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Carnosine-Like Immunoreactivity in the Central Nervous System of Rats During Postnatal Development

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ABSTRACT

In the nervous system of adult rodents, the aminoacylhistidine dipeptides (carnosine and/or homo-carnosine) have been shown to be expressed in three main populations of cells: the mature olfactory receptor neurons, a subset of glial cells, and the neuroblasts of the rostral migratory stream. The current study analyzed the distribution of these dipeptides during postnatal development within the rat brain and spinal cord focusing on their pattern of appearance in the glial cells. Double staining methods using antibodies against carnosine and some markers specific for immature (vimentin) and mature (glial fibrillary acidic protein and Rip) glial cell types were used. Glial immunostaining for the aminoacylhistidine dipeptides appears starting from postnatal day 6 and reaches the final distribution in 3-week-old animals. The occurrence of carnosine-like immunoreactivity in astrocytes lags behind that in oligodendrocytes suggesting that, as previously demonstrated by in vitro studies, oligodendrocytes are also able to synthesize carnosine and/or homocarnosine in vivo. Furthermore, the spatiotemporal patterns observed support the hypothesis that the production of these dipeptides coincides with the final stages of glia differentiation. In addition, a strong carnosine-like immuno-reactivity is transiently seen in a small population of cells localized in the hypothalamus and in the subfornical organ from birth to postnatal day 21. In these cells, carnosine-like immunoreactivity was not colocalized with any of the glial specific markers used. Moreover, no evidence for colocalization of carnosine and gonadotropin-releasing hormone (GnRH) has been observed.

Indexing terms: aminoacylhistidine dipeptides; oligodendrocytes; astrocytes; brain; spinal cord

Carnosine (β-alanyl-L-histidine) and other related aminoacylhistidine dipeptides, such as homocar-nosine (γ-aminobutyryl-L-histidine) and anserine (β-alanyl-N-methyl-L-histidine), are present in the nervous system of different classes of vertebrates (Crush, 1970; Bonfanti et al., 1999). These pepti-des are synthesized by the enzyme carnosine synthetase (Kalyankar and Meister, 1959; Horinishi et al., 1978; Ng and Marshall, 1978). Many hypotheses have been proposed to explain their biological functions (Margolis, 1980; Quinn et al., 1992; Hipkiss, 1998). In vitro, these substances act as anti-oxidants, free radical scavengers, and metal ion chelating agents; nevertheless, their precise physio-logical role in the nervous system remains unknown (Bonfanti et al., 1999).

Carnosine and homocarnosine, but not anserine, are present in the mammalian central nervous sy-stem (CNS; Margolis and Grillo, 1984; Biffo et al., 1990). Quantification of these dipeptides within the rodent nervous system revealed high concentrations of carnosine in the olfactory epithelium (OE) and bulb (OB; Margolis, 1974), and lower concentrations of both carnosine and homocarnosi-ne in the brain and spinal cord (Pisano et al., 1961; Abraham et al., 1962; Margolis, 1974; Cairns et al., 1988).

Immunohistochemical studies, by using a rabbit polyclonal antiserum that recognizes both carnosi-ne and homocarnosine, showed that carnosine-like immunoreactivity (-LI) is present in the primary receptor neurons (ORNs) of the OE and in a large population of glial cells

widely distributed throughout the white and gray matter of the rodent brain (Biffo et al., 1990). In

the olfactory system, both the perikarya and cell processes of mature ORNs, including their axonal projections to the OB, show carnosine-LI. A population of carnosine-like immunopositive extramucosal cells which appear to be comigrating with gonadotropin-releasing hormone (GnRH)-positive neurons along the olfactory pathway from the olfactory placode to the OB have been reported during fetal development but are undetectable in the adult (Tarozzo et al., 1995a).

Carnosine-LI has been described to occur in subpopulations of both astrocytes and oligodendrocytes of adult rat brain (Biffo et al., 1990; De Marchis et al., 1997). By contrast, immunocytochemical studies performed in cultured glial cells showed that, in vitro, the presence of carnosine-LI is restricted to a subset of mature oligodendrocytes (De Marchis et al., 1997). In agreement with these findings, previous studies have demonstrated that, in vitro, carnosine biosynthesis is confined to oligodendroglia (Hoffmann et al., 1996).

Other cell populations in the CNS that are highly positive for carnosine-LI include ependymal cells and specialized radial-glial-derived cells, such as hypothalamic tanycytes (Peretto et al., 1998), and the cerebellar Bergman glia (Biffo et al., 1990). In addition, carnosine-LI is present in the subependymal layer (SEL) of adult rodents starting from the third postnatal week of life when the glial and neuronal components of the SEL achieve their mature organization in chains of migrating neuroblasts within glial tubes (Peretto et al., 1998).

The purpose of the present study was to analyze the onset and development of carnosine-related dipeptides (carnosine and/or homocarnosine) expression within the rat brain and spinal cord, with particular attention to the pattern of carnosine-LI during glial maturation in different regions of the CNS.

It is well established that the production of both types of macroglial cells starts prenatally from multipotent stem cells of the ventricular/subventricular zone, and that dif-ferentiation into mature functional elements occurs only postnatally (Jacobson, 1991). Accordingly, our analysis focused on brains and spinal cords obtained from rats of different postnatal ages (P1, P3, P6, P9, P12, P16, P21, P30, P90) and used double staining methods to characterize the antigenic phenotype of the carnosine-like immunoreactive cells.

Immature and mature glial cell populations were identified by their immunoreactivity to vimentin (Dahl et al., 1981), glial fibrillary acidic protein (GFAP; Bignami and Dahl, 1977), and Rip (Friedman et al., 1989).

MATERIALS AND METHODS
Tissue preparation

Brains and spinal cords were obtained from 27 male Wistar rats (Charles River, Italy) and killed on postnatal days (P) 1, 3, 6, 9, 12, 16, 21, 30, and 90 (adult; three animals for each age). All experiments were performed in accordance with the current Italian law, under authorization of the Italian Ministry of Health, D.L. 116/92. Animals were deeply anesthetized with intraperitoneal sodium pentobarbital (pentothal sodium, Gellini, Milan, Italy; 60 mg/100 g, i.p.) and then intracardially perfused with a heparinized saline solution (25 IU/ml in 0.9% NaCl, for 2–3 minutes), followed by a freshly prepared solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After dissection, brains and spinal cords were postfixed overnight in the same fixative. The tissues were subsequently rinsed in PB, cryoprotected in increasing concentrations of sucrose (7.5%, 15%, 30%) and frozenliquid nitrogen-cooled isopentane at -70°C. Serial parasagittal and coronal cryostat sections (10 –12 µm) were collected onto gelatin-coated slides, and then used for immunohistochemistry.

Immunohistochemistry

Immunohistochemical reactions were performed by using single and double immunofluorescence methods and the indirect peroxidase procedure. All incubations were run at room temperature and all washes were in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 10 minutes, repeated twice. The tissue sections were treated with primary antibodies diluted in 0.01 M PBS, pH 7.4, containing 0.1% Triton X-100 and 1% nonimmune serum of the donor species of the secondary antiserum. Immunohistochemical controls included incubation of sections in carnosine antiserum preincubated with 10 µM bovine serum albumin (BSA)-carnosine conjugate or in the absence of the primary antibodies.

Antibodies. The following primary antibodies were used: (1) anti-carnosine, a polyclonal rabbit serum preabsorbed with BSA-glycine and BSA-histidine conjugates to increase the specificity of the reaction (F. Margolis, Baltimore, MD), diluted 1/1,000; (2) 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; (3) anti-vimentin, a monoclonal IgG (Dako, Denmark) diluted 1/600; Rip, a monoclonal IgG (S. Hockfield, DSHB, Iowa City, IA) diluted 1/100; anti-GnRH, a polyclonal rabbit IgG (R. Benoit, Hopital Général de Montreal, Quebec) diluted 1/10,000.

Immunofluorescence. Affinity-purified goat antimouse IgG (Fab-specific, Sigma, St. Louis, MO 1/40) coupled to fluorescein isothiocyanate (FITC), and Texas Red-avidin (Vector, Burlingame, CA, 1/400) conjugated to goat anti-rabbit biotinylated secondary antibody (Vector, 1/200) were used. Sections were mounted in a solution of PBS/glycerol (9/1).

Peroxidase staining. Peroxidase reactions were developed by using the biotin-avidin system (Vector) with 3, 3'-diaminobenzidine as a chromogen. Slides were then
dehydrated and mounted in DPX (Fluka, Milan, Italy).

Photography. The tissue was evaluated and photographed with a conventional epifluorescence microscope Axioskop 20 (Zeiss, Thornwood, NY) equipped with a MC100 camera (Zeiss).

RESULTS
Carnosine-LI in the developing rat spinal cord

The spinal cord of each animal was divided into cervical, thoracic, and lumbar levels. The different levels showed similar patterns of staining at each of the postnatal ages studied. In the first postnatal ages analyzed (P1, P3), no carnosine-LI was observed. At these same ages, strong vimentin immunoreactivity was seen, accompanied by a small number of GFAP-immunopositive astrocytes and rare Rip-immunopositive cell processes and somata in the forming white matter (data not shown). Carnosine-LI was first seen at P6, when an increasing number of glial cells expressed GFAP and Rip. At this age, a discrete number of round cells were stained for carnosine in the forming white and gray matter (Fig.1a). The immunostaining was confined to the cell somata (both nucleus and cytoplasm). The distribution of carnosine-LI was not uniform, showing a ventrodorsal gradient, with most of the cells localized in the ventral horns and central gray matter, and with only rare immunopositive cell bodies present in the dorsal horns (see schematic drawing, Fig.1a). The immunoreactivity was completely absent in the dorsal and ventral roots of the spinal cord. Double staining on individual sections, by using anti-carnosine together with anti-GFAP or Rip antibodies, revealed that at P6 carnosine expression is confined to Rip-immunopositive oligodendrocytes (data not shown). However, beginning at this postnatal age, in addition to the glial staining described above, carnosine-LI was observed in the ependyma lining the central canal.

At P9, the number of carnosine-like immunoreactive elements has increased (Fig.1b). The majority of the carnosine-like immunoreactive cells, both in the white and gray matter, were also immunopositive for Rip (Fig. 2a–d); however, some carnosine/GFAP double- labeled astrocytes were also observed (Fig. 2e,f).

Between P16 and P21 (Fig.1c), an increasing number of carnosine-like immunopositive astrocytes was present both in the gray and white matter, accompanied by a progressive extension of the stai-

ning to radially oriented cell processes in the white matter. The pattern of distribution observed at P21 was similar to that of adult animals in which carnosine-LI was localized to numerous cell somata of interfascicular oligodendrocytes and to both cell somata and processes of a large number of astrocytes. In the gray matter, carnosine-like immunoreactive astrocytes were primarily stained in the cell somata with little or no staining in cell processes. In animals from 1-month-old until adulthood, the intensity of the carnosine-LI decreased for each cellular element. Preincubation of the anti-carnosine serum with the BSA-carnosine conjugate completely abolished the immunoreactivity within the spinal cord at every age.
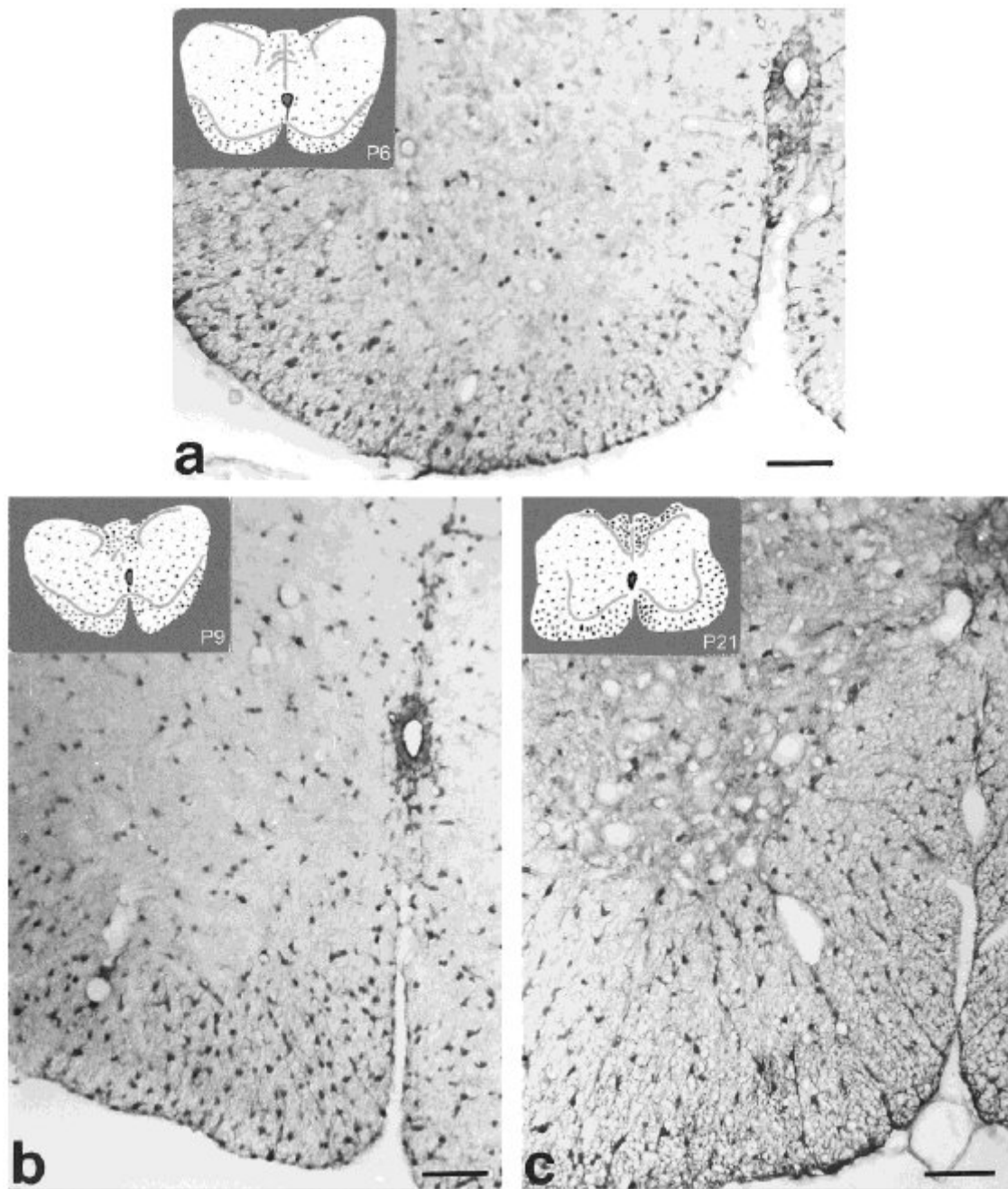
Fig. 1. Coronal sections through the rat thoracic spinal cord showing carnosine-like immunoreactivity (LI) at postnatal day (P)6 (a), P9 (b), and P21 (c). For each age, schematic drawings illustrate the gradual development of carnosine-LI during the first 3 postnatal weeks (see text for details). Scale bars = 64 μm.

Carnosine-LI in the developing rat brain

In the developing rat brain, the pattern of staining for carnosine-related dipeptides demonstrated the existence of two different classes of immunopositive cells: the first was represented by a heterogeneous and widely distributed population of glial cells, whereas the second was characterized by a more intense immunostaining concentrated in small populations of spatially restricted cell bodies immunonegative for glial markers. In addition, we observed an abundant system of immunopositive nerve fibers. For all these cases, the immunolabeling was completely abolished after preincubation of the antiserum with the BSA-carnosine conjugate.

Development of carnosine-LI in glial cells. As in the spinal cord, no glial elements exhibited carnosine-LI at P1 and P3. Carnosine-LI first appeared at P6, in a population of cells widely distributed throughout the basal brainstem (Fig. 3). In coronal sections of the pontine area and the medulla, strong immunoreactivity was localized in the gray matter and in fiber tracts such as the inferior cerebellar peduncle (Fig. 4) and the spinal tract of the trigeminal nerve. This strong immunoreactivity was evident in small round cells and only occasionally in their processes (Fig. 4c). The majority of the carnosine-like immunoreactive cells were clearly Rip-immunopositive (Fig. 4), and no GFAP colocalization was observed, suggesting that these cells are mature oligodendrocytes. Other than these cells, only a subset of columnar ependymal cells lining the fourth ventricle exhibited carnosine-LI. At P9, the expression of carnosine-related dipeptides was evident along the brainstem reaching the tegmentum (Figs. 3, 5). A large number of cells colocalized carnosine-LI and immunoreactivity for Rip (Fig. 5a,b), but at this age, a discrete number of carnosine/GFAP double-labeled cells were also observed (Fig. 5c,d). An intense carnosine-LI was also present in numerous glial-like cells of the pineal gland, in a subset of ependymal cells of the cerebral aqueduct, and of the epithalamic third ventricle.

By P12, the ependymal staining for carnosine has extended to the lateral ventricles and to the hypothalamic third ventricle, where tanycytes of the lateral wall of the infundibular recess were strongly stained. At this age, carnosine immunostaining extended in a dorsorostral direction, in the cerebellum, the dorsal mesencephalon, and in restricted regions of the prosencephalon. The number of stained cells decreased from the more caudoventral regions to the dorsorostral regions, where only rare immunoreactive elements were present (Fig. 3). In the cerebellum, the carnosine-LI was uniformly distributed throughout the white matter, with strong immunopositivity in numerous small round cell bodies (Fig. 6a). A few labeled cell somata, which were Rip-immunopositive oligodendrocytes, were also evident in the granule cell layer (Fig. 6). At the same age, positive cell bodies were visualized in the inferior colliculus, along with small numbers in the superior colliculus, the thalamus, and in the mammillary body of the hypothalamus. More rostrally, the expression of carnosine was observed in a discrete number of cell bodies localized in white matter regions such as the corpus callosum, the anterior commissure, the lateral olfactory tract, and less robustly, the optic tract. Diffuse immunostaining was also evident in the caudate-putamen and rarely in the fundus of the striatum.

Subsequently, between P16 and P21, the number of positive cells increased and carnosine-LI was more extensively distributed in glial elements of the white and gray matter (Fig. 3). An increasing number of carnosine-like immunoreactive cells had an astroglial-like stellate morphology, with some processes lightly stained. As expected, a mixture of carnosine/Rip and carnosine/GFAP double-labeled cells were observed. At these ages, strong staining is also present in the tanycytes lining the circumventricular organs. Furthermore, in accordance with previous immunocytochemical studies (Peretto et al., 1998), an intense immunostaining concentrated in the subependymal layer of the lateral ventricles was evident. In 1-month-old animals, the intensity of carnosine-LI in glial cells was weaker in comparison with the staining observed in younger animals.

Carnosine-LI in other cell types. From the earliest developmental ages investigated, P1, P3, to P21, we observed a strong carnosine-LI in isolated or restricted groups of cells mainly localized in the hypothalamus and in the subfornical organ (SFO; Fig. 7). The carnosine-LI in these cells was particularly intense, exceeding that observed for glial cells during postnatal development. Within

the anterior hypothalamus, a small number of scattered carnosine-like immunoreactive cells lay at the ventral boundary of the medial and lateral preoptic regions. Some isolated, thin, and beaded nerve fibers were also found in these areas. At the level of the suprachiasmatic nuclei, in a ventrolateral position, rare, isolated carnosine-like immunoreactive cells were seen, whereas a more concentrated number was present in the tuberal hypothalamus, in a ventromedial position (Fig.7a,b). In addition, a large number of immunopositive beaded processes occurred in the outer layer of the median eminence, whose terminals were in direct contact with the capillaries of the hypophysial portal vessels (Fig. 7a).

Besides the hypothalamic localization, a discrete number of carnosine-like immunopositive cells and irregularly oriented, thin, beaded nerve fibers were seen in the SFO, a midline sagittal structure that bulges into the lumen of the third ventricle at the level of the interventricular foramina (Dellmann, 1998; Fig.7c). These cells possessed a round, elongated perikaryon having one or two thin processes that in certain cases seemed to be in direct contact with the cerebrospinal fluid. Furthermore, a few scattered carnosine-like immunopositive cell bodies, with one short prominent process oriented dorsoventrally, were found at the level of medial and lateral septum and in the paraventricular nucleus of the thalamus. Carnosine-LI was never observed to colocalize with any of the glial specific markers (anti-GFAP, anti-vimentin, or Rip), at different levels of the prosencephalon in these restricted populations of cells. The pattern of carnosine-LI in these regions shows interesting similarities with the previously described distribution of GnRH immunoreactivity (Merchenthaler et al., 1984). However, we have not observed any evidence for colocalization of carnosine-LI and GnRH.
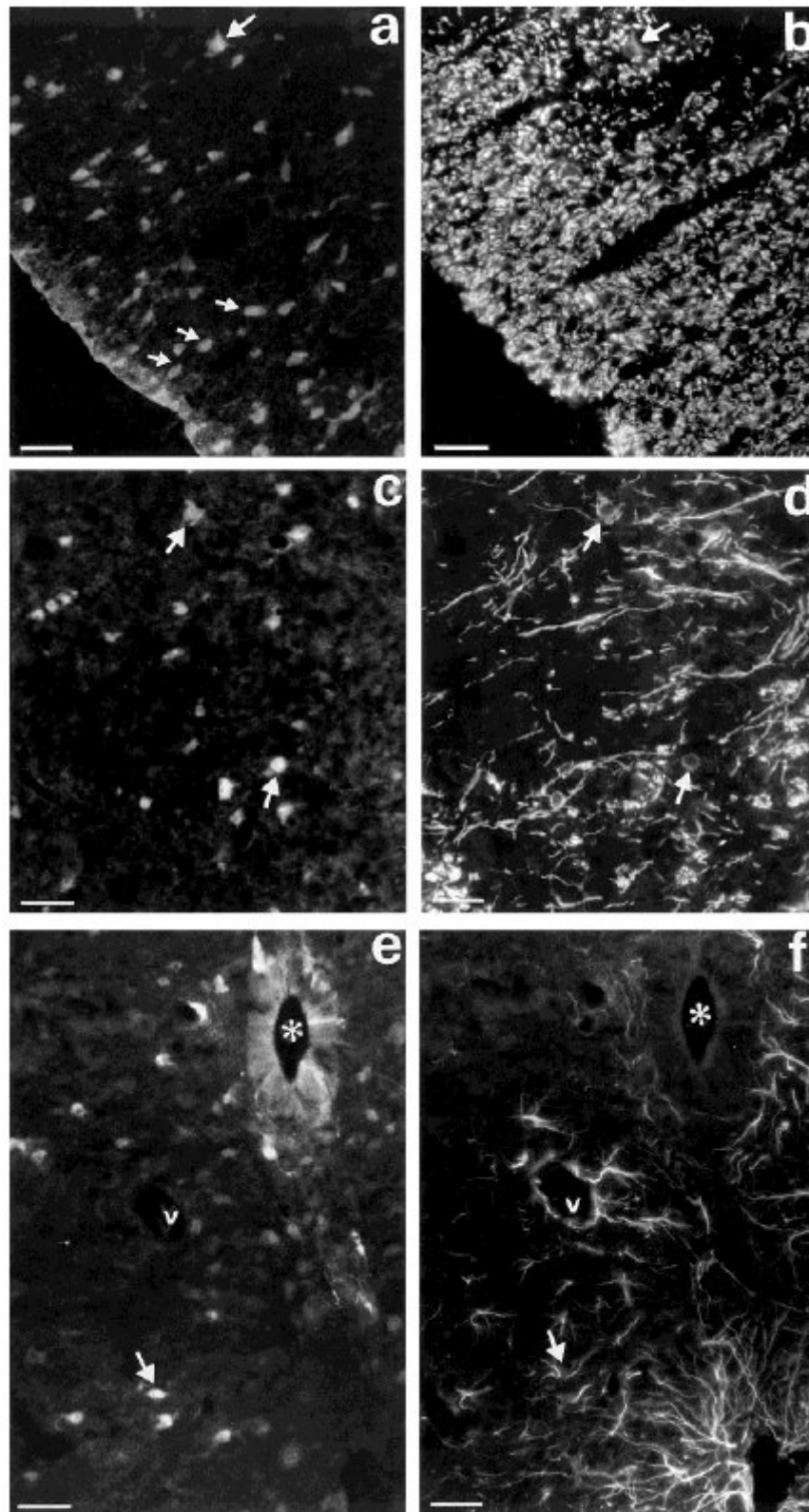
Fig. 2. Coronal sections of the thoracic rat spinal cord at postnatal day (P)9. In a, small arrows indicate some carnosine-like immunoreactive cells aligned in rows in the white matter. a–d: Simultaneous localization of carnosine (a,c) and Rip (b,d). Both in the white (a,b) and gray (c, d) matter, carnosine-like immunoreactivity (LI) is mainly localized in Rip-immunopositive cells (see arrows for example). e,f: Simultaneous localization of carnosine (e) and glial fibrillary acidic protein (GFAP; f). At this age, a restricted number of cells are double-labeled for carnosine and GFAP. Arrow (e,f) indicates one double-labeled cell; asterisk indicates central canal; v, blood vessel. Scale bars = 32 μm.
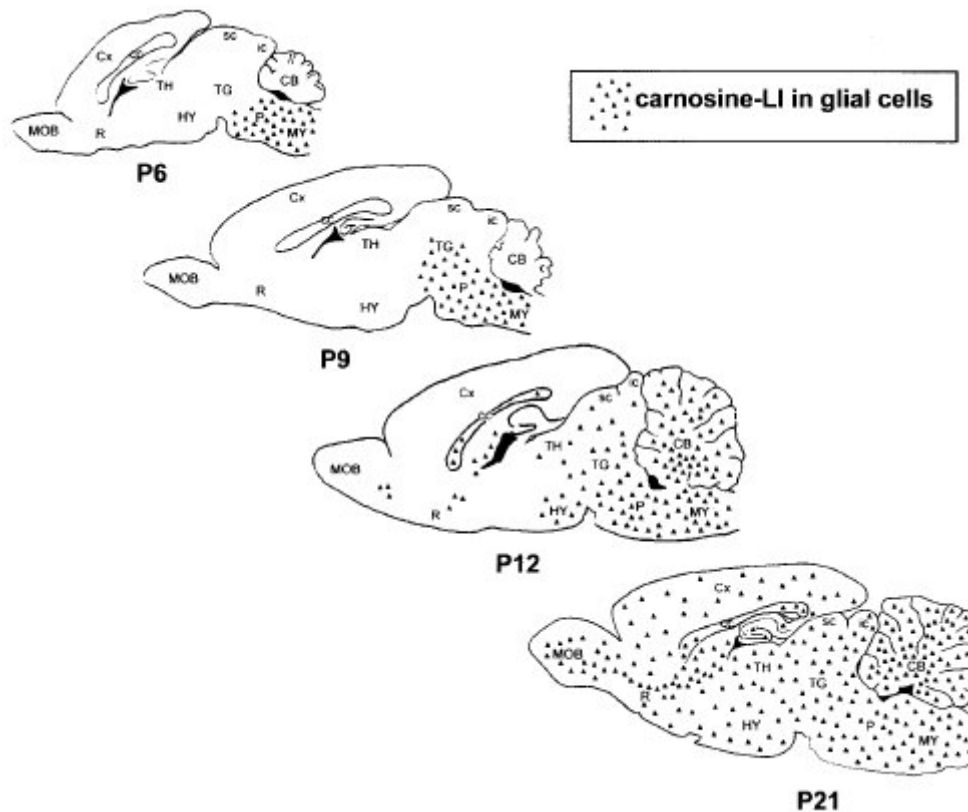
Fig. 3. Schematic drawings illustrating the gradual development of carnosine-like immunoreactivity (LI) in glial cells in the rat brain during the first 3 postnatal (P6 –21) weeks. CB, cerebellum; cc, corpus callosum; Cx, cortex; HY, hypothalamus; ic, inferior colliculus; MOB, main olfactory bulb; MY, medulla; P, pons; R, rinencephalon; sc, superior colliculus; TG, tegmentum; TH, thalamus.

Carnosine-LI in nerve fibers. At the most rostral region of the brain, as previously reported, a strong immunostaining for carnosine was observed in the glomerular layer of the main (MOB) and accessory olfactory bulb (AOB), where the axons of the ORNs reach their target (Biffo et al., 1990). In addition, some short, scattered, beaded immunoreactive processes were observed in the external plexiform layer of the caudal MOB and AOB. Furthermore, a large number of carnosine-like immunoreactive thin, beaded fibers were seen running throughout the olfactory tubercle (Fig.7d). In cross- sections through the middle part of the olfactory tubercle, where it joins the ventral surface of the frontal lobe, we observed the presence of these immunoreactive processes in the molecular layer of the piriform cortex.

As described in the previous paragraph, immunoreactive processes were also found in the thalamic and hypothalamic regions, with the highest concentration in the median eminence (Fig. 7a). The intensity of immunostaining found in all these nerve processes was comparable to that observed for the restricted population of cells immunonegative for glial markers. Carnosine-LI in glomerular layers of the MOB and AOB persists until adulthood; by contrast, the other immunoreactive nerve processes were only visible until P21. After this age, only rare short processes of the above-mentioned regions of the brain exhibited carnosine-LI.
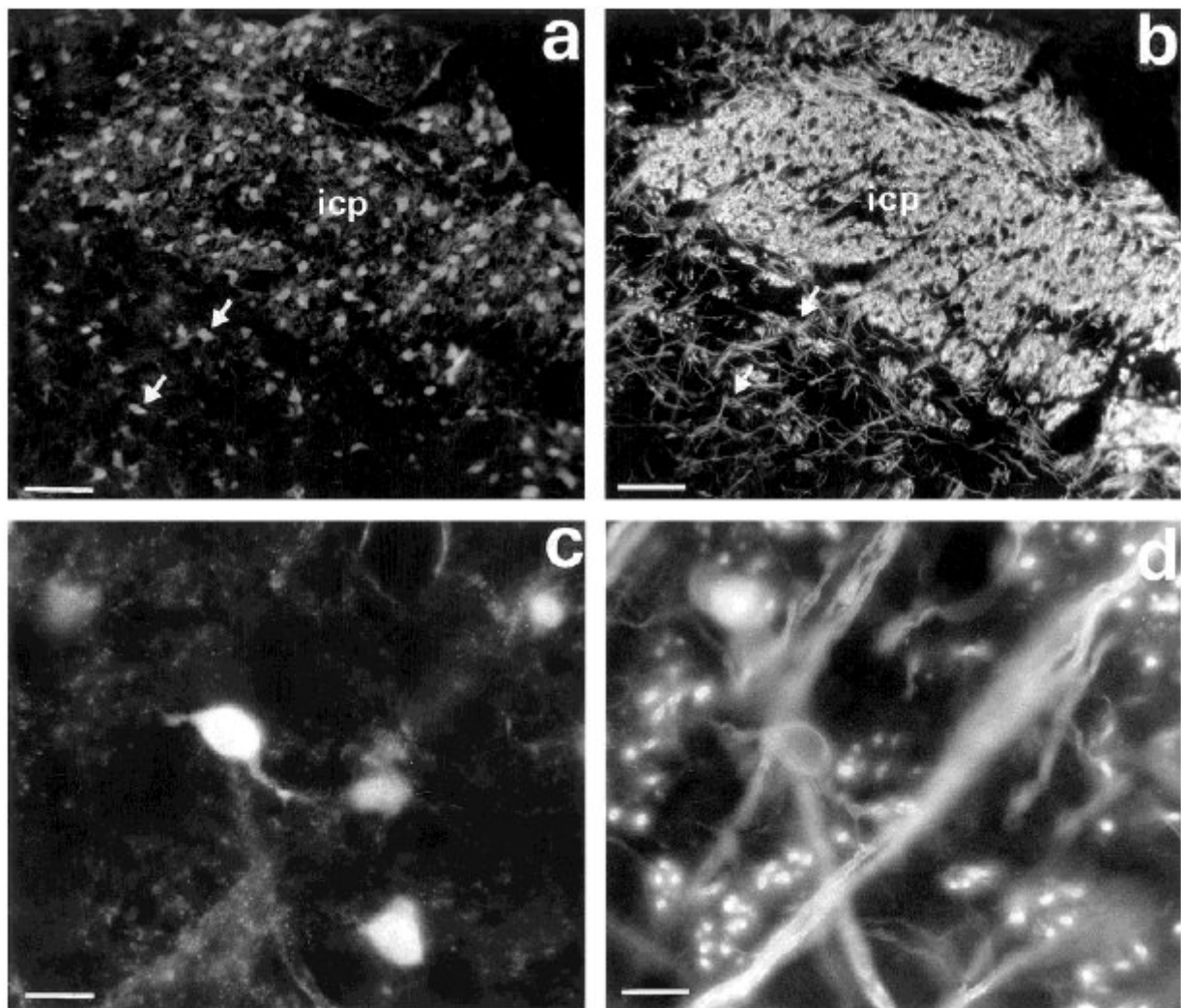
Fig. 4. Coronal sections of postnatal day (P)6 rat basal brainstem. Simultaneous localization of carnosine (a,c) and Rip (b,d). a,b: The pattern of distribution of carnosine-like immunoreactive cells is similar to that of Rip-immunopositive oligodendrocytes. In both the gray and the white matter, the majority of the carnosine-like immunopositive cells is double-labeled for Rip (see arrows for example). c,d: High-power view illustrating two carnosine/Rip-immunopositive oligodendrocytes in the gray matter. Note that the short faintly carnosine-like immunopositive cell process (c) is strongly labeled for Rip and terminates in myelin sheaths (d). icp, inferior cerebellar peduncle. Scale bars = 64 μm in a,b; 13 μm in c,d.

DISCUSSION
Specificity of anti-carnosine serum

The anti-carnosine antiserum used in this study has been well characterized in previous biochemical and immunohistochemical studies (Biffo et al., 1990; Artero et al., 1991). This polyclonal rabbit antiserum was raised against carnosine conjugated to various carrier proteins (excluding BSA), and cross-reacts with homocarnosine and anserine (Biffo et al., 1990). A number of studies employing different analytical methods established that carnosine and homocarnosine are present in the mammalian brain, whereas anserine is absent (Margolis and Grillo, 1984; Biffo et al., 1990). Carnosine is highly concentrated (2 nmol/mg of tissue) in the OB of rodents, whereas lower concentrations of carnosine (0.01– 0.2 nmol/mg) and homocarnosine (0.05– 0.2 nmol/mg) occur in the brain and in the spinal cord (Pisano et al., 1961; Abraham et al., 1962; Margolis, 1974; Cairns et al., 1988).These data permit us to consider the immunoreactivity herein reported, referred to as carnosine-LI, to represent the presence of carnosine and/or homocarnosine. Accordingly, in the present study, the ami-

noacyl-histidine dipeptides carnosine and homocarnosine are referred to as carnosine-related dipeptides. After liquid-phase preincubation of the antiserum with a BSA-carnosine conjugate, the immunoreactivity is completely abolished in the brain and spinal cord, for each of the different postnatal stages analyzed.
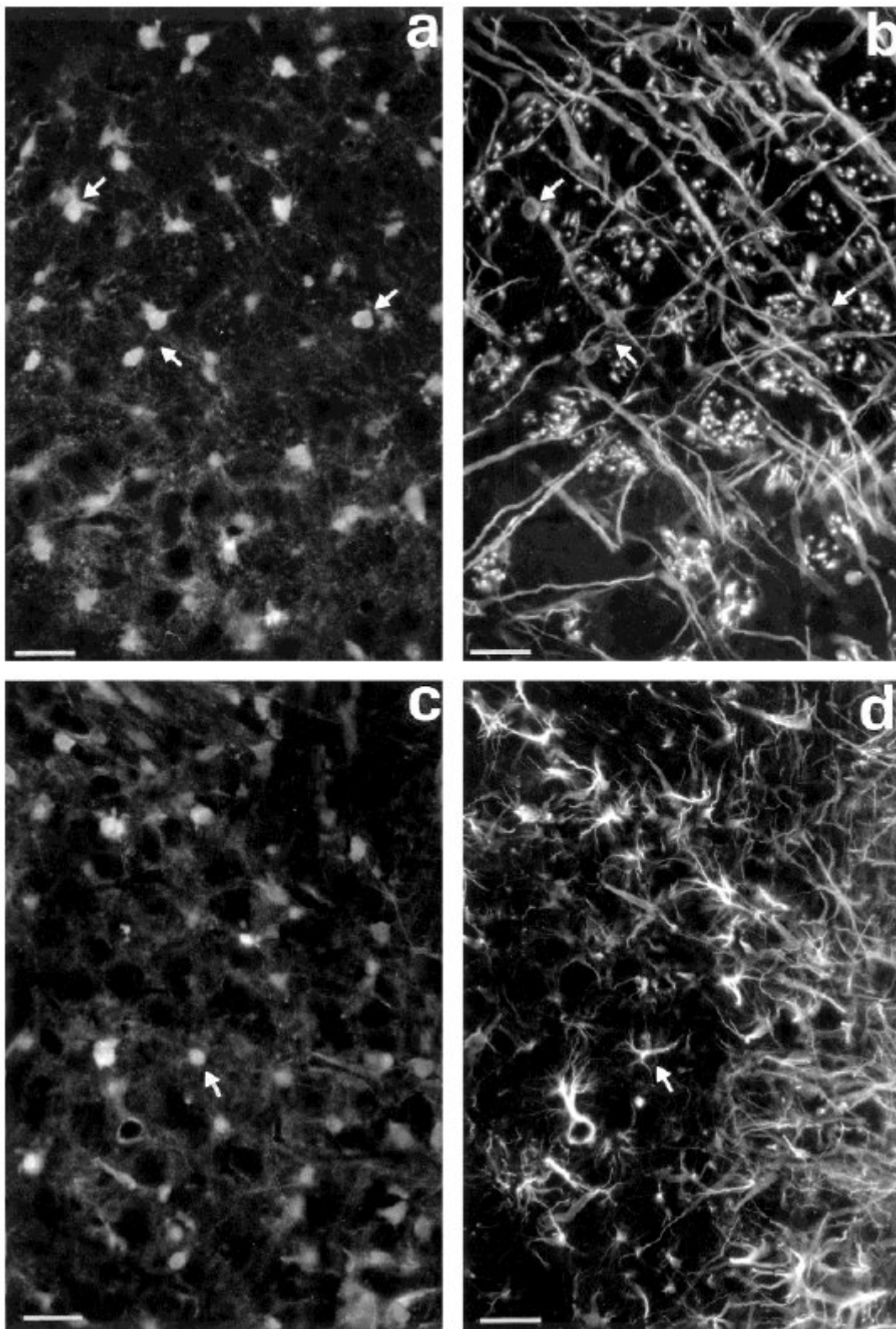

Fig. 5. Coronal sections of postnatal day (P)9 rat brain at the level of the tegmentum. a,b: Simultaneous localization of carnosine (a) and Rip (b). c,d: Simultaneous localization of carnosine (c) and glial fibrillary acidic protein (GFAP; d). Arrows indicate some of the double-labeled cells. Scale bars = 64 μm.
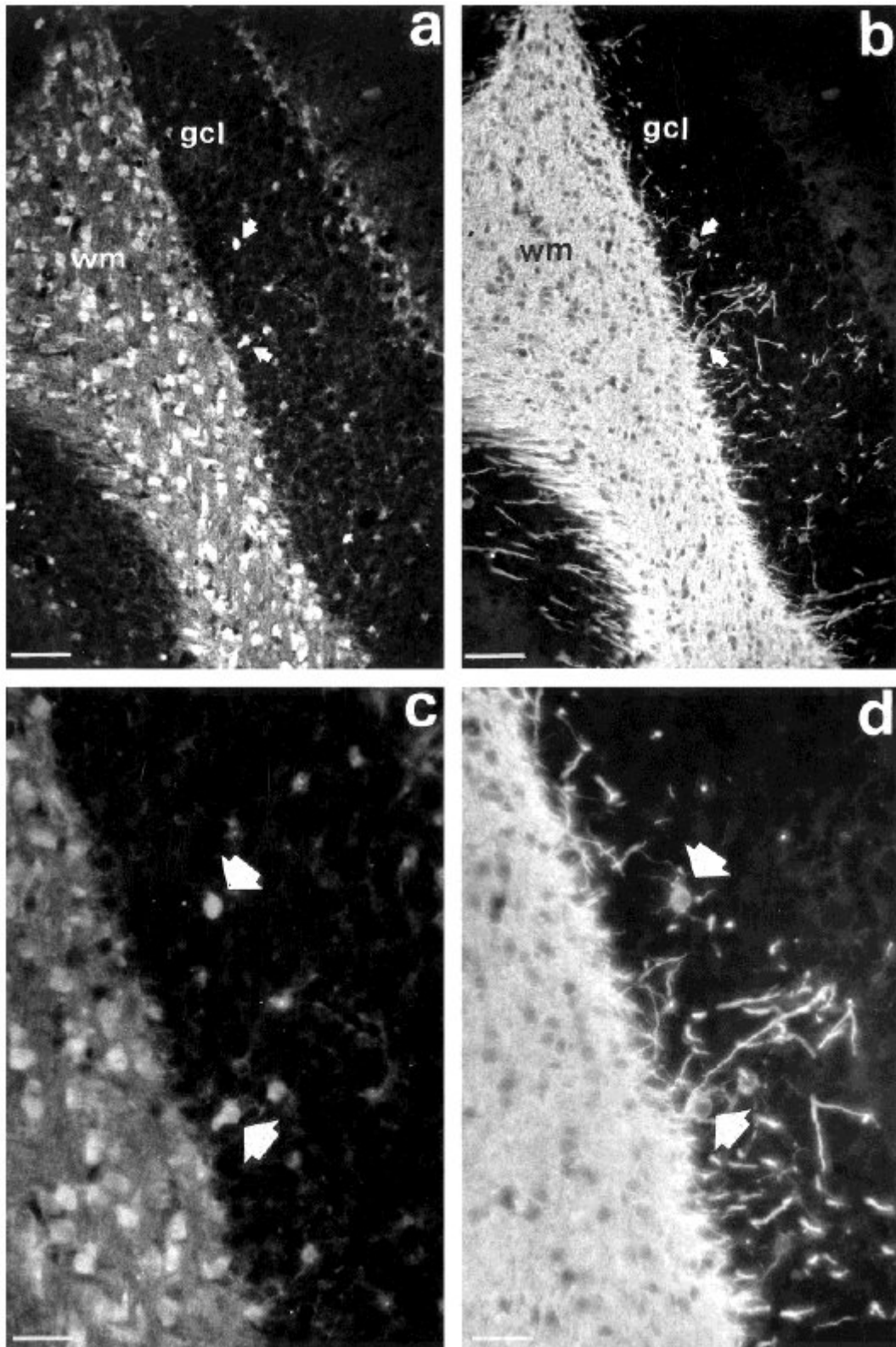
Fig. 6. Coronal section of postnatal day (P)12 rat brain at the level of the cerebellum. a,b: Simultaneous localization of carnosine (a) and Rip (b). In the white matter, strongly stained with Rip antibody, numerous carnosine-like immuno-reactive cells are present. Some double-labeled carnosine/Rip-immunoreactive cells are also evident in the granule cell layer. Arrows indicate two double-labeled cells. c,d: High-power view of the two double-labeled cells in a and b; gcl, granule cell layer; wm, white matter. Scale bars = 64 μm in a,b; 30 μm in c,d.
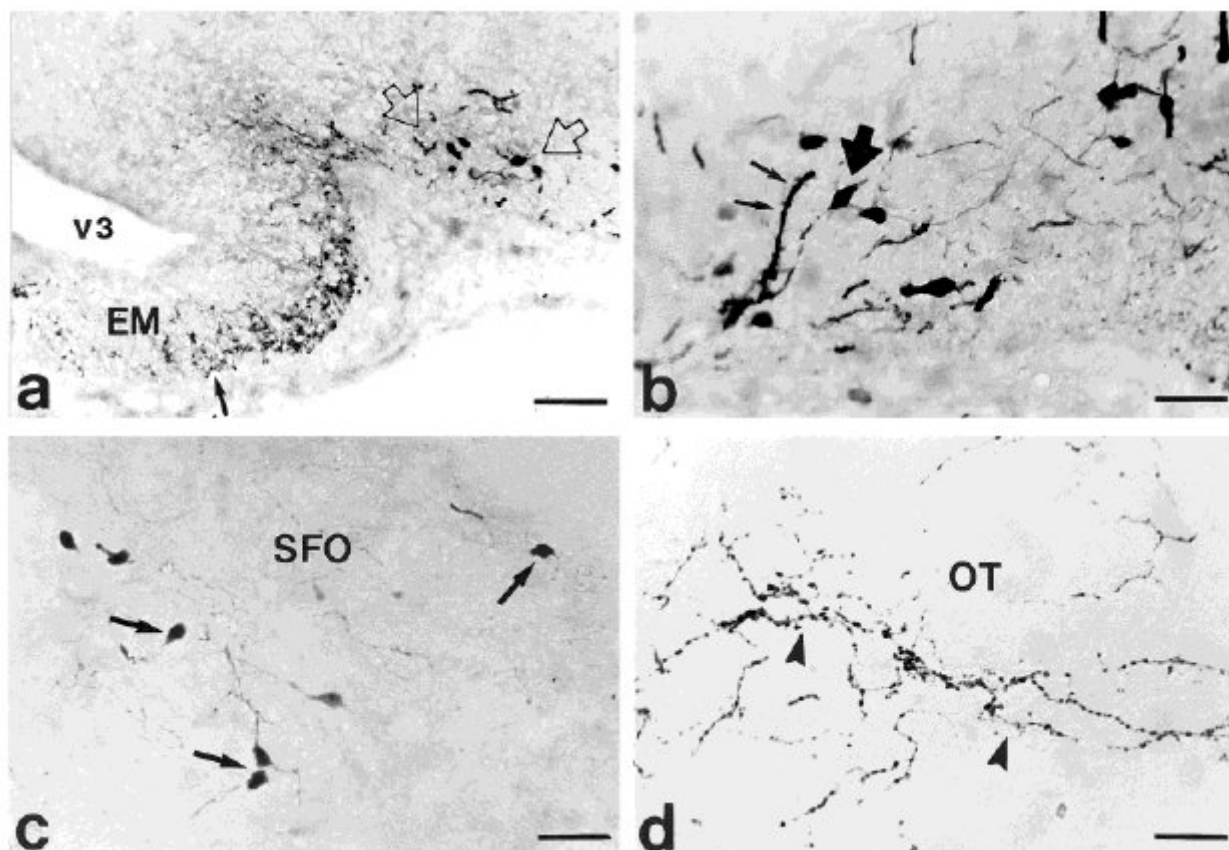
Fig. 7. Coronal (a– c) and sagittal (d) sections of postnatal day (P)3 rat brain at the level of the tuberal hypothalamus (a,b), the subfornical organ (c), and the olfactory tubercle (d). a: Carnosine-like immunoreactivity (LI) is present in nerve processes running in the outer part of the median eminence (black arrow indicates immunopositive beads in direct contact with the capillaries of the hypophysial portal vessel) and in cellular bodies and processes (empty arrows) localized in the ventromedial part of the tuberal hypothalamus (see also b). In b, a high-power view of this region in another section shows strongly labeled cells, having round, fusiform (thick arrow) or irregularly shaped cell bodies with thick, coarse processes (small black arrows). In c carnosine-like immunopositive cells are in the subfornical organ. The cellular body of these cells is strongly labeled (arrows), whereas the nerve processes are only faintly stained. d: Sagittal section showing numerous beaded carnosine-like immunoreactive fibers (see text for details). EM, median eminence; SFO, subfornical organ; OT, olfactory tubercle; v3, third ventricle. Scale bars = 64 µm in a; 32 µm in b– d.

Cellular localization of carnosine-LI

Our results show a dense carnosine-like immunoreactivity in different cell populations of the developing CNS. The immunopositive cells appeared to be labeled in both the nucleus and the cytoplasm. However, this nuclear staining is probably due to the diffusion of immunoreactive material from the cytoplasm to the nucleus and may be a fixation artifact.

Carnosine-LI in glial cells of the developing CNS. Carnosine-LI is detectable in glial cells of both the brain and spinal cord of rats beginning at the end of the first postnatal week of life. The localization of carnosine-related dipeptides in glial cells of the rodent brain has been previously described (Biffo et al., 1990; De Marchis et al., 1997). On the other hand, this is the first immunohistochemical evidence of the occurrence of carnosine-LI in the spinal cord, for which only quantitative biochemical data were previously available (Osborne et al., 1974; Ng and Marshall, 1978).

In previous in vivo studies, we have demonstrated that the localization of carnosine-related dipeptides in adult brain glia involves two distinct cell types: oligodendrocytes and astrocytes (De Marchis et al., 1997). Furthermore, in vitro experiments showed that oligodendrocytes are able to synthesize these dipeptides, whereas astrocytes are able to take up these molecules from the medium and eventually release them (Hoffmann et al., 1996). After uptake, carnosine is not hydrolyzed by astroglia,

in which it could be recovered from the cell extract in unmetabolized form (Schulz et al., 1987). The two processes, carnosine uptake and release are reported to be mediated by a high-affinity energy-proton-dependent transport system (Schulz et al., 1987; Yamashita et al., 1997). These results raise the question of whether these two cell types display the same distinct exclusive capacities in vivo. In an attempt to resolve this question, monoclonal antibodies raised against the enzyme carnosine synthetase, which can synthesize both carnosine and homocarnosine (Margolis et al., 1987), were tested in immunocytochemistry but have been ineffective (personal observations). In the current study, we approached the problem analyzing the distribution of these dipeptides during postnatal development within the rat brain and spinal cord, focusing on their pattern of appearance in glial cells. Analyzing the developmental profile, we demonstrate that carnosine-LI appears sequentially in the two principal types of glial cells and is first evident in subpopulations of mature oligodendrocytes. This observation strongly supports the hypothesis that also in vivo oligodendrocytes are able to synthesize carnosine and/or homocarnosine. However, the unresolved question is whether the astrocytes, which in subsequent stages manifest carnosine-LI, can themselves synthesize the dipeptides or instead take up these molecules after their release by mature oligodendrocytes. In vitro studies demonstrated not only that carnosine is actively synthesized by these latter cells, but also that the dipeptide is released into the culture medium (Hoffmann et al., 1996). Interestingly, a glutamate receptor-mediated release of carnosine, dependent on elevated intracellular Ca2$^+$, has been demonstrated in oligodendrocytes (Bakardjiev, 1998). This glial cell type possesses several neurotransmitter receptors and responds to glutamate with depolarization and elevated intracellular Ca2$^+$ (Kastritsis and McCarthy, 1993; Berger et al., 1994). This suggests a possible role for carnosine-related dipeptides in glial-neuronal interactions. Another site of carnosine-related dipeptide localization to be considered is that of the ependymal cells. From the very first ages at which carnosine-LI is present in oligodendrocytes (P6), an immunopositivity is observed in the same regions, in a subset of ependymal cells. The occurrence of carnosine-LI in ependyma, which has been previously described to occur also in adult animals, together with the carnosine localization described in the neuroblasts and in the glia of the SEL, are of particular interest considering the potential role recently suggested for these cell types as stem cell compartments of the adult CNS (Morshead et al., 1994; Johansson et al., 1999; Doetsch et al., 1999). The occurrence of carnosine-related dipeptides in the ependymal cells, which line the cerebral ventricles and the central canal of the spinal cord, might be related to their close anatomical and functional relationship with the cerebrospinal fluid (Bruni, 1998) which contains a low concentration of the aminoacylhistidine dipeptides (Sano, 1969).

It is well known that during development, the differentiation of both types of macroglial cells into mature functional elements, as well as the maturation of ependymal cells, takes place during the first 3 postnatal weeks of life (Jacobson, 1991). In particular, immunocytochemical analyses show that the maturation of oligodendroglia occurs during this period along a caudorostral gradient, from spinal cord to forebrain, and along a ventrodorsal gradient within the CNS in general (Coffey and McDermott, 1997). The developmental spatiotemporal pattern observed for carnosine-LI strongly suggests a correlation between the wave of oligodendrocyte maturation and the occurrence of carnosine-related dipeptides in both principal types of glial cells and ependymal cells. This correlation, together with the exclusive localization of carnosine-LI in differentiated cells (De Marchis et al., 1997), supports the hypothesis that the production of these dipeptides is associated with the onset of glial function. However, progressively, only a subpopulation of mature glial cells and a subset of ependymal cells are carnosine-like immunopositive, suggesting that a differential expression of these molecules may be related to the existence of functionally heterogeneous populations within the two principal types of glial cells and in the ependyma (Wilkin et al., 1990; Bruni, 1998). Moreover, after the first 3 weeks of life, when carnosine-LI achieves a widespread distribution in the whole CNS, the staining obtained with anti-carnosine is more diffuse and weak in comparison with that observed in younger animals, suggesting a decrease in the level of carnosine related dipeptide expression.

Carnosine-LI in other cells types and nerve processes of the developing CNS. Earlier immunohistochemical studies on the localization of the carnosine-related dipeptides in the nervous system

of adult mammals revealed that, apart from the glial immunostaining, carnosine-LI is present in a cell population that is located outside the brain, the mature ORNs (Biffo et al., 1990). These neurons project their carnosine-like immunoreactive axons from the OE to the main and accessory OB. From an ontogenetic point of view, the occurrence of carnosine-LI in the rat OE was detected starting from embryonic day 16 (E16; Biffo et al., 1992). Approximately at the same time (E14 –19), a number of cells that are carnosine-immunopositive were described along the olfactory nerves and in the OB (Tarozzo et al., 1994). These cells, which are present only during fetal life, were considered to migrate together with other populations of migrating cells (such as GnRH-positive neurons) toward the ventromedial aspect of the presumptive OB, arising from the olfactory pit. Carnosine-immunopositive cells outside the OE were also reported in neonatal (P1–7) pups of the marsupial, Monodelphis domestica (Tarozzo et al., 1995b). It has been suggested that these transitory cells could play a role in guiding the olfactory fibers and in the establishment of their final connections in the OB.

In this study, starting from the first postnatal day of life, in addition to the carnosine-like immunoreactive ORN projections in the OB glomeruli, we observed a carnosine-LI in nerve processes penetrating into the external plexiform layer. The occurrence of such ectopic sensory fibers has been previously demonstrated during the mouse olfactory system development (Monti Graziadei et al., 1980). As suggested by the authors, these fibers could have an inductive influence upon their target during ontogenesis. A system of fibers running throughout the ventromedial aspect of the OB, the olfactory tubercle and in more caudal regions of the basal prosencephalon, with a high concentration of terminals in the median eminence, was also observed. Our developmental analysis shows that this staining is transitory, in fact after P21, only rarely are carnosine-like immunoreactive processes found in these same regions.

In addition, during the same period of time (P1–21), we observed intense carnosine-LI in isolated or small groups of cells in restricted regions of the diencephalon such as the ventromedial hypothalamus, and in the SFO. These cells are immunonegative for glial-specific markers (GFAP, Rip, and vimentin) and no direct evidence has been presented to identify the neuronal nature of these cells. Further analysis needs to be carried out on this transient population of cells to understand whether they change their phenotype, switching to the expression of other neuropeptides, or alternatively are selectively eliminated. Based on the spatiotemporal pattern of localization, we hypothesize that, as for the previously described carnosine-like immunoreactive embryonic cells (Tarozzo et al., 1994), these cell populations might also derive from the olfactory placode. Moreover, the distribution observed for these carnosine-like immunopositive cell populations and fibers is similar to that previously described for another group of well-characterized migrating neurons, which express GnRH (Merchenthaler et al., 1984). We observed strong morphological similarities but no colocalization between carnosine-like and GnRH-immunolabeled cells, which have a more extended localization in the brain. Therefore, although the fate and the final phenotype of such carnosine-immunopositive cells still remain obscure, our results support the hypothesis that this developmentally regulated population of cells, together with other previously described cell types (Tarozzo et al., 1995a), could contribute to drive the organization of the prosencephalon and eventually to the formation of the central neuroendocrine system.

Conclusions

The present study analyzes the postnatal development of carnosine-LI in the rat CNS. Our results demonstrate that the expression of carnosine-related dipeptides is developmentally regulated and specifically localized in glial cell populations and ependymal cells of the brain and spinal cord. Furthermore, a transient expression of these molecules is demonstrated in putative neuronal cell bodies and fibers in restricted areas of the prosencephalon.

Several studies on the biological functions of these dipeptides reported that they possess a wide range of properties that enable them to act as antioxidants, metal chelators, free radical scavengers, and inhibitors of protein glycosylation to prevent several types of cell damage (Quinn et al., 1992; Hip-

kiss, 1998). In this context, the developmentally regulated expression of these molecules in glial and ependymal cells, which are critically involved in functional activities of the brain, may correlate with the level of their metabolic activity and of the related oxidative metabolism. It is also intriguing that carnosine-LI occurs in the ependymal cells and SEL precursors, which are considered as the potential stem cell compartment of the adult brain (Morshead et al., 1994; Johansson et al., 1999; Doetsch et al., 1999).

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