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Identification of the glial cell types containing carnosine-related peptides in the rat brain

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Abstract

The cellular localization of carnosine-like immunoreactivity was investigated in the adult rat forebrain and in glial cell cultures obtained from newborn rat brain. Using double staining methods, we showed that in vivo carnosine-like immunoreactivity was occurring in a large number of both glial fibrillary acidic protein (GFAP)-positive astrocytes and 2¢3¢-cyclic nucleotide 3¢-phosphodiesterase (CNP)-positive oligodendrocytes. In vitro, the carnosine-immunoreactive staining was restricted to a subpopulation of completely differentiated oligodendrocytes, whereas no reaction was detected in immature oligodendrocytes and in astrocytes. These observations could have profound physiopathological implications considering the role suggested for carnosine and related peptides as endogenous antioxidants, free radical scavengers and anti-glycating agents of the central nervous system (CNS).

Keywords: Carnosine; Central nervous system; Rat; Oligodendrocytes; Astrocytes; Cell culture; Immunocytochemistry

Carnosine (β-alanyl-L -histidine) and the related aminoacylhistidine dipeptides, homocarnosine (β-aminobutyryl-L histidine) and anserine (β-alanyl-N -methyl-L -histidine) are present in the muscle and nervous tissue of many vertebrates [9]. High amounts of carnosine and homocarnosine have been detected in the mammalian brain, whereas anserine is absent [2,9,17]. Carnosine is particularly abundant in the olfactory mucosa, where it is located in the olfactory receptor neurons (ORNs), and in the olfactory bulb, the target of the axons arising from ORNs [8]. Several data suggest that carnosine could act as a neuromodulator in the primary olfactory system [9].

Besides its localization in ORNs and their axonal projections, carnosine and/or homocarnosine have been found within glial cells, which, from their morphology and distribution, were identified as astrocytes and cerebellar Bergmann glia [2]. Biosynthesis of aminoacylhistidine dipeptides by primary cultures of central nervous system (CNS)-derived glia has also been demonstrated [1].

However, a precise characterization of glial cell types expressing carnosine-related peptides is lacking. This identification is important in order to envisage the biological functions played in glial cells, considering the role ascribed to

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carnosine-related peptides as intracellular pH buffers, metal ion chelators, free radical scavengers, antioxidants and anti-glycating agents [13,15]. Accordingly, we investigated the biochemical phenotype of carnosine-containing cells in the rat CNS, through double immunocytochemical methods, both in vivo and in vitro.

In vivo experiments were carried out on adult Wistar rats (Charles River). The animals were deeply anesthetized with intraperitoneal sodium pentobarbital (Pentothal Sodium, Gellini, 60 mg/100 g) and intracardially perfused with a heparinized saline solution (25 IU/ml in 0.9% NaCl, during 2–3 min), followed by a freshly prepared solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The tissues were subsequently rinsed in PB, cryoprotected, frozen at - 70° C and cut in serial coronal sections (8 µm).

In vitro experiments were carried out using glial cultures obtained from newborn Sprague–Dawley rats (Charles River). Primary cultures of mixed glia were obtained from the cerebral cortex of 1- to 2-day-old rats, using a modified method of McCarthy and De Vellis [10], as previously described [11]. Separate cultures of type 1 and type 2 astrocytes were prepared as previously described [12], cultured in Dulbecco's modified Eagle's medium (DMEM; Biochrom K.G., Berlin, Germany) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA). After 5 days in vitro (DIV) the cells were fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 20 min at 4° C, then rinsed in phosphate-buffered saline (PBS).

To prepare oligodendrocyte enriched cultures, primary cultures of mixed glia were obtained as described for astrocytes with the differences that the whole brain, rather than the cerebral cortex, and calf serum (10%) instead of fetal calf serum, were used [12]. The cultures of oligodendrocytes were grown in a chemically defined medium (insulin 5 µg/ml; transferrin 10 m g/ml in DMEM with 50 units/ml penicillin and 50 µg/ml streptomycin at pH 7.2). This medium was changed every 2 days; after 5 DIV, the cultures were fixed as described above. The characterization of the astrocyte and oligodendrocyte enriched cultures was performed as previously described [12].

For immunocytochemical studies all incubations were run at room temperature and washes were in PBS for 10 min. The tissue sections were treated with primary antibodies diluted in a PBS solution with 0.1% Triton X-100 and 1% non-immune serum of the same donor species of the secondary antiserum. The cultured cells were permeabilized and the non-specific binding was blocked by a 20 min incubation with PBS containing 0.3% Triton X-100 and 1% nonimmune serum of the same donor species of the secondary antibody. Single and double labelings were carried out by using immunofluorescence methods.

The primary antibodies used were: polyclonal rabbit anticarnosine (diluted 1:1000) which cross-reacts with carnosine, anserine and homocarnosine; anti-anserine (diluted 1:400), which is highly specific for anserine, provided by Dr. F.L. Margolis [2]; monoclonal mouse A2B5, marker of

the oligodendrocyte-type 2 astrocyte (O-2A) progenitors [3,16] (diluted 1:60); anti-myelin basic protein (a-MBP, diluted 1:20); anti-glial fibrillary acidic protein (a-GFAP, diluted 1:30); anti-2¢ 3¢ -cyclic nucleotide 3¢ -phosphodiesterase (a-CNP, diluted 1:200) from Boehringer Mannheim Biochemica; monoclonal mouse LB1 (anti-ganglioside GD3 , undiluted); anti-galactocerebroside (a-GC, undiluted) provided by Dr. G. Levi [6]. Secondary biotinylated antirabbit and anti-mouse antibodies (diluted 1:250) were from Vectastain; anti-mouse FITC (diluted 1:40) was from Boehringer Mannheim Biochemica; anti-rabbit FITC (diluted 1:50) was from Dako. The incubation time was overnight for the

primary and 1 h for the secondary antibody.

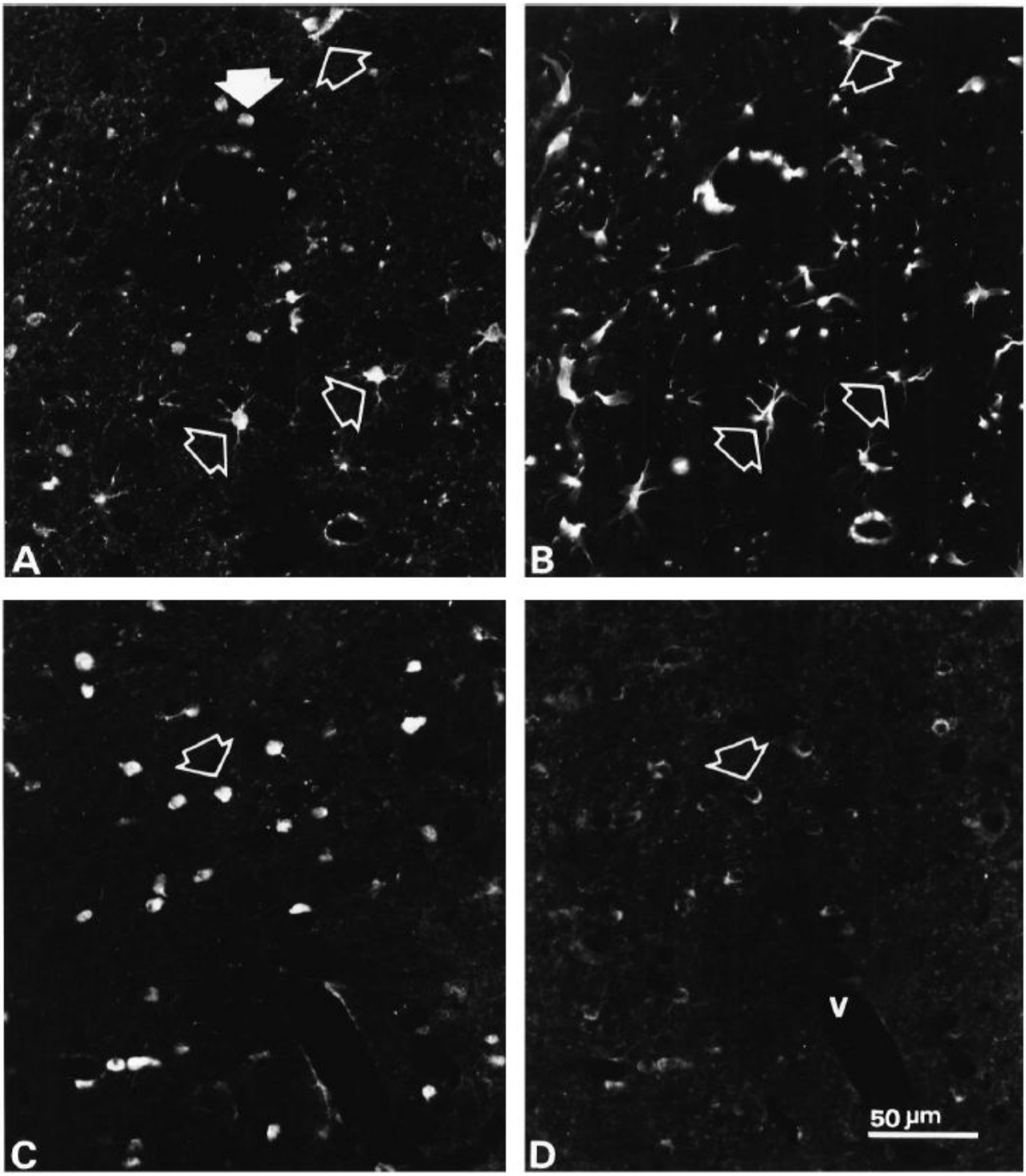

Fig. 1. Coronal sections of adult rat forebrain. Double simultaneous immunostaining with anti-carnosine (A,C) and anti-GFAP (B) or anti-CNP (D) antibodies. Some of the double labeled cells are indicate with empty arrow heads (A–D). In (A), full arrow head indicates a carnosineimmunopositive cell immunonegative for GFAP. V, Blood vessel.

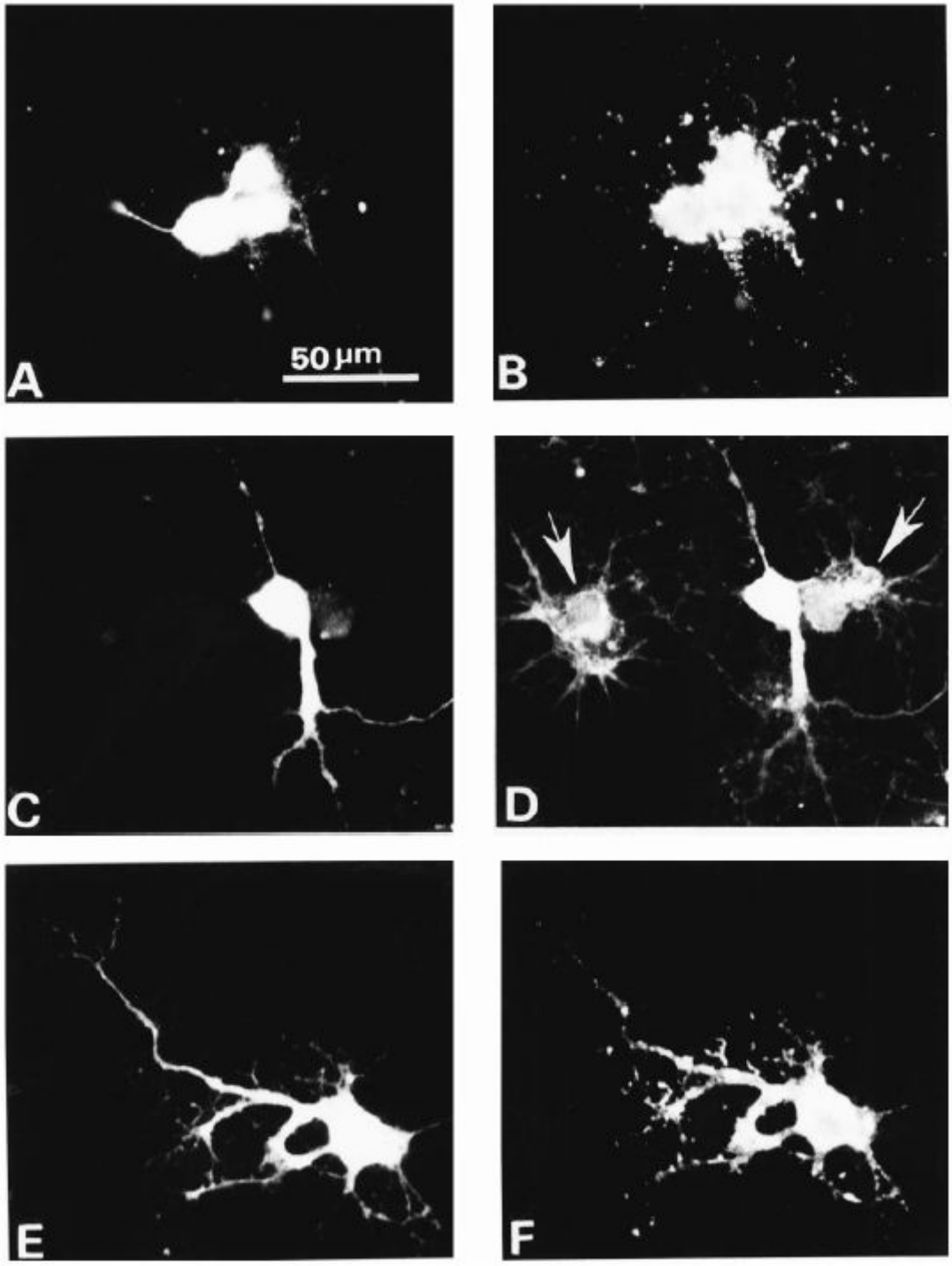

Fig. 2. Oligodendrocyte cultures double immunostained with anticarnosine (A,C,E) and anti-GC (B), anti-CNP (D) or anti-MBP (F) antibodies. In (D), arrows indicate two CNP-immunopositive cells immunonegative for carnosine.

In sections of the rostral forebrain of adult rats, a widespread carnosine-like immunoreactivity (-LI) was associated with cells of a glial-like morphology (Fig. 1A,C). Besides the glial localization, carnosine-LI was seen in the ORNs projections to the olfactory bulb, as previously described [2], and in migratory cells of the subependymal layer [7] and its rostral extension toward the olfactory bulb (P. Peretto et al., submitted to Neuroscience).

In glial cell cultures obtained from newborn rat brain, strongly labeled carnosine-like immunoreactive (-LIR) cells were found (Fig. 2A,C,E).

Immunostaining for carnosine both in vivo and in vitro was specific, since the reaction with the antiserum was abolished by liquid phase absorption with 0.1 μM carnosine–bovine serum albumin (BSA) conjugate.

Since the antiserum did not discriminate between carnosine, homocarnosine or anserine [2], the immunostaining observed (referred to as carnosine-like) could reflect the presence of one out of the three dipeptides. However, carnosine and homocarnosine have been described in the rodent brain, while anserine was undetectable [2]. Using a specific antiserum directed against anserine we confirmed that, also in vitro, no staining was detectable, suggesting that the immunoreactivity reflects the presence of carnosine and/or homocarnosine. In the rodents' brain, according to previous biochemical investigations [8], the values reported for carnosine were in the range of 0.01 to 0.2 nmol/mg of tissue, except for the olfactory bulb where this dipeptide reaches a concentration of 2 nmol/mg, probably due to the massive occurrence of the carnosine projections from the ORNs. The values reported for homocarnosine in the whole brain were in the range of 0.05–0.2 nmol/mg.

In vivo, by double simultaneous immunostaining, we observed that a conspicuous number of the CNS glial cells was immunopositive when treated with anti-carnosine serum. Process-bearing carnosine-LIR cells were doublelabeled for GFAP, a specific marker for astroglia (Fig. 1A,B), confirming the previous studies by Biffo et al. [2]. Moreover, we demonstrated that many carnosine-LIR cells with a simple round shape, localized in white and gray matter, were GFAP-negative, but they were stained by antibodies specific for the oligodendrocyte-marker CNP (Fig. 1C,D).

Interestingly enough, the in vitro immunolocalization pattern was rather different. Using primary cultures of mixed glia, we identified isolated or small clusters of carnosine-LIR cells. Double simultaneous stainings showed that the carnosine-LIR cells were GFAP-negative (data not shown). These observations suggested that the carnosine-LI in vitro could be restricted to oligodendrocytes.

To support this assumption, selective enriched cultures of astrocytes and oligodendrocytes were analyzed. Immunostaining with anti-carnosine serum on enriched cultures of astrocytes gave no results. By contrast, strong immunolabelings were found in enriched cultures of oligodendrocytes. In these cultures, where oligodendrocytes are present at different levels of cell maturation, a few carnosine-LIR cells, with a morphology varying from bipolar to a more complex branched shape, were encountered (Fig. 2A,C,E).

To test whether the presence of carnosine was correlated to a particular stage of the cell maturation, double labelings, using oligodendrocyte stage-specific markers, were performed. No colocalizations were observed between carnosine-LI and A2B5, or LB1, markers of oligodendrocyte precursors. Colocalizations with carnosine-LI occurred using anti-GC, anti-CNP and anti-MBP antibodies (Fig. 2). These antisera are raised against myelin-specific antigens sequentially expressed during oligodendrocyte differentiation and preserved in mature oligodendrocytes [4].

Carnosine-LIR cells were positive for each of these myelin specific markers. In particular, all the carnosine-LIR cells were always positive for MBP, one of the last antigens expressed by oligodendrocytes during cell maturation [4]. However, carnosine-like- and myelin-specific antigenimmunopositive cell populations did not completely overlap, since many oligodendrocytes did not stain for carnosine (Fig. 2C,D), showing that, also in vitro, only a subpopulation of differentiated oligodendrocytes was carnosine-LIR.

Carnosine is a powerful antioxidant, free radical scavenger and anti-glycating

agent [13,15]. In this contest, its localization in differentiated oligodendrocytes, both in vivo and in vitro, is of the highest interest, considering that these cells are susceptible to cell death due to glutamate exposure and/or reactive oxidative intermediates [14].

Biosynthesis of carnosine by glial cells in primary cultures has been previously demonstrated, whereas no significant synthesis of homocarnosine was described [1]. Furthermore, in agreement with our findings, a recent study has demonstrated that carnosine synthesis, in vitro, is confined to oligodendrocytes, and a correlation between carnosine biosynthesis and the differentiation of oligodendrocytes was also suggested [5]. Contrary to the synthesis, however, carnosine-uptake was described to be restricted to astrocytes [5], where we did not observe any carnosine-LI.

From all these data, it can be deduced that in vitro the immunoreactive peptide is mainly carnosine, and that it can be only synthesized by differentiated oligodendrocytes. Moreover, the absence of staining in astrocytes indicates that, in our conditions, these cells contain a low level of aminoacylhistidine dipeptides, or none at all. It is possible that the amount of carnosine produced by oligodendrocytes in culture does not reach the level necessary for its uptake in astrocytes.

In addiction, we suggest that the differences observed between the in vivo and in vitro labeling-patterns might be due to (1) the different age of animals considered in our experiments (neonatal in vitro, and adult in vivo), (2) a reduction of the functional interrelationships between the different glial cell types and/or (3) the lack of glial-neuronal interactions occurring in our culture system. Experiments are in progress to study the presence of carnosine both in vivo, during ontogeny, and in vitro, using a co-culture system of different types of glial cells and neurons.

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