the aim to investigate the presence of the carnosine-LI cells in the different SEL compartments, we performed double labellings using carnosine and either glia-specific antigens or markers which identify cells of the RMS (see for example Refs 11, 12, 14, 16, 22, 26, 36 and 41). We used antibodies raised against the glial fibrillary acidic protein (GFAP 8), an astrocytic marker highly expressed in the glial tubes of adult rodents [14,22,26,36] and the cytoskeletal protein vimentin, which is abundant in immature glial cell populations, ependymal cells, Bergmann glia and developing radial glia [37] and has been described to persist in astrocytes of the rat glial tubes, during adulthood. [36] To identify the migrating cells, antibodies raised against the polysialylated form of the neural cell adhesion molecule (PSA-NCAM [40]), the neuron-specific, class III β-tubulin, [32] and the phosphoprotein stathmin [45] were used. Furthermore, the immunocytochemical detection of systemically- administered bromodeoxyuridine (BrdU) was employed to detect *in vivo* the actively proliferating cells.

Finally, in order to define the temporal pattern of carnosine appearance in the rat forebrain, the study was extended to the postnatal period.

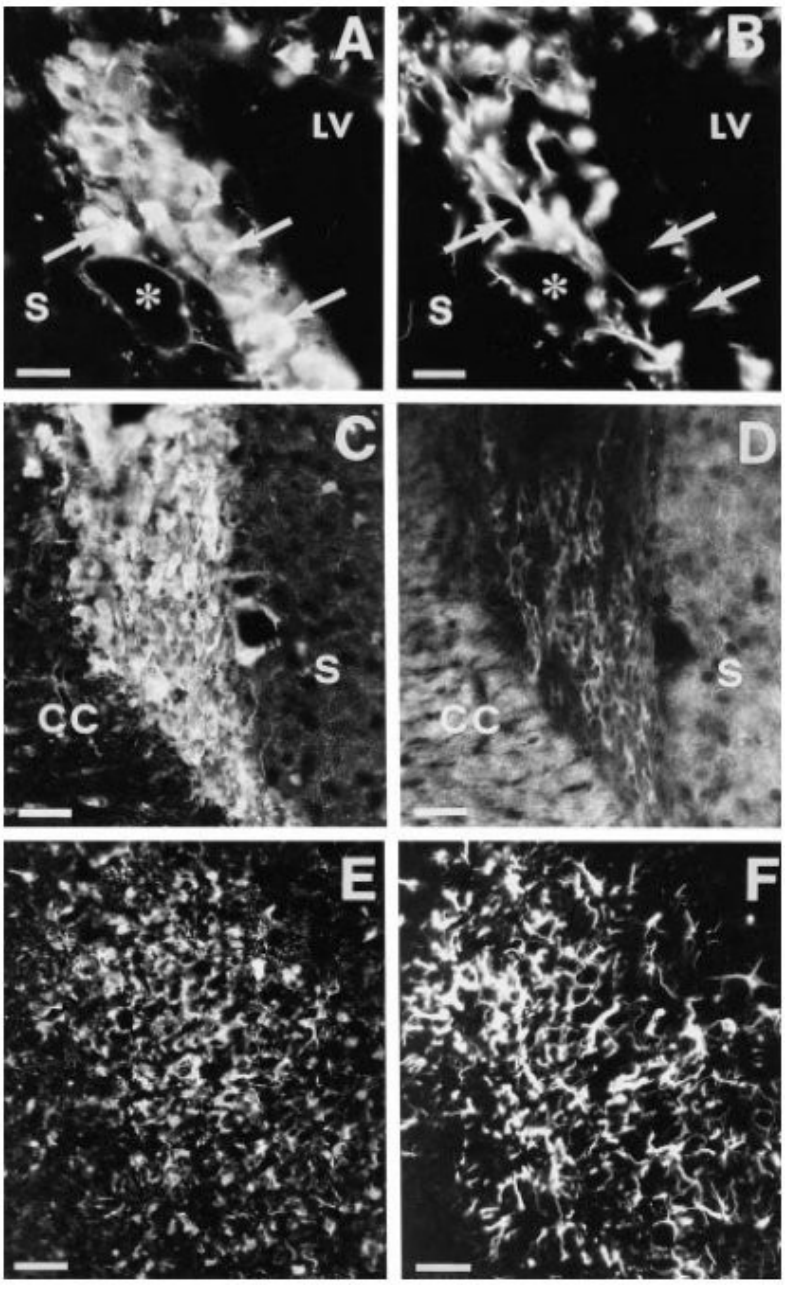

Fig. 3. Distribution of carnosine-LI in the SEL of the adult mouse (coronal sections). (A,B) Double labelling for carnosine (A) and GFAP (B) in the dorsolateral corner of the lateral ventricle (LV). Carnosine is highly expressed in the SEL (adjacent to the striatum, S), including many glial cells (note the double labelling with GFAP) and subependymal cells which fill the lumina of the glial tubes (arrows). A blood vessel is indicated by the asterisk. (C,D) Simultaneous localization of carnosine (C) and class III β-tubulin (D) in the first part of the SEL rostral extension (vertical arm). Note that carnosine is highly expressed in the whole SEL area, whereas the immunoreactivity for class III β-tubulin is restricted to chains of subependymal cells and appears to be diffuse in the surrounding, mature gray (striatum) and white (corpus callosum) matter. (E,F) Double labelling for carnosine (E) and GFAP (F) in the SEL of the olfactory bulb. The dipeptide is prevalently associated to astrocytic glial cells and processes of very ill-defined glial tubes. Scale bars: A,B=7.5 μm; C–F=15 μm.

EXPERIMENTAL PROCEDURES

Tissue preparation

Brains were obtained from 18 young (postnatal day (P)2, P5, P9, P13, P17, P21, P25, P30, P60; two animals for each age) and 12 adult (three- to five-months-old) Wistar rats (Charles River, Italy), and three adult (three-months-old) CD-1 mice (Charles River, Italy). All experiments were performed in accord with the current Italian law, under authorization of the Italian Ministry of Health, n. 600.8/ 24433/82.20/AG1826. Animals were deeply anaesthetized with intraperitoneal sodium pentobarbital (Pentothal Sodium, Gellini, Italy; 60 mg/100 g, i.p.) and then perfused intracardially first with an heparinized saline solution (25 IU/ml in 0.9% NaCl, during 1–3 min) followed by a freshly prepared solution of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After dissection, brains were postfixed overnight in the same fixative, cryoprotected in ascending sucrose solutions, frozen in liquid nitrogen- cooled isopentane at 70 C, and cryostat sectioned in series (6–8 μm). Coronal and parasagittal sections were collected onto gelatine-coated slides.

Bromodeoxyuridine labelling

Nine postnatal (one for each age) and six adult rats were injected intraperitoneally with 2 mg BrdU/100 g body weight in 0.1 M Tris, pH 7.4; then were perfused transcardially, after 1 h (postnatal rats), 1 h, five or 15 days (adult rats) survival. Animals killed after 15 days, underwent two subsequent BrdU administrations, separated by an interval of 24 h.

Antibodies

The following primary antisera and antibodies were used: (i) anti-carnosine, a polyclonal rabbit IgG (F. Margolis, Nutley, U.S.A.), diluted 1/1000; (ii) anti-anserine, a polyclonal rabbit IgG (F. Margolis, Nutley, U.S.A.), diluted 1/400; (iii) anti-bromodeoxyuridine, a monoclonal IgG (anti-BrdU, Boehringer, Germany), diluted 1/50; (iv) anti-glial fibrillary acidic protein, a polyclonal rabbit IgG (anti- GFAP, Dako, Denmark) and a monoclonal IgG (Boehringer, Germany), diluted 1/600 and 1/20, respectively; (v) anti-vimentin, a monoclonal IgG (Dako, Denmark), diluted 1/600; (vi) anti-PSA-NCAM, a monoclonal immunoglobulin M (IgM) raised against the capsular polysaccharides of meningococcus group B that share α-2,8- PSA residues with PSA-N-CAM (for further details on production and specificity, see Ref. 39), diluted 1/1000; (vii) anti-stathmin, a polyclonal rabbit IgG (A. Sobel, Paris, France), diluted 1/1000; (viii) anti-class III -tubulin, a monoclonal IgG (Sigma, U.S.A.), diluted 1/400.
All the antibodies were diluted in a solution of 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.1% Triton X-100.

Immunocytochemistry

Immunocytochemical reactions were carried out by using single and double immunofluorescence methods and/or indirect peroxidase procedure. After a 10 min wash in 0.01 M PBS, sections were incubated overnight at 4 C in a solution of PBS/Triton X-100 containing normal serum and primary antibodies. Detection of BrdU immunoreactivity required treatment with 2 M HCl for 1 h at 37 C, prior to incubation in primary antibodies.

Immunofluorescence. Affinity-purified goat anti-mouse IgG (Fab-specific, Sigma, U.S.A., 1/40) and sheep anti- mouse Ig (Boehringer, Germany, 1/50) or swine anti-rabbit IgG (Dako, Denmark, 1/50) immunoglobulins coupled to fluorescein isothiocyanate (FITC), swine anti-rabbit (Dako, Denmark, 1/50) coupled to rhodamine, and Texas Red–avidin (Vector, U.K., 1/500), served as immunolabels. Sections were mounted in a solution of PBS/glycerol (9/1) and examined with a conventional

epifluorescence microscope, using appropriate filters.

Peroxidase staining. Peroxidase reactions were developed using the biotin–avidin system (Vector, U.K.), with 3,3 - diaminobenzidine as a chromogen. Slides were then dehydrated and mounted in DPX (Raymond A. Lamb, U.K.).
For double stainings, a combination of two indirect immunofluorescence procedures, using FITC+rhodamine or FITC+Texas Red-conjugated secondary antibodies, or a sequential localization of immunoperoxidase and immunofluorescence methods, were used.
Immunocytochemical controls included: (i) incubation of sections in carnosine antiserum pre-absorbed with 10 μM of bovine serum albumin (BSA)–carnosine conjugated peptide; (ii) omission of primary antibodies; and (iii) incubation of sections in primary antibodies followed by inappropriate immunolabels.

RESULTS

Carnosine-like immunoreactivity in the subependyma layer of adult rodents
In accordance with previous immunocytochemical studies, carnosine-LI was observed in sections of the rat and mouse brain, associated with: a wide population of stellate, glial-like cells, the glial coverage of blood vessels, ependymal cells and tanycytes surrounding the ventricles, and fibres of the olfactory receptor neurons reaching the glomerular layer of the olfactory bulb. In the adult forebrain, between the lateral ventricle and the olfactory bulb, a dense carnosine-LI was detected in the strip of tissue corresponding to the SEL of the lateral ventricle and its rostral extension (Fig. 2A–C). After preabsorbtion of the anti-carnosine serum on BSA-conjugated peptide, immunoreactivity was abolished within the SEL and elsewhere (Fig. 2E,F). No immunoreactivity was observed in the rodent brain by using an antiserum which recognizes anserine.
In single carnosine immunostainings performed all along the SEL, the immunoreactivity was associated to a high number of small, tightly-packed cells, showing a cytoplasmic and (some of them) a faint nuclear reaction (Figs 2A,C, 3A,C, 4A,C,F, 6A).
We examined the distribution of carnosine-LI at different levels of the RMS (see Fig. 1). At the level of the lateral ventricle, the immunoreactivity was mainly detected in the subependyma of the anterior horn (Figs 2A, 3A). In more posterior and ventral parts of the ventricular wall, in which the SEL is limited to scattered groups of subependymal cells, only small clusters of carnosine-LI cells were observed (not shown). A striking carnosine-LI was also present throughout the SEL rostral extension (Figs 2C, 3C, 4A,F, 6A). In coronal sections of its horizontal arm, wherein an optimal view of the glial tubes and chains of migrating cells can usually be obtained (see Fig. 1), the carnosine-LI cells did not appear to be exclusively associated with one of these compartments. By contrast, in the peripheral part of the SEL, where some chains are separated from each other by the presence of mature neuropil (see Ref. 36), some isolated clusters of 3–5 carnosine-LI cells could be appreciated (Fig. 4C). Within each of these clusters the cells displayed a large nucleus and a thin rim of cytoplasm. In sagittal views, some of the carnosine-LI cells showed elongated cell bodies with ellipsoid nuclei (Fig. 2A) and, in favourable sections, also elongated processes reminiscent of leading or trailing processes. Clusters of tightly-packed, carnosine-LI cells were frequently observed in close apposition to the wall of blood vessels located within the SEL or in its immediate vicinity (Fig. 4D). These clusters were different from the thin glial coverage (carnosine- and GFAP-positive) detectable on blood vessels throughout the brain tissue. Along the SEL rostral extension, the immunoreactivity was also present in residual ependymal cells (Figs 2C, 4A,F; see Ref. 36), which can be recognized for their larger size and very strong carnosine immunoreactivity.

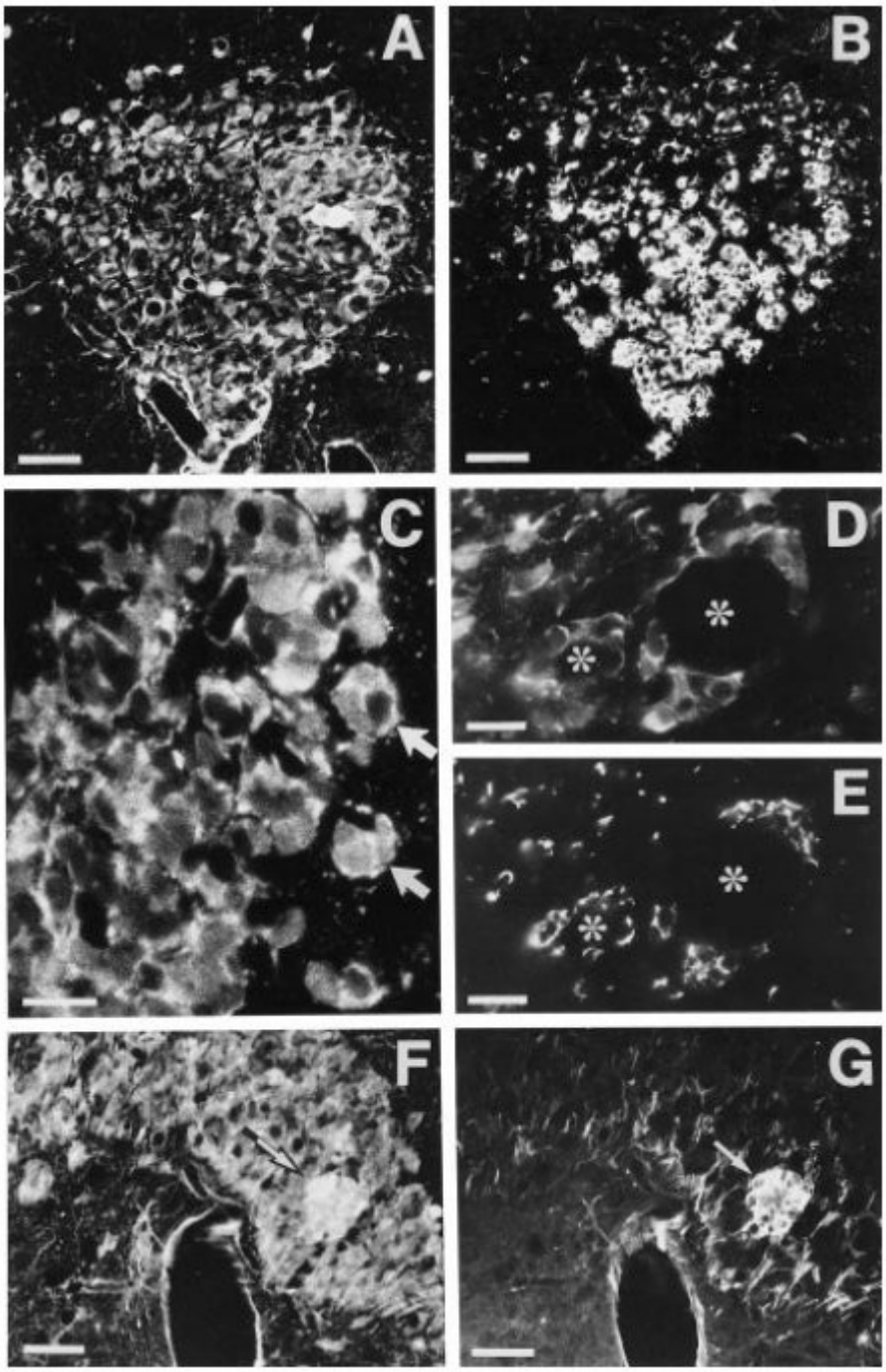

Fig. 4. Immunoreactivity for carnosine and other antigens in the SEL rostral extension of the adult rat (coronal sections). (A,B and D,E) Simultaneous localization of carnosine (A,D) and PSA-NCAM (B,E). Note a partial overlap between carnosine-LI and clusters of subependymal cells revealed with PSA-NCAM. A group of residual ependymal cells is highly immunoreactive for carnosine (in A, on the right), but not for PSA-NCAM (in B). In (D,E) clusters of globose cells, immunoreactive for the two antigens and in close contact with two blood vessels (asterisks). (C) In favourable sections, carnosine-LI is associated to typical clusters of subependymal cells (arrows) in the peripheral part of the SEL, composed of tightly-packed cells with a large nucleus and a thin halo of cytoplasm. A faint immunoreactivity is also visible within some nuclei. (F,G) Double labelling for carnosine (F) and vimentin (G), showing a group of residual ependymal cells (arrow). In the SEL, vimentin is restricted to the glial wall of the tubes, whereas carnosine-LI virtually fills the area. Scale bars: A,B,F,G=30 μm; C=10 μm; D,E=15 μm.

In the olfactory bulb, an accumulation of carnosine-LI cells was also visible in the SEL (Figs 2B, 3E), although the cell density appeared to be reduced, a feature which was particularly evident in the mouse. These cells were distributed in a wide area corresponding to the enlarged, less tightlypacked SEL, and most of them were clearly recognizable as stellate glial cells. Thus, in the olfactory bulb, it was not easy to clearly identify a limit between the carnosine-LI cells in the SEL and those scattered in the surrounding tissue, also characterized by a stellate, astrocytic-like morphology.

Double labelling experiments with carnosine and glial markers. In carnosine/GFAP double labellings it appeared evident that a great number of carnosine-LI cells detected in the SEL filled the lumina of the glial tubes (Figs 2C,D, 3A,B, 6A). Moreover, an overlapping of the two antigens was observed in the glial meshwork forming the tubes (Fig. 6A). Such a distribution was detected all along the system, from the lateral ventricle to the olfactory bulb, although, in the latter, carnosine

appeared to be prevalently expressed by astrocytes rather than subependymal cells (Figs 2B, 3E). In the mature tissue surrounding the SEL, the GFAP/carnosine double labellings revealed that most of the GFAP-positive, stellate astrocytes also express carnosine, although some carnosine-LI, GFAP-negative cells, characterized by a round-shaped cell body and scarce processes were also detected (not shown).
Single vimentin staining confirmed, in the rat, the occasional occurrence of residual ependymal cells within the SEL, recognizable as clusters of globose, vimentin-positive (GFAP-negative) cells intermingled with the glial tubes (which also express vimentin in the rat: Fig. 4G; see Ref. 36). As expected, the clusters of ependymal cells were also strongly immunoreactive for carnosine (Fig. 4F,G). Such preparations confirmed that the great majority of carnosine-LI subependymal cells contained withinb the glial tubes are vimentin immunonegative, thus indicating that most of these cells do not correspond to residual ependyma.

Double labellings with carnosine and markers of the rostral migratory stream. n carnosine/PSA-NCAM double labellings, only a partial overlapping was detected all along the SEL of the lateral ventricle and its rostral extension. The chains of migrating cells identified with the PSA-NCAM antibody frequently included carnosine-LI cells (Fig. 4A,B), particularly in the posterior part of the SEL. However, carnosine-LI was also present in structures which did not take part in these chains, corresponding to the glial cells described above. Toward the olfactory bulb, an overlap between the two antigens was still observed in the SEL, whereas it was not detectable in the olfactory bulb, outside of the SEL (Fig. 6B). At this level, corresponding to the radially-oriented part of the migratory stream, carnosine-LI was restricted to stellate, glial-like cells (Figs 2B, 3E, 6B). On the whole, the partial overlapping of the two antigens was limited to the chains of cells located in the SEL and contained into the glial tubes, including the clusters of cells associated to the blood vessels of the SEL (Fig. 4D,E). A pattern of distribution very similar to that described above was observed in double labellings for carnosine and stathmin (not shown), another marker specifically associated with cells of the RMS.[16] As expected, a striking co-existence of PSA-NCAM and stathmin with carnosine-LI was also detectable in the olfactory afferent fibres of the glomerular layer, in the olfactory bulb.
Again, a similar pattern of distribution was observed in double labellings with β-tubulin which, in the SEL, revealed the chains of migrating cells, in great majority overlapping with the mass of carnosine-LI cells (Fig. 3C,D). As expected, the immunoreactivity for -tubulin was also detectable in the surrounding, mature nervous tissue.

Carnosine-like immunoreactivity and markers of the rostral migratory stream in the postnatal rat forebrain

Carnosine-like immunoreactivity. From the earliest developmental stage investigated in this study (P2), carnosine immunoreactivity was consistently detected in the olfactory receptor neurons of the nasal mucosa and in their axonal projections to the olfactory bulb glomerular layer. Up to P9 (Fig. 5A), in the anterior forebrain the immunoreactivity was restricted to such primary afferent fibres, whereas no immunoreactivity was detectable in the SEL area (Fig. 5A,B). At the ventricular level, carnosine immunoreactivity was observed at P9 in ependymal but not subependymal cells. On the whole, in the rat brain, if one excludes the ependyma and the primary olfactory projections, carnosine-LI was first detected at P13–P17, as a faint reaction in some glial-like cells. At P17, this pattern of immunostaining was more evident, involving a considerable number of astrocytic-like cells, scattered in the olfactory bulb and forebrain. Many of these cells, strongly immunoreactive for carnosine, were detected in the white matter of the olfactory tract and optic chiasm, and to a lesser extent in the external perimeter of the SEL. Between P21 and P25 virtually all the SEL area was filled by a great number of tightly-packed, carnosinepositive cells, making it difficult to distinguish between glial and subependymal cells (Fig. 5C). Starting from P25 and in the subsequent postnatal

stages examined, the distribution of carnosine-LI in the SEL was overlapping to that described in the adult.

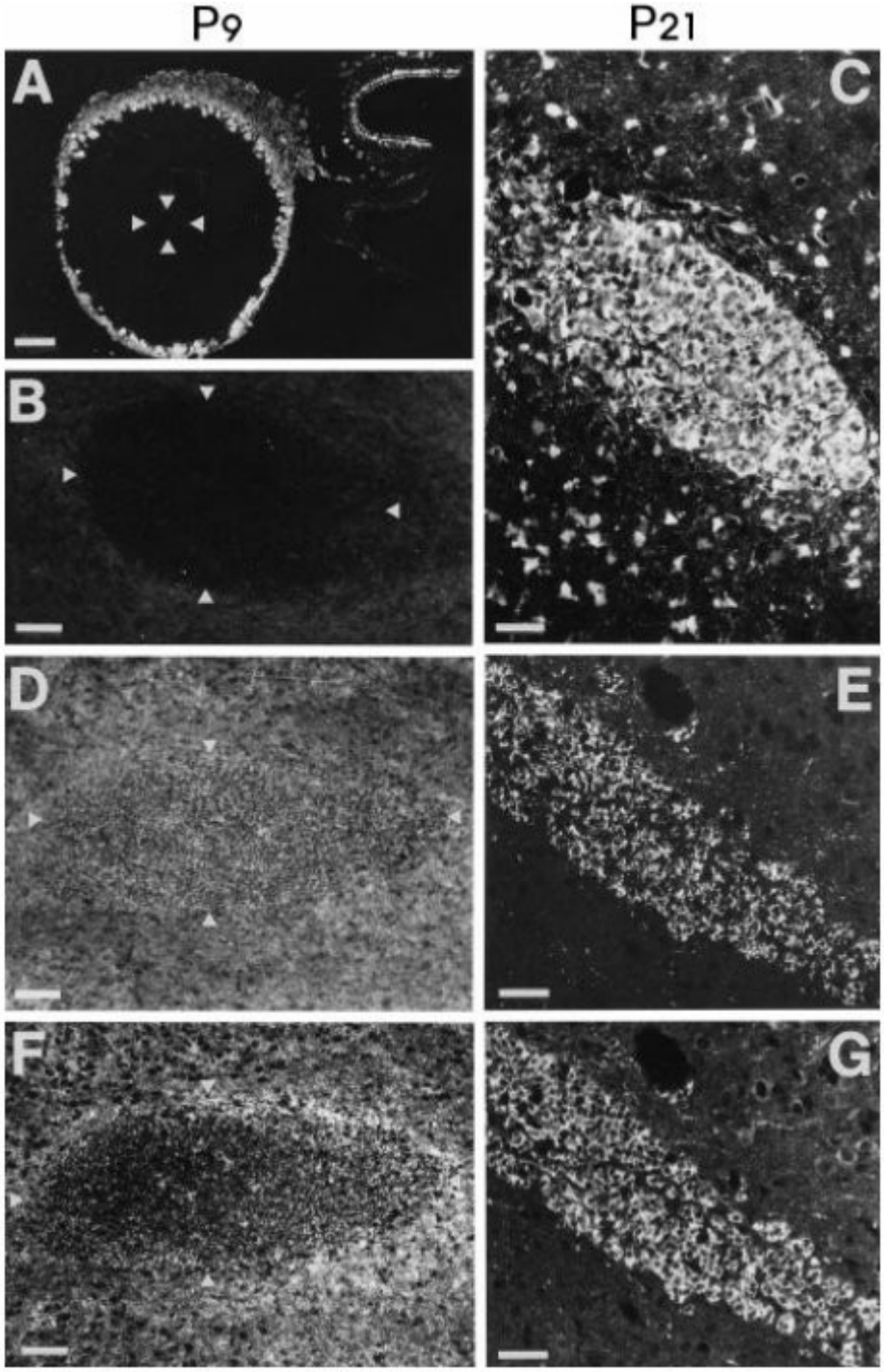

Fig. 5. Immunoreactivity for carnosine (A–C) and markers of the rostral migratory stream (D–G) in the postnatal rat forebrain: (A,B,D,F) animals killed at P9; (C,E,G) animals killed at P21; coronal sections of the SEL rostral extension, except for A (olfactory bulb and mucosa). Very strong carnosine-LI is visible at P9 in olfactory receptor neurons (A, top right) and in their axonal projections to the olfactory bulb glomerular layer (A, left). No carnosine-LI is detectable at P9 in the SEL (area indicated by arrowheads), even in the SEL rostral extension (in B). At P21 (C), strong carnosine-LI is present in the SEL and in astrocytes of the surrounding tissue. PSA-NCAM (D,E) and stathmin (F,G) immunoreactivities appear to be rather diffuse and uniform both within and outside the SEL at P9 (D,F), whereas, at P21 (E,G) these antigens are restricted to the SEL area and are associated to clusters of subependymal cells. Scale bars: A=200 μm; B,D,F=60 μm; C,E,G=30 μm.

Double labelling experiments with carnosine and bromodeoxyuridine

Adult rats. In sections of the rat forebrain, 1 h or five days after BrdU injection, a great number of newly-generated cells which had incorporated BrdU in the nucleus were detected in the SEL. In particular, as previously described,[36] after five days survival most of the migrating cells were concentrated in the horizontal arm of the SEL rostral extension (Fig. 6C). In carnosine/BrdU double labellings performed at this level, it appeared evident that many BrdU-positive nuclei belong to carnosine-LI cells, both after 1 h and five days survival (Fig. 6C). However, due to the pattern of carnosine staining, it was difficult to exactly ascertain if all the cells whose nuclei had incorporated

BrdU were also carnosine-LI. In animals killed 15 days after BrdU injection, no double labellings with carnosine-LI (glial) cells were observed in the olfactory bulb, outside the SEL area (not shown).

Postnatal rats. At earlier stages (up to P13) many BrdU-positive nuclei were observed both in the SEL area and in the surrounding tissue (expecially in the olfactory bulb). Starting from P17–P21 they were progressively concentrated within the SEL, although some of them were still detected in the outside tissue (Fig. 6D). At these stages, corresponding to the first appearance of carnosine-LI in the SEL (see above), a complete co-expression of carnosine and BrdU was visible in the same cells within the SEL area, but not in the outside tissue (Fig. 6D). From P25–P30 the newly generated cells were detected exclusively in the SEL, with a distribution similar to that described in the adult.

DISCUSSION

Specificity of the anti-carnosine serum

The polyclonal antiserum used here to localize carnosine has been well characterized in previous immunocytochemical and biochemical studies.[2,5–7,21,42] This antiserum can react with different aminoacyl dipeptides such as carnosine, homocarnosine and anserine.5 Anserine has been described in avian brains20 and in mammalian retinas,[29] but it is absent in the mammalian brain.[3–5,29,34,49] This was confirmed by the absence of immunoreactivity after using the anti-anserine serum on our specimens. Thus the immunoreactivity detected in the present study must be ascribed to carnosine and/or homocarnosine, and for this reason it has been referred to as carnosine-LI. However, previous biochemical studies demonstrated that carnosine is the dipeptide expressed in rodent olfactory neurons and that it is present at high levels in the olfactory bulb (for review see Ref. 36). After liquid-phase absorptions of the antiserum with a BSA-conjugate dipeptide, the immunoreactivity was completely abolished in the SEL, as well as in other brain regions.

Carnosine-like immunoreactivity in the adult rodent forebrain

In the present study, in agreement with previous immunocytochemical studies,5 we observed a selective localization of this dipeptide in specific neuronal and glial cell populations of the rodent nervous system, and we report for the first time a dense carnosine-LI in certain periventricular regions of the adult forebrain. The immunoreactivity appeared to be highly concentrated in the SEL, a site of active cell proliferation persisting during adulthood.[1,44] A great deal of attention has been recently focused on this area of the rodent brain, since the huge amount of undifferentiated cells which are generated herein undergo a long distance migration to their final destination in the granule and glomerular layers of the olfactory bulb.[25,27] The entire population of these migrating cells forms the RMS; on the other hand, the SEL (including its rostral extension) is an identifiable anatomical region in which the first, tangentially-oriented part of the RMS is contained. Accordingly, we analysed in detail the distribution of carnosine-LI within the entire system, to elucidate the relationships existing between: (i) the distribution of carnosine-LI and the different compartments of the SEL; (ii) the distribution of carnosine-LI and the entire RMS.

Carnosine-like immunoreactivity in the subependymal layer: glial tubes and subependymal cells

The SEL is composed of small, tightly-packed cells apparently forming an homogeneous tissue, which actually involves two distinct cellular compartments:(i) the newly-generated, migrating cells, which are referred to as subependymal cells and are organized to form chains, and (ii) the astrocytes of the glial tubes, in which the chains of subependymal cells are contained (see Fig. 1). From the results of the present study it appeared clear that carnosine-LI was associated to both these cellular

compartments. In single staining preparations using the anti-carnosine serum, we observed a pattern of immunoreactivity which was different from that obtained to visualize either

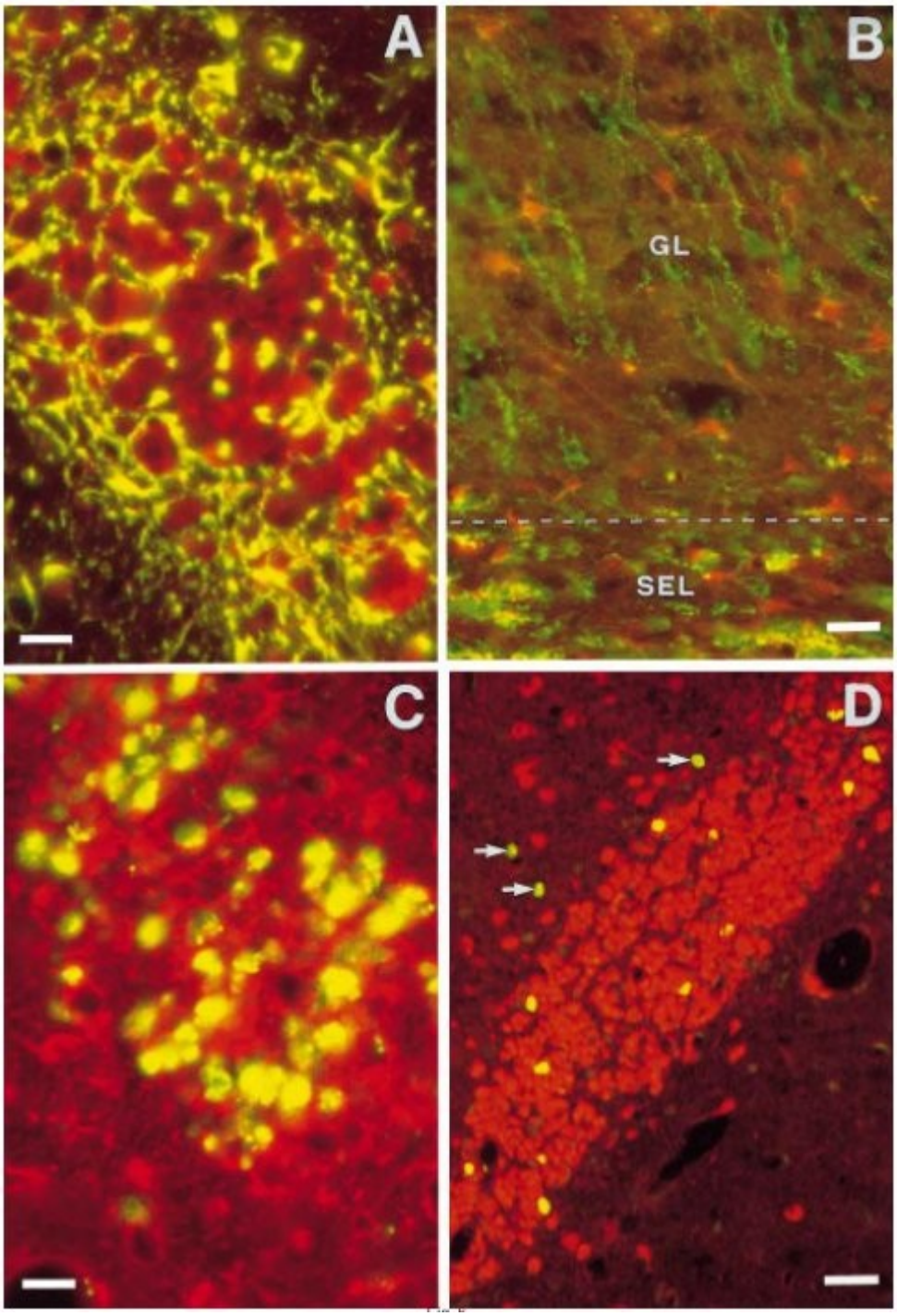

Fig. 6. Simultaneous localization of carnosine-LI and GFAP (A), PSA-NCAM (B), BrdU (C,D), in the adult (A,B,C) and postnatal (D) rat forebrain. (A,C,D) coronal sections of the SEL rostral extension; (B) parasagittal section in the olfactory bulb. (A) carnosine-LI cells (red) fill the lumina of GFAP-positive (green) glial tubes, which mostly appear yellow since also immunoreactive for carnosine. (B) In the olfactory bulb carnosine-LI (red) is associated to scattered, stellate-shaped glial cells whereas it is absent in radially-oriented, PSA-NCAM-positive (green) neuroblasts. A partial overlap between the two antigens (yellow) is restricted to tangentially-oriented chains of the SEL. The dotted line marks the limit between the SEL area and the granular layer (GL). (C) Carnosine-LI cells (red) show a BrdU nuclear staining (green; appearing yellow for the overlap), five days after peritoneal injection. (D) A huge number of tightly-packed, carnosine-LI cells (red) in the SEL at P21. Double labelling with BrdU (green) reveals a complete co-existence (yellow) in these cells. Three BrdU-positive nuclei which not co-localize with carnosine are also visible outside the SEL (arrows). Scale bars: A,C=20 μm; B,D=40 μm.

the astrocytic glia or the subependymal cells (see schematic representation in Fig. 7 and Table 1). With glial markers (GFAP, vimentin, S-100; see Refs 14, 22, 26 and 36 and this study) the immunostaining is restricted to the glial tubes, which appear immunonega tive within their lumina. On the other hand, the molecules expressed by the undifferentiated, migrating cells (PSA-NCAM, stathmin, class III β-tubulin, see Refs 11, 16 and 41 and this study) are selectively associated to well-recognizable clusters (or chains, in parasagittal sections) of tightly-packed cells. By contrast, carnosine-like immunoreactivity observed along the SEL appeared as a dense reaction, rather uni formly distributed through the tightly-packed tissue forming the subependyma and apparently including the glial tubes and their content. This was confirmed by double labelling preparations

using carnosine and several combinations of such markers, in which carnosine-LI was overlapping both with astrocytes of the glial tubes and with subependymal cells. The overlap with subependymal cells was more evident in the posterior/medium part of the SEL, including the lateral ventricle and most of the rostral extension. In more anterior parts, corresponding to the SEL of the olfactory bulb, the overlap was mainly restricted to astrocytes. In comparison, this aspect was more evident in the mouse and represented the main difference observed among the two rodent species examined.

The presence of carnosine in astrocytes of the glial tubes was expected since the dipeptide is abundant in most of the astrocytic cells of the adult brain,[5] although its function has not yet been elucidated. However, the glial cells of the SEL, at least in the rat, also express vimentin,[36] a cytoskeletal protein usually abundant in immature cells, such as radial glia,[9,18] and in several adult cell populations which also are immunoreactive for carnosine, namely the cerebellar Bergmann glia, tanycytes, ependymal cells and radial glia-like cells in the hypothalamic supraoptic nucleus (see Refs 5, 10, 37, and this study). It has been suggested that vimentin expression could be related to the connections with increased extracellular fluid compartments or direct contact with cerebrospinal fluid.[37] Indeed, the SEL originates from the primitive subventricular zone, thus retaining embryonic features as revealed in ultrastructural studies by several cytological features and by the tendency of the cells to shrink during fixation, leaving enlarged extra cellular spaces.[22,26,36]

Interestingly, our study shows that carnosine also is highly expressed in newly-generated cells which fill the lumina of the glial tubes. We demonstrated this using different approaches. Firstly, we show that in this compartment of the SEL carnosine is co-expressed with: (i) PSA-NCAM, an embryonic cell surface molecule which has been demonstrated to act as a permissive factor allowing this particular type of cell migration[35]; (ii) class III β-tubulin, a cytoskeletal protein early expressed by undifferentiated neuronal precursors32 and abundant in cells of the RMS[14,22,36]; and (iii) stathmin, a cytosolic phosphoprotein abundantly expressed during development, which selectively stains the chains of migrating cells in the SEL of the adult rat.[16] Moreover, the observation of carnosine-LI cells whose nuclei were BrdU-immunoreactive shows that the dipeptide is actually expressed by newly-generated cells, since no BrdU staining is detectable in glial cells of the SEL during adulthood.[26,36] The high concentration of these double-labelled cells in the horizontal arm of the SEL rostral extension five days after BrdU injection, confirms that most of them are migrating cells (see also Ref. 36). Finally, we exclude that the carnosine-LI, non-glial cells of the SEL are residual ependymal cells. Indeed, since the SEL rostral extension originates from the obliteration of the primitive olfactory ventricle, some clusters of ependymal cells can be occasionally observed at this level, intermingled with the subependymal cells filling the glial tubes.[36] Although carnosine immunostaining also revealed these clusters, they were not relevant

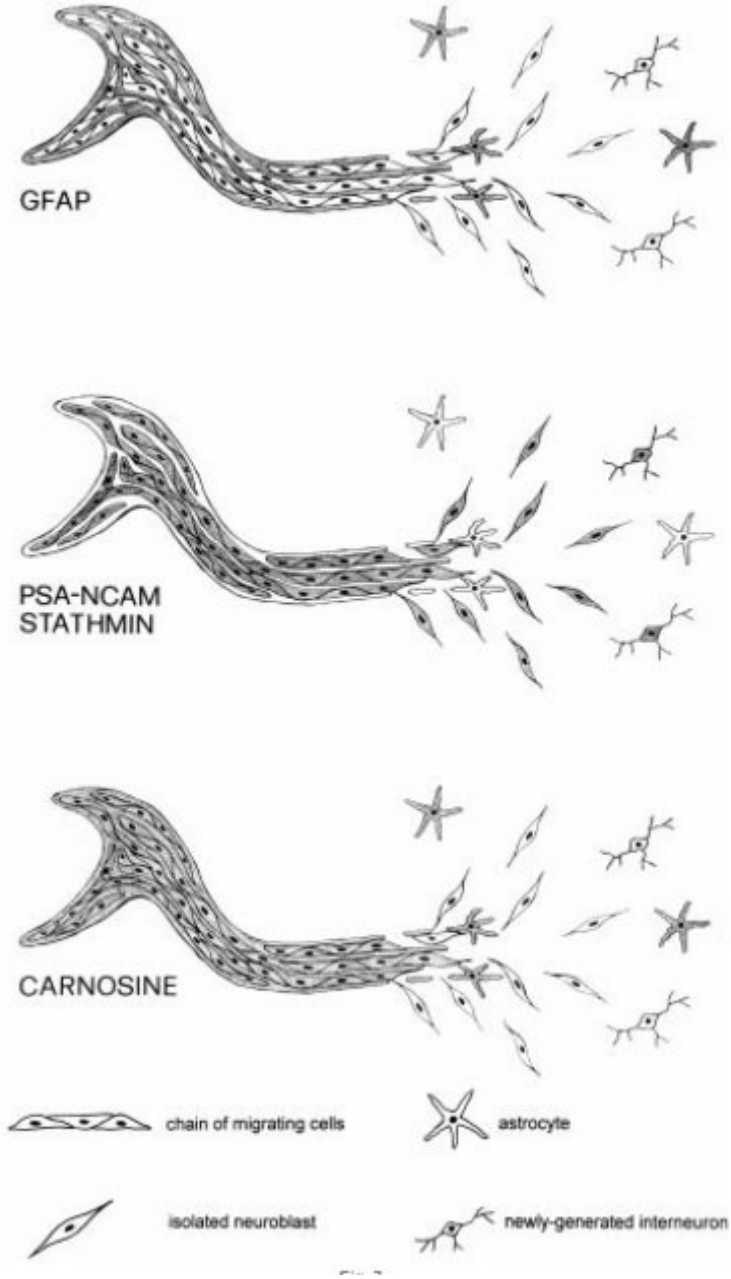

Fig. 7. Schematic drawing showing the pattern of distribution of different molecules (in grey) in the SEL and in the whole rostral migratory stream (RMS) of the adult rat. Glia-specific antigens, such as GFAP, are restricted to the astrocytic glial tubes, which are visible through the entire SEL area, although they became ill-defined in the olfactory bulb. ''Embryonic'' molecules, such as PSA-NCAM and stathmin, are considered markers of the entire RMS, revealing both newly-generated cells forming chains in the SEL (subependymal cells) and those which spread radially in the olfactory bulb as isolated neuroblasts. Carnosine-LI is detectable in the whole SEL area, associated both with astrocytes of the glial tubes and with chains of subependymal cells, but not with migrating neuroblasts in the olfactory bulb. Outside the SEL, carnosine and GFAP are associated to stellate-shaped glial cells.

Table 1. Distribution of carnosine-like immunoreactivity and neuronal and glial markers in the forebrain of adult rodents

	Carnosine	PSA-NCAM Stathmin	β-Tubulin	GFAP	Vimentin (rat)
SEL: astrocytes of the glial tubes	+			+	+
SEL: chains of subependymal cells	+	+	+		
Migrating neuroblasts in the olfactory bulb		+	+		
Ependyma/residual ependyma	+				+
Mature tissue surrounding the SEL	glial cells		neuropil	astrocytes	

to the total pattern of immunoreactivity, and were easily recognizable for their larger size and strong vimentin immunoreactivity (an antigen which is not expressed in the chains of subependymal cells).

Carnosine-like immunoreactivity is restricted to the tangentially-oriented part of the rostral migratory stream

As stated above, the RMS can be divided into two parts characterized by important differences concerning cell migration: in the first part, occurring within the SEL, the cells are organized to form tangentially-oriented chains, contained within astrocytic glial tubes,[14,19,22,26,36] whereas, in the second part, occurring outside the SEL, through the olfactory bulb, the cells move as isolated, radially-oriented neuroblasts, without any relationship to the glia. Several molecules have been found to be associated to the cells of the RMS, most of which are antigens usually expressed in undifferentiated cells during development.[11,16,31,41,46] In particular, the ‘‘embryonic’’ glycoprotein PSA-NCAM[40] and the phosphoprotein stathmin[45] are selective markers for the migrating cells all along the migration route, including the radially-oriented, bipolar-shaped cells in the olfactory bulb[11,12,16,41] (see Fig. 7 and Table 1). By contrast, carnosine-like immunoreactivity was restricted to the newly-generated cells contained within the SEL, suggesting that the dipeptide is transiently expressed by cells moving through the tangentially-oriented part of the RMS. Since these cells (forming chains within the glial tubes) differ from those localized outside the SEL (migrating in the olfactory bulb, not organized in chains and not enwrapped by glial structures), thus it is possible to speculate that the transient expression of the dipeptide in tangentially-migrating cells could be related either to their organization in chains or to their relationships with glial tubes. In our opinion, the former hypothesis appears more likely since it has been recently shown in vitro that cells of the RMS typically undergo chain migration, also in a glia-independent manner.[48]
To get more insights about this hypothesis, we examined the distribution of carnosine-LI and that of chain migration markers, PSA-NCAM and stathmin, in the postnatal rat. It is well known that carnosine-LI is expressed in the olfactory epithelium from embryonic day 16.[6,30] By contrast, in the forebrain, apart from the olfactory neuron axonal projections in the external part of the olfactory bulb, we were able to first detect a faint immunoreactivity for the dipeptide only at P9/P13, in glial cells. In the SEL, a strong reaction comparable in density and pattern of distribution to that described in the adult and involving the newly-generated subependymal cells, was observed starting from P21. Interestingly enough, this stage coincided with the first visualization of PSA-NCAM- or stathmin-positive chains of subependymal cells, whereas in earlier postnatal stages these antigens were rather uniformly diffuse in the SEL and abundantly expressed elsewhere. Since long distance, tangential cell migration has been demonstrated to occur in the rat forebrain during the postnatal period,[27] our observations indicate that in the RMS important changes in the organization of the migrating cells occur around the third postnatal week, and that such changes are coincident with the appearance of carnosine in this cell population. This is confirmed by ongoing studies on the glial organization in the postnatal rat forebrain (Peretto et al., in preparation), showing that changes involving the cells of the RMS at this stage are strictly related with modifications of the astrocytic glia.

CONCLUSIONS

In the present study, the immunoreactivity for carnosine and the structurally-related peptide homo-carnosine has been described in the SEL of adult rodents, both in astrocytes of the glial tubes and in newly-generated, migrating cells of the RMS. In the past, these peptides were detected in the olfactory receptor neurons of the nasal mucosa and in brain astrocytes.[5] The SEL and the olfactory neuro epithelium share important similarities: both regions are related to the olfactory system and are characterized by striking structural plasticity during adulthood, involving neurogenesis. The

SEL is a site of persisting cell proliferation[1,44] containing a population of stem cells[33,47] which give rise to neurons and glial cells, in vivo.[25,27] However, the newly-generated cells of the SEL differ from the olfactory receptors since they undergo a long distance migration prior to their differentiation.

We show here that carnosine-like immunoreactivity: (i) is transiently expressed by the undifferentiated subependymal cells, when they are organized to form chains in the tangential part of the RMS, (ii) this dipeptide is not detectable in the cells of the RMS, when they migrate radially into the olfactory bulb; (iii) in the rat, the appearance of carnosine in the SEL is delayed at the third posnatal week, in coincidence with the first morphological evidence for chain migration. These results suggest that carnosine, in the RMS, rather than being involved in the processes of cell renewal, could be associated with the particular type of tangential chain migration occurring in the SEL, within astrocytic glial tubes.

Acknowledgements - The authors thank F. Margolis for kindly providing the anti-carnosine serum, and G. Rougon for her generous gift of anti-PSA-NCAM antibody. We also thank F. Scaranari for his excellent photographic expertise. This work was supported by grants of the Ministero dell'Universita` e della Ricerca Scientifica e Tecnologica (MURST) and the Consiglio Nazionale delle Ricerche (CNR).

REFERENCES

1. Altman J. (1969) Autoradiographic and histological studies of postnatal neurogenesis. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J. comp. Neurol. 137, 433–458.
2. Artero C., Martì E., Biffo S., Mulatero B., Andreone C., Margolis F. L. and Fasolo A. (1991) Carnosine in the brain and olfactory system of amphibia and reptilia: a comparative study using immunocytochemical and biochemical methods. Neurosci. Lett. 130, 182–186.
3. Bauer K., Salnikow J., DeVitry F., Tixier-Vidal A. and Kleinkauf H. (1979) Characterization and biosynthesis of β-aminoacyl amino acids from rat brain and the C-6 glioma cell line. J. biol. Chem. 254, 6402–6407.
4. Bauer K., Hallermayer K., Salnikow J., Kleinkauf H. and Hamprecht B. (1982) Biosynthesis of carnosine and related peptides by glial cells in primary cultures. J. biol. Chem. 257, 3593–3597.
5. Biffo S., Grillo M. and Margolis F. L. (1990) Cellular localization of carnosine-like and anserine-like immunoreactivities in rodent and avian central nervous system. Neuroscience 35, 637–651.
6. Biffo S., Marti E. and Fasolo A. (1992) Carnosine, nerve growth factor receptor and tyrosine hydroxylase expression during the ontogeny of the rat olfactory system. J. chem. Neuroanat. 5, 51–62.
7. Biffo S., Sassoe′-Pognetto M., Verdun di Cantogno L., Perroteau I. and Fasolo A. (1992) Bulbectomy enhances neurogenesis and cell turnover of primary olfactory neurons but does not abolish carnosine expression. Eur. J. Neurosci. 4, 1398–1406.
8. Bignami A., Eng L. F., Dahl D. and Uyeda C. T. (1972) Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. Brain Res. 43, 429–435.
9. Bignami A., Raju T. and Dahl D. (1982) Localization of vimentin, the non-specific intermediate filament protein, in embryonic glia and in early differentiating neurons. Devl Biol. 91, 286–295.
10. Bonfanti L., Poulain D. A. and Theodosis D. T. (1993) Radial-glia-like cells in the supraoptic nucleus of the adult rat. J. Neuroendocrinol. 5, 1–5.
11. Bonfanti L. and Theodosis D. T. (1994) Expression of polysialylated neural cell adhesion molecule by proliferating cells in the subependymal layer of the adult rat, in its rostral extension and in the olfactory bulb. Neuroscience 62, 291–305.
12. Bonfanti L., Peretto P., Merighi A. and Fasolo A. (1997) Newly-generated cells from the rostral migratory stream in the accessory olfactory bulb of the adult rat. Neuroscience 81, 489–502.
13. Boulder Committee (1970) Embryonic vertebrate central nervous system: revised terminology. Anat. Rec. 166, 257–261.
14. Brannon Thomas L., Gates M. A. and Steindler D. A. (1996) Young neurons from the adult subependymal zone proliferate and migrate along an astrocyte, extracellular matrix-rich pathway. Glia 17, 1–14.
15. Brunjes P. C. and Frazier L. L. (1986) Maturation and plasticity in the olfactory system of vertebrates. Brain Res. Rev. 11, 1–45.
16. Camoletto P., Peretto P., Bonfanti L., Manceau V., Sobel A. and Fasolo A. (1997) The cytosolic phosphoprotein stathmin is expressed in the olfactory system of the adult rat. NeuroReport 8, 2825–2829.
17. Crush K. G. (1970) Carnosine and related substances in animal tissues. Comp. Biochem. Physiol. 34, 3–30.
18. Dahl D., Rueger D. and Bignami A. (1981) Vimentin, the 57000 molecular weight protein of fibroblast filaments, is the major cytoskeletal component in immature glia. Eur. J. Cell Biol. 24, 191–196.

19. Doetsch F. and Alvarez-Buylla A. (1996) Network of tangential pathways for neuronal migration in adult mammalian brain. Proc. natn. Acad. Sci. U.S.A. 93, 14,895–14,900.
20. Fisher D. E., Amend J. F. and Strumeyer D. H. (1977) Anserine and carnosine in chicks (Gallus gallus), rat pups (Rattus rattus) and ducklings (Anas plathyrhynchos): comparative ontogenetic observations. Comp. Biochem. Physiol. 56B, 367–370.
21. Jackson M. C. and Lenney J. F. (1996) The distribution of carnosine and related dipeptides in rat and human tissues. Inflamm. Res. 45, 132–135.
22. Jankovski A. and Sotelo C. (1996) Subventricular zone-olfactory bulb migratory pathway in the adult mouse: cellular composition and specificity as determined by heterochronic and heterotopic transplantation. J. comp. Neurol. 371, 376–396.
23. Kishi K. (1987) Golgi studies on the development of granule cells of the rat olfactory bulb with reference to migration in the subependymal layer. J. comp. Neurol. 258, 112–124.
24. Kohen R., Yamamoto Y., Cundy K. C. and Ames B. N. (1988) Antioxidant activity of carnosine, homocarnosine and anserine present in muscle and brain. Proc. natn. Acad. Sci. U.S.A. 85, 3175–3179.
25. Lois C. and Alvarez-Buylla A. (1994) Long-distance neuronal migration in the adult mammalian brain. Science 264, 1145–1148.
26. Lois C., Garcia-Verdugo J. and Alvarez-Buylla A. (1996) Chain migration of neuronal precursors. Science 271, 978–981.
27. Luskin M. B. (1993) Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron 11, 173–189.
28. Margolis F. L. (1980) Carnosine: an olfactory neuropeptide. In The Role of Peptides in Neuronal Function (eds Baker J. L. and Smith T.), pp. 545–572. Dekker, New York.
29. Margolis F. L. and Grillo M. (1984) Carnosine, homocarnosine and anserine in vertebrate retinas. Neurochem. Int. 6, 207–209.
30. Margolis F. L., Grillo M., Kawano T. and Farbman A. I. (1985) Carnosine synthesis in olfactory tissue during ontogeny: effect of exogenous -alanine. J. Neurochem. 44, 1459–1464.
31. Mendez-Otero R. and Cavalcante L. A. (1996) Expression of 9-O-acetylated gangliosides is correlated with tangential cell migration in the rat brain. Neurosci. Lett. 204, 97–100.
32. Menezes J. R. L. and Luskin M. B. (1994) Expression of neuron-specific tubulin defines a novel population in the proliferative layers of the developing telencephalon. J. Neurosci. 14, 5399–5416.
33. Morshead C. M., Reynolds B. A., Craig C. G., McBurney M. W., Staines W. A., Morassutti D., Weiss S. and van der Kooy D. (1994) Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron 13, 1071–1082.
34. Nadi N. S. and Margolis F. L. (1978) A simple method for the elimination of amine contaminants in buffers for single column amino acid analysis in physiological samples at picomole levels. Analyt. Biochem. 91, 180–185.
35. Ono K., Tomasiewicz H., Magnuson T. and Rutishauser U. (1994) N-CAM mutation inhibits tangential neuronal migration and is phenocopied by enzymatic removal of polysialic acid. Neuron 13, 595–609.
36. Peretto P., Merighi A., Fasolo A. and Bonfanti L. (1997) Glial tubes in the rostral migratory stream of the adult rat. Brain Res. Bull. 42, 9–21.
37. Pixley S. K. R. and De Vellis J. (1984) Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. Devl Brain Res. 15, 201–209.
38. Quinn P. J., Boldyrev A. A. and Formazuyk V. E. (1992) Carnosine: its properties, functions and potential therapeutic applications. Molec. Aspects Med. 13, 379–444.
39. Rougon G., Dubois C., Buckley N., Magnani J. L. and Zollinger W. (1986) A monoclonal antibody against Meningococcus group B polysaccharides distinguishes embryonic from

adult N-CAM. J. Cell Biol. 103, 2429–2437.
40. Rougon G. (1993) Structure, metabolism and cell biology of polysialic acids. Eur. J. Cell Biol. 61, 197–207.
41. Rousselot P., Lois C. and Alvarez-Buylla A. (1995) Embryonic (PSA) N-CAM reveals chains of migrating neuroblasts between the lateral ventricle and the olfactory bulb of adult mice. J. comp. Neurol. 351, 51–61.
42. Sassoe˙-Pognetto M., Cantino D., Panzanelli P., Verdun di Cantogno L., Giustetto M., Margolis F. M., De Biasi S. and Fasolo A. (1993) Presynaptic colocalization of carnosine and glutamate in olfactory neurons. NeuroReport 5, 7–10.
43. Skulachev V. P. (1978) Membrane-linked energy buffering as the biological function of Na+/K+ gradient. Fedn Eur. biochem. Socs Lett. 87, 171–179.
44. Smart I. (1961) The subependymal layer of the mouse brain and its cell production as shown by radioautography after thymidine-H3 injection. J. comp. Neurol. 116, 325–338.
45. Sobel A. (1991) Stathmin: a relay phosphoprotein for multiple signal transduction? Trends biochem Sci. 16, 301–305.
46. Xu W. and Emson P. C. (1997) Neuronal stem cells express vesicular monoamine transporter 2 immunoreactivity in the adult rat. Neuroscience 76, 7–10.
47. Weiss S., Reynolds B. A., Vescovi A. L., Morshead C., Craig C. G. and van der Kooy D. (1996) Is there a neural stem cell in the mammalian forebrain? Trends Neurosci. 19, 387–393.
48. Wichterle H., Garcia-Verdugo J. M. and Alvarez-Buylla A. (1997) Direct evidence for homotypic, glia-independent neuronal migration. Neuron 18, 779–791.
49. Wideman J., Brink L. and Stein S. (1978) New automated fluorometric peptide microassay for carnosine in mouse olfactory bulb. Analyt. Biochem. 86, 670–678.