

# Cholinergic innervation of parvalbumin- and calbindin-containing neurones in the hippocampus during postnatal development of the rat brain

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*Immunohistochemical study of the cholinergic innervation of the parvalbumin- and calbindin-containing cells in the hippocampus was conducted on 30 rat brains of various postnatal ages: P0, P4, P7, P14, P21, P30, P60 and P180. Sections with double immunostaining for vesicular acetylcholine transporter (VAChT; the marker of cholinergic cells, fibres and terminals) and parvalbumin (PV) or calbindin (CB) were analysed using confocal laser-scanning microscope. Obtained data demonstrate that the pattern of cholinergic innervation of calbindin- and parvalbumin-immunoreactive hippocampal neurones shows some differences. During development as well as in the adult species cholinergic terminals preferentially innervate CB-containing neurones, while cholinergic terminals on PV-containing cells were observed rarely. Cholinergic endings on the CB-ir neurones are localised both on their somata and dendrites, whereas on PV-ir cells they form synaptic contact predominantly with processes. In spite of the unquestionable cholinergic influence particularly on CB-ir cells, the number of cholinergic endings suggests that this input seems not to be crucial for the activity of the studied cell populations.*

**key words:** hippocampus, development, calbindin, parvalbumin, cholinergic system

## INTRODUCTION

Calcium-binding proteins are intracellular calcium acceptors belonging to two different families: EF-hand proteins and annexins. The annexins family is characterised by proteins that bind calcium in the presence of phospholipid-containing membranes. EF-hand proteins may function mainly as a calcium “buffer”, decreasing the free cytoplasmic concentration of this ion [19] and contributing to calcium homeostasis [40]. EF-hand “buffer” proteins, such

as parvalbumin (PV), calbindin D28k (CB) or calretinin (CR), are specifically observed in well-defined subpopulations of neurones belonging to multiple functional systems [19].

In the hippocampus, a critical area for learning and memory, parvalbumin and calbindin — markers of different subpopulations of GABAergic interneurons [8, 30, 42] — act differently by GABA<sub>A</sub> and GABA<sub>B</sub> receptor-mediated neuronal response, respectively [31, 34]. Their expression in the developing

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hippocampus is characterised by a distinct temporal pattern [3–5, 27, 39]. It seems probable that these proteins play a different functional role during development: PV is thought to be involved in the maturation of inhibitory circuitry, CB in the production, stabilisation, maintenance of cytoskeletal elements and neuronal migration [19].

The hippocampus is under the influence of various neurotransmitter systems. Its neurones receive the most intense cholinergic innervation from the medial septum/diagonal band complex [1, 25]. The endings of cholinergic septohippocampal fibres are localised in all the hippocampal cell layers [13, 14] and the cholinergic system has been implicated as crucial in learning and memory processes [26]. Furthermore, during development, the cholinergic system plays an important role in the proliferative processes and axon guidance [23, 24].

Understanding the targeting specificity of septal cholinergic afferents with subpopulations of GABAergic interneurons could have important implications for hippocampal function. Some evidence indicates that functionally and morphologically distinct subtypes of interneurons might modulate selective types of hippocampal-dependent memory processes [18, 22, 35]. Thus in the present study we aimed to investigate the developmental relationship between the cholinergic innervation and the neurones containing parvalbumin and calbindin in the hippocampus, using immunohistochemical methods. For the detection of cholinergic fibres and terminals, we used a specific antibody against vesicular acetylcholine transporter (VACHT) [33].

## MATERIAL AND METHODS

The material consisted of 30 Wistar rat brains of various postnatal ages: P0, P4, P7, P14, P21, P30, P60 and P180. Care and treatment of the animals were in accordance with the guidelines for laboratory animals established by the National Institutes of Health as well as by the Local Ethical Committee of the Medical University of Gdańsk. All animals were deeply anaesthetised with lethal doses of Thiopental (80 mg/kg of body weight), then transcardially perfused with 0.9% solution of NaCl with heparin, followed by 4% solution of paraformaldehyde in 0.1 M phosphate buffer (pH 7.4; 50–250 ml). The brains were postfixed in 4% paraformaldehyde fixative for 3–4 hours, and then kept in 0.1 M phosphate buffer containing 10% sucrose (overnight at 4°C) and 30% sucrose (until sunk).

Coronal 40- $\mu$ m-thick, serial sections of the brain were cut on JUNG 1800 cryostat (Leica, Germany). The free-floating sections were blocked for 1 hour with 3% NGS containing 0.4% Triton X-100 and then incubated for 48 hours in 4°C with the mixture of the anti-VACHT and anti-parvalbumin or anti-calbindin antibodies diluted in 3% NGS (Table 1). After multiple rinses in PBS, the sections were incubated (2–3 hours, at room temperature) with the mixture of the appropriate secondary antibodies conjugated with the FITC or Cy3 (Table 1).

The specificity of staining was checked according to the procedure described by Wouterlood et al. [41].

The histological sections were studied under the MicroRadiance AR-2 (Bio-Rad, UK) confocal laser-scanning microscope equipped with an Argon two-line (488 and 514 nm) laser. The former line was applied to excite the fluorescein isothiocyanate (FITC), whereas the latter to excite Cy3. For 3D reconstruction the image analysis program LaserSharp 2000 v. 2.0 (Bio-Rad, UK) was used. Digital images were obtained using Laser Pic v. 4.0 (Bio-Rad, UK) software.

For subdivision of the hippocampal formation we applied the criteria used by Sloviter [36] and Jiang and Swann [21] (Fig. 1).

## RESULTS

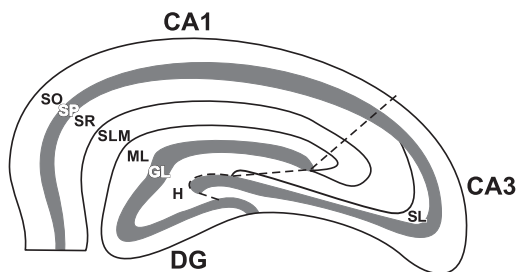
### PV-immunoreactivity

In rats of P0, P4 and P7 groups, no PV-immunoreactivity (PV-ir) was detected in the hippocampus. On P14 PV-ir elements were observed in both dentate gyrus and hippocampus proper. At this stage

**Table 1.** Specifications and dilutions of the primary and secondary antibodies

Antibodies	Manufacturers	Dilution
Goat anti-VACHT ( <i>polyclonal</i> )*	Chemicon	1:1000
Mouse anti-Parvalbumin ( <i>monoclonal</i> )*	Sigma	1:500
Mouse anti-Calbindin-D (28 kD; <i>monoclonal</i> )*	Sigma	1:100
Cy <sup>TM</sup> 3-conjugated Donkey anti-Goat**	Jackson ImmunoResearch	1:1000
FITC-conjugated Donkey anti-Mouse**	Jackson ImmunoResearch	1:100

\* primary antibodies, \*\* secondary antibodies



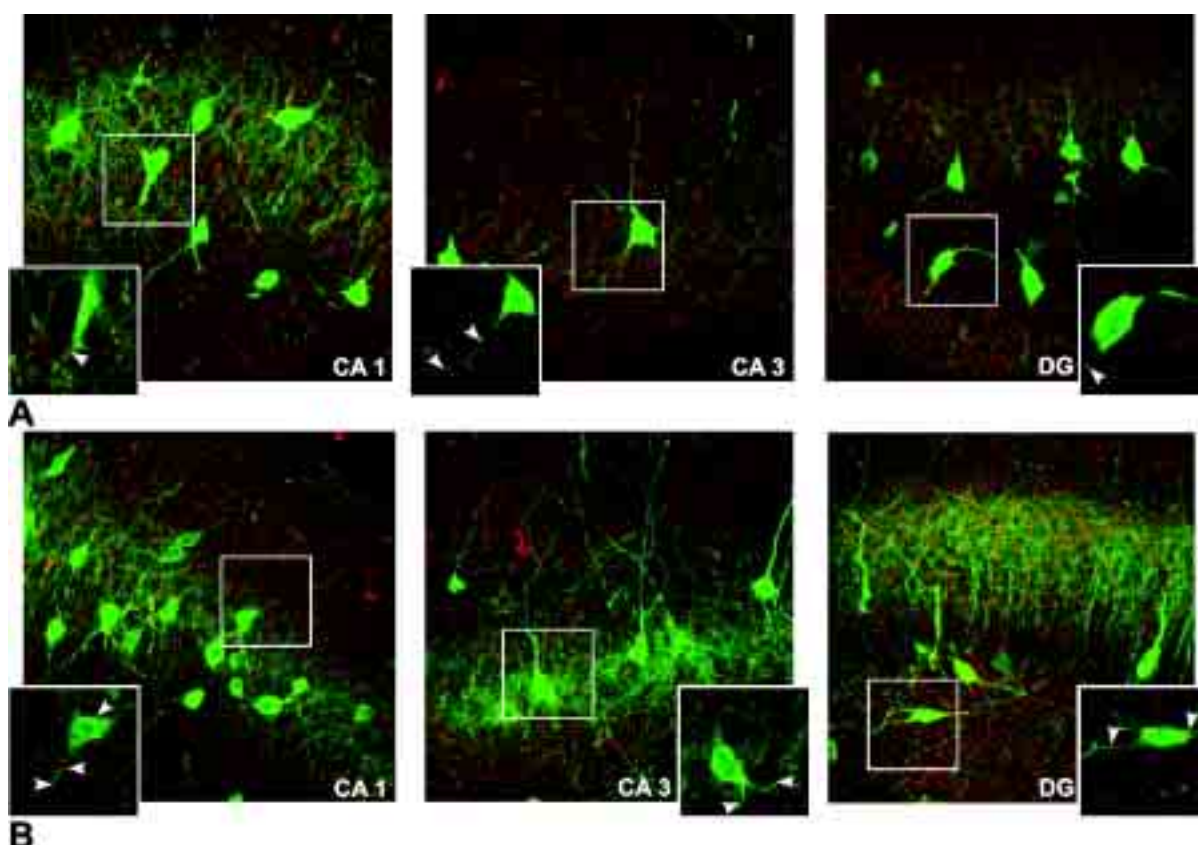
**Figure 1.** Rat hippocampus. Schema. Dotted lines separate sector CA1 from CA3 and CA3 from dentate gyrus (DG); SO — stratum oriens, SP — stratum pyramidale, SR — stratum radiatum, SLM — stratum lacunosum-moleculare, ML — molecular layer of dentate gyrus, GL — granule cell layer, H — hilus of dentate gyrus, SL — stratum lucidum (based on Jiang and Swann [21])

both PV-ir somata and fibres in the hippocampus proper were mainly located in the stratum pyramidale and oriens (Fig. 2A). In the dentate gyrus the majority of PV-ir somata were observed in the infragranular region of hilus whereas PV-ir fibres and puncta were dense in the granule cell layer (Fig. 2A).

During the next stage the number of PV-immunoreactive somata and fibres increased and at P21 they assumed a level and distribution characteristic for adult brain. From P21 the pattern of PV-immunoreactivity was similar to that previously described [10, 30, 32]. In the hippocampus proper, PV-immunoreactivity was observed in CA1 and CA3 sectors. PV-ir neurones were of various sizes and shapes; multipolar or bipolar cells prevailed (Fig. 2B). In the dentate gyrus, PV-positive elements were mainly observed in the granular layer and in neighbouring regions (Fig. 2B). Most of them were of fusiform or pyramidal shape; some horizontal basket cells were also observed (Fig. 2B). The densest plexus of PV-ir terminals occurred in the principal cell layers (pyramidal and granular; Fig. 2B). It frequently formed “baskets” surrounding cells immunonegative for parvalbumin (Fig. 2).

#### Cholinergic innervation of parvalbumin positive cells

A study of double immunolabelled sections with anti-VACHT and anti-parvalbumin antibodies indi-



**Figure 2.** PV-positive cells (green) and VACHT-positive fibres and terminals (red) in sectors CA1 and CA3 of the hippocampus proper and in the dentate gyrus (DG) at stages: P14 (A) and P21 (B). White arrowheads indicate VACHT-positive puncta forming synaptic contact with PV-ir neurones; P — postnatal day.

cated that VAcHT positive puncta constituted terminals on PV-ir cells (Fig. 2); their amount increased to P21 and then this pattern of immunoreactivity did not change considerably. 3D-reconstruction of double labelled sections showed that cholinergic innervation of PV-ir cells in the hippocampus is rather scanty; only a small population of hippocampal PV-ir neurones (predominantly in the dentate gyrus) possessed cholinergic endings located mainly on their processes (Fig. 2), while sporadically on their somata. In the principal cell layers VAcHT-ir and PV-ir elements formed "baskets" often surrounding the same cells immunonegative for parvalbumin (Fig. 2).

### CB-immunoreactivity

During the first four days of postnatal life, CB-immunoreactivity was observed throughout the whole hippocampus, but predominantly in the pyramidal layer of CA1 sector and in the granule cell layer of dentate gyrus (Fig. 3A). CB-ir neurones were also found in other strata (mainly in the stratum radiatum of the hippocampus proper and in the hilus of dentate gyrus); most of them showed non-pyramidal morphology.

During the next stage the general pattern of distribution of CB-immunoreactivity was sustained but the number of immunoreactive cells (especially in the granule cell layer) increased. The cells were stained more intensely (Fig. 3B, C). At the same time the development of CB-immunoreactive processes was also observed (strong CB-immunoreactivity characterised especially the mossy fibres of CA3 sectors; Fig. 3C) so at P21 they showed mature arborisation (Fig. 3D).

From P21 morphology, the distribution and level of CB-ir elements in hippocampus did not change significantly and were similar to that previously described in adult species [16, 36]. In the hippocampus proper both pyramidal cells (mostly in CA1 sector) as well as interneurones (in all subfields) were CB-immunoreactive (Fig. 3D). In the dentate gyrus strong CB-immunoreactivity was observed mainly in neurones of the granule cells layer, although some CB-ir cells were observed also in the molecular layer and hilus (Fig. 3D).

### Cholinergic innervation of calbindin positive cells

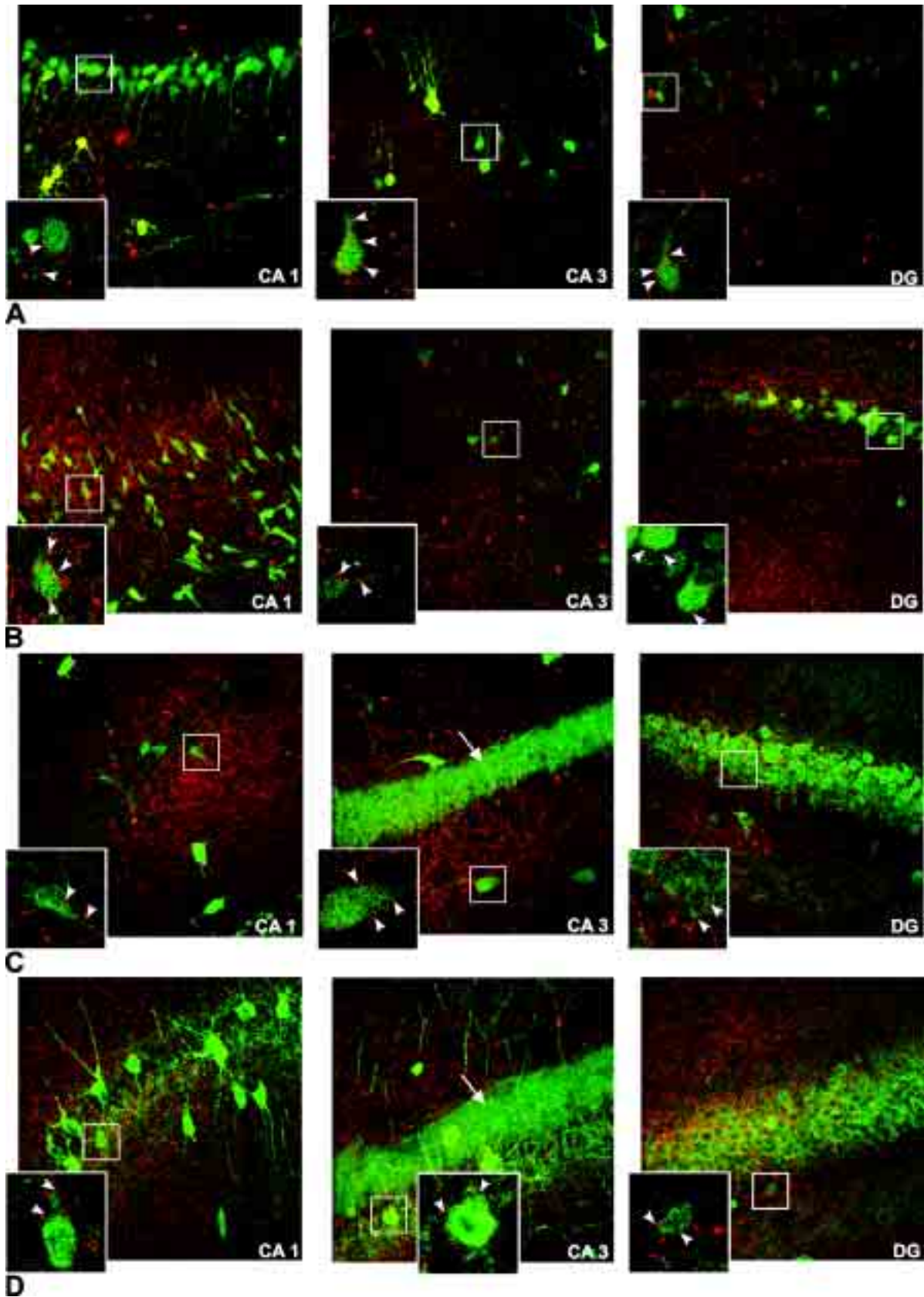
3D-reconstruction of double immunostained sections has shown that as early as on P0 VAcHT-ir puncta formed synaptic contact with CB-ir neurones, both in hippocampus proper and in dentate gyrus (Fig. 3A). Then, along with the development of CB-ir cells and

cholinergic network, the number of cholinergic endings on CB-ir elements increased, so in adult species CB-ir neurones possessed quite a few cholinergic endings located on their soma and along dendrites (including their initial parts). Cholinergic fibres innervated both principal (pyramidal and granular) and non-principal CB-ir cells (Fig. 3). In the principal cell layers VAcHT-ir and CB-ir elements formed "baskets" often surrounding the same cells immunonegative for calbindin (Fig. 3).

## DISCUSSION

The results of the present study suggest that the pattern of cholinergic innervation of calbindin- and parvalbumin-immunoreactive hippocampal neurones shows some differences. During development and in adult brain, cholinergic terminals preferentially innervate CB-containing neurones compared to PV-ir cells. Moreover, distribution of VAcHT-ir terminals on the cells is dissimilar. Cholinergic endings on CB-ir neurones are localised on both their somata and dendrites, whereas on PV-ir cells they form synaptic contact predominantly with processes.

Parvalbumin-containing neurones in the hippocampus appeared relatively later. Then the density of PV-immunoreactive somata and fibres increased and at P21 they assumed a level and distribution characteristic for adult brain. This observation confirms the prior studies with both immunohistochemical [5, 37] and *in situ* hybridisation methods [9]. In the rat hippocampus low hybridisation was observed at P10–P12. Between P12 and P16 the number of PV mRNA-positive cells increased markedly within all hippocampal subfields, reaching an adult-like distribution of labelled cells by P18. At this time the functional maturation of the hippocampal system, correlated with the developmental expression of parvalbumin, seems to be reached [5, 27, 37]. The influence of cholinergic innervations on PV-ir hippocampal neurones during development and in adult is rather limited. This is confirmed by both the low number of VAcHT-ir endings observed on PV-ir cells and their location (mainly on processes). According to Dougherty and Milner [11] septal cholinergic afferent makes predominantly axodendritic synapses upon hippocampal PV-ir neurones. Such a dendritic contact may exert a less rapid and potent effect on the cell than perikarial contact [11, 38]. Also dendritic synapses influence the efficacy of specific types of afferent innervation [29], whereas somatic synapses regulate action potential firing.



**Figure 3.** CB-positive cells (green) and VAcHT-positive fibres and terminals (red) in sectors CA1 and CA3 of hippocampus proper and in the dentate gyrus (DG) at stages: P0 (**A**), P7 (**B**), P14 (**C**), P21 (**D**). White arrowheads indicate VAcHT-positive puncta forming synaptic contact with CB-ir neurones; arrows indicate CB-positive mossy fibres; P — postnatal day.

It seems that the subpopulation of PV-ir interneurons is under the control of systems other than the cholinergic one. PV-immunoreactive population appears to be a major target for GABAergic septohippocampal fibres [12]. According to Fukuda et al. [15] PV-ir GABAergic neurons receive a significantly larger GABAergic input than PV-negative GABAergic neurons. Among hippocampal interneurons PV-ir cells are also unique in their tendency to receive a higher density of GABAergic input on their somata. Activation of this projection would increase hippocampal excitability through the disinhibition of principal cells [6].

The other source of GABAergic innervation of PV-ir cells is the terminals of remaining hippocampal intrinsic neurons (including PV-ir) [15]. Physiological studies have raised the possibility that non-pyramidal neurons are crucially involved in the induction and maintenance of membrane potential oscillations in pyramidal neurons [7]. It seems most probable that the activity of hippocampal GABAergic neurons is influenced by GABAergic connections with both intrinsic and septal neurons, leading to synchronous oscillations in the principal neurons.

PV-ir neurons in the hilus are targets of mossy fibre collaterals [28, 32] — they may participate in feed-back inhibition of the granule cells. PV-ir neurons in CA3 sector have also synaptic contact with mossy fibres and may participate in feed-back circuit inhibiting CA3 pyramidal cells [10].

From the beginning of the observation period VAcHT-ir puncta formed synaptic contacts with CB-ir neurons in the hippocampus. During the development, in spite of the increase in the number of synaptic contacts between them, the distribution of immunoreactivity has not changed — it was still present on somata and along dendrites of CB-ir cells.

It seems that CB-ir neurons receive more intensive cholinergic input than PV-ir cells.

During development, CB is presumed to play a role in cellular development and neuronal plasticity by interfering with the intracellular calcium buffering and transport [2].

The presence of VAcHT-ir endings on CB-containing cells already at early postnatal period may suggest the influence of the cholinergic system on the development.

Primary targets of CB-ir interneurons are probably distal dendrites of pyramidal cells [16]. Correlation of the anatomical, neurochemical and electrophysiological data suggests that CB-containing neurons would be involved in feed-forward, GABA<sub>B</sub> receptor mediated inhibition in the distal dendritic tree [16]. This inhibition may be under the control/

/modulation of septal cholinergic projection and/or serotonergic projection from the median raphe nucleus, selectively innervating only CB-ir interneurons in the hippocampus [17, 20].

In the principal cell layers VAcHT-ir terminals, along with PV-ir and CB-ir elements, formed “baskets” often surrounding the same cells immunonegative for PV and CB, respectively — this observation suggests that septal cholinergic afferents and PV-ir and/or CB-ir cells could modulate the same principal cells. Moreover, the proximity of cholinergic terminals and PV-ir and CB-ir endings raises the possibility that PV-ir and CB-ir cells may modulate septal cholinergic hippocampal afferents or *vice versa* [11].

To sum up, our results suggest that: 1) populations of interneurons containing distinct calcium binding proteins, parvalbumin and calbindin, also differ in regard to their cholinergic innervations, 2) cholinergic input (in spite of its unquestionable influence particularly on CB-ir cells) seems not to be crucial for the activity of interneurons. It seems probable that the cholinergic system affects hippocampal activity rather by direct contact with principal cells or by other intrinsic subpopulation such as GABAergic neurons containing NPY which are preferentially innervated by cholinergic septal afferents [11].

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