Biosynthesis of carnosine in primary cultures of rat olfactory bulb

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Abstract

Primary cultures of glia cells obtained from adult rat olfactory bulb synthesize carnosine (β-alanyl histidine). The rate of synthesis increases the older the culture is and is enhanced by the addition of dibutyryl cyclic-AMP (dBcAMP) to the medium. Millimolar concentrations of this agent intensify galactocerebroside (GalC) staining compared to control cultures. Removal of GalC positive cells through antibody and complement cell killing decreases carnosine synthesis to a minimum. Cultures prepared from olfactory bulb of new-born rats contain neuron specific enolase (NSE) positive neurons and GalC positive ensheathing cells. Such cultures produce carnosine. When switched to nerve growth factor (NGF) depleted medium containing dBcAMP the share of neurons in the culture decreases drastically with time and concomitantly an increase of the relative rate of carnosine synthesis is observed. After 1 week in such medium the cultures contain almost no NSE positive cells. Virtually all cells express glial fibrillary acidic protein (GFAP) and are GalC positive. These data suggest that carnosine is synthesized by the ensheathing cells of the olfactory bulb and not by olfactory neurons.

Keywords: Carnosine; Synthesis; Olfactory bulb; Ensheathing cells; Cell culture; Rat

Carnosine is present at a low level throughout the mammalian central nervous system (CNS) while significant amounts of this peptide are found in the olfactory mucosa and bulb [9]. Initial data suggested that carnosine played a role as a neurotransmitter in olfaction [10]. Later studies colocated it with glutamate in olfactory sensory neurons and proposed a neuromodulatory role [13]. Concerning the site of synthesis in the CNS, it has been reported that primary cultures of glia cells from rodent brain manifest the ability to synthesize carnosine [3]. Recently, it could be demonstrated that the synthesis is a property of the oligodendrocytes of brain and spinal cord [8]. The question arises as to the cell type in the olfactory bulb responsible for such synthesis. The present study suggests that carnosine is synthesized from the ensheathing cells of the olfactory bulb.

Cultures of glia cells were prepared from whole olfactory bulbs of adult (3 months old) male Sprague–Dawley rats according to Ramón-Cueto and Nieto-Sampedro [11]. The cells were suspended in Dulbecco’s modified Eagle’s medium and Ham’s F12 medium, 1:1 (DMEM/F12) supplemented with 10% fetal calf serum (FCS; all purchased from GIBCO, Eggenstein, Germany) and plated on poly-d-lysine-coated dishes at a density of 2.10^5 cells/cm^2. The medium was renewed every 2 days. Eight days after plating, the medium was replaced with 1:1 mixture of the initial medium with oligodendrocyte differentiation medium described by Barres et al. [2], supplemented with 0.5 mM dibutyryl cyclic-AMP (dBcAMP; Fluca, Neu-Ulm, Germany), referred to as dBcAMP-containing medium. The cultures were fed every 2 days. Removal of galactocerebroside (GalC)-positive cells from such cultures was accomplished after 9 days in dBcAMP-containing medium by antibody-mediated cell killing as described [8].

Rat olfactory neurons were prepared from 3 day old pups by a modification of a procedure described by Ronett et al. [12]. After digestion the cells were suspended in modified Eagle’s medium containing d-valine (MDV), 10% dialyzed FCS (dFCS), 5% heat inactivated horse serum (all obtained from GIBCO, Eggenstein, Germany), 10 μM cytosine arabinoside (CA; Sigma, Deisenhofen, Germany) and 25 ng/ml nerve growth factor (NGF) (Boehringer Mannheim, Mannheim, Germany). The cell suspension was filtered through 25 μm nylon mesh and plated at a density of 5.10^5 cells/cm² on laminin coated (Sigma, Deisenhofen, Germany) dishes. Cell viability was above 94%. After 24 h the medium was renewed with MDV containing 15% dFCS, NGF and CA.
Cells were fed every day with fresh medium. After 5 days in vitro the cultures were switched to dBcAMP-containing medium used for ensheathing cells from adult rat olfactory bulb and maintained for a week. This medium was renewed every second day.

The cells grown on poly-d-lysine or laminin coated coverslips were characterized by using the indirect immunofluorescence method. The primary antibodies used were: monoclonal mouse anti-vimentin (1:40) from Sigma, Deisenhofen, Germany, mouse anti-GalC (1:20) and anti-myelin basic protein (MBP) (1:200) from Boehringer Mannheim, Mannheim, Germany; polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) (1:200) from Dakopatts, Glostrup, Denmark and rabbit anti-neuron specific enolase (NSE) (1:2000) from Polysciences Europe GmbH, Eppelheim, Germany. Secondary antibodies from Dianova, Hamburg, Germany were used at dilutions: goat-anti-mouse-IgG-fluoroprobe 570 (1:200) and anti-rabbit-IgG labeled with fluoroprobe 570 or 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein (DTAF; 1:300). The cells were permeabilized in 0.1% Triton X-100 and blocked with horse serum. The incubation time was 1 h for the primary and 30 min for the secondary antibody. The coverslips were mounted with glycerol/phosphate-buffered saline (PBS) (1:1) and examined under an Olympus BH-2 microscope with fluorescence optics.

The biosynthesis of carnosine was studied after incubating the cells for 3 h at 37°C with culture medium containing 10 μM \([^3H]β\)-alanine (specific radioactivity 50 mCi/mmol; DuPont de Nemours, Bad Homburg, Germany). Medium and cell extract were analyzed separately as previously described [14] and the data presented as a sum of both values. DNA was measured fluorimetrically using H33258 fluorophore and calf thymus DNA as standard according to Downs and Willinger [7].

Glia cells obtained from rat adult olfactory bulb were cultured in DMEM/F12 medium with 10% FCS for 8 days. Almost all cells were vimentin (data not shown), GFAP and some of them GalC positive at that time (Fig. 1A–C) and already showed a certain level of carnosine biosynthesis (Table 1). After 8 days the medium was replaced with dBcAMP-containing medium and the cells were maintained for different periods of time in it. An increase in carnosine synthesis could be observed (Table 1) which correlated with an increase of the intensity of GalC staining (Fig. 1D–F). Treatment of such cultures with anti-GalC antibody followed by agar-absorbed rabbit complement dropped the level of carnosine synthesis from 13.0 ± 1.8 pmol.h\(^{-1}.mg\^{-1}\) DNA to 2.2 ± 0.3 pmol.h\(^{-1}.mg\^{-1}\) DNA.

Dissociated olfactory tissue from 3 day old rats consisted of small round neuronal cells which were NSE positive. Some of them grew over flattened non-neuronal cells of

Fig. 1. Primary cultures of adult rat olfactory tissue. The cells were kept 8 days in DMEM/F12 medium with 10% FCS (A–C). After 9 additional days in dBcAMP-containing medium the intensity of GalC staining increases strongly (D–F). (A,D) Phase contrast; (B,E) GFAP labeling; (C,F) GalC labeling. Bar 150 μm.
The process of differentiation of ensheathing cells in vitro is still not very well understood. The source of tissue (embryonic, new-born, adult olfactory bulb) as well as the culture conditions could influence the cell phenotype [1,5,6,11]. One set of data shows that co-culturing of these cells with dorsal root ganglion neurons was effective in inducing myelin sheath formation around the neurites [4]. Nevertheless, the expression of GalC and MBP positive phenotype in neuron-free cultures is still a subject of discussion. Doucette and Devon [6] have reported that ensheathing cells express GFAP and GalC but not MBP when the intracellular level of cAMP is elevated. By following a modified version of the procedure by Ramón-Cueto and Nieto-Sampedro [11], it was possible to obtain GFAP and weakly GalC positive cells from adult rat olfactory bulb in serum-containing medium. The intensity of GalC staining increased substantially in dBcAMP-containing medium. Parallel to the differentiation of the cells, an increase of carnosine synthesis was observed (Fig. 1D–F; Table 1) which dropped drastically after removal of GalC positive cells through antibody and complement mediated cell killing. When the primary cultures were obtained from olfactory bulb of neonatal rats the ensheathing cells were well.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Total time in culture (days)</th>
<th>Total carnosine synthesized pmol h⁻¹ (µg DNA)⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F12 with 10% FCS</td>
<td>8</td>
<td>6.5 ± 1.1</td>
</tr>
<tr>
<td>dBcAMP-containing medium</td>
<td>10</td>
<td>7.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>8.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>10.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>13.0 ± 0.5</td>
</tr>
</tbody>
</table>

After 8 days in DMEM/F12 medium with 10% FCS the medium was replaced by dBcAMP-containing medium. The carnosine synthesized was measured at the indicated periods of time.
Differentiated because of the co-culture with olfactory bulb neurons. Removal of the neurons from such cultures affected the morphology of the ensheathing cells but not the expression of GFAP and GalC (Fig. 2). The biosynthesis of carnosine increased substantially at the same time which suggests that this type of cell but not olfactory neurons synthesizes carnosine.


Table 2

<table>
<thead>
<tr>
<th>Medium</th>
<th>Total time in culture (days)</th>
<th>Total carnosine synthesized pmol h⁻¹ (μg DNA)⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDV with NGF</td>
<td>3</td>
<td>7.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>dBcAMP-containing</td>
<td>8</td>
<td>17.2 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>28.5 ± 2.2</td>
</tr>
</tbody>
</table>

Neonatal rat olfactory cells were cultured for 5 days in MDV medium with 15% dialyzed FCS, 25 ng/ml NGF and 10 μM cytosine arabinoside and after that time switched to dBcAMP-containing medium used for culturing the cells from adult rat olfactory bulb.

At the indicated periods of time the carnosine synthesized was measured as described above.