

Cholinergic endings on various neurones containing calcium binding proteins and glutamic acid decarboxylase in the hippocampus of the rat

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Immunohistochemical study of the cholinergic innervation of the hippocampal cells containing glutamic acid decarboxylase (GAD) and calcium binding proteins: parvalbumin (PV), calbindin D28k (CB) and calretinin (CR) was conducted on 5 adult rat brains. Analysis of sections with double immunostaining for vesicular acetylcholine transporter (VAChT; the marker of cholinergic cells, fibres and terminals) and respectively either GAD or PV, CB, CR, using confocal laser-scanning microscope shows that the intensive cholinergic innervations receive GAD, PV and CB-positive hippocampal cells. Cholinergic afferentations of the CR-positive neurones are considerably fewer.

key words: hippocampus, calcium-binding proteins, calbindin-D28k, calretinin, parvalbumin, GAD, cholinergic system

INTRODUCTION

The hippocampus is a prominent limbic structure that plays an essential role in the control of cognitive-emotive behaviour, especially in regard to learning and memory [18,21,40]. Neurones in the hippocampus are divided into two subpopulations: (1) principal neurones, i.e. the pyramidal neurones of the hippocampus proper and the granule cells of the dentate gyrus and (2) nonprincipal neurones i.e. nonpyramidal and nongranule cells [8,20,24]. Principal neurones are generally homogeneous and regarded to be glutamatergic, whereas the vast majority of hippocampal nonprincipal neurones are heterogeneous and they are assumed to be GABAergic [30]. The latter are distributed all over the hippocampal layers, and are considered as interneurones (local circuit neurones). GABAergic neurones in both the hippocampus proper and dentate gyrus expressed immunoreactivity for the calcium-binding proteins. However,

parvalbumin-, calbindin D28k- and calretinin-positive interneurones form distinct (or only partially overlapping) subpopulations [16,17,19,29,33,38] which probably fulfil somewhat different functions.

Because the most dramatic disturbance in learning and memory performance has been observed after cholinergic lesions, which affect the hippocampus, it seems likely that the cholinergic system is essential for the function of this structure [2]. The cholinergic fibres in the hippocampus mainly arise from neurones in the medial septum/diagonal band complex [1,26] and are localised in all the hippocampal cell layers (although their highest concentration was observed in the vicinity of the pyramidal and granular layers) [8,11].

In the present study we investigated the correlation between the cholinergic innervation and neurones containing γ -aminobutyric acid (GABA), parvalbumin (PV), calbindin D28k (CB) and calretinin (CR)

in the hippocampus using immunohistochemical methods. For detection of cholinergic fibres and terminals we used specific antibody against vesicular acetylcholine transporter (VACHT). As a marker of GABAergic cells we used antibody against GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD).

MATERIAL AND METHODS

The material consisted of 5 adult rat brains from the Wistar strain. 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 Nembutal (80 mg/kg of body weight), then transcardially perfused with 0.9% solution of NaCl with heparin, followed by 4% paraformaldehyde solution in 0.1M phosphate buffer (pH 7.4). 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- μ m-thick, serial sections of the brain were cut on JUNG 1800 cryostat (Leica, Germany). The following primary antibodies were used for immunohistochemical double staining: goat anti-VACHT with combination of either rabbit anti-GAD or rabbit anti-calretinin, mouse anti-parvalbumin and mouse anti-calbindin D28k. Specifications and dilutions of the primary antibodies are shown in Table 1. The secondary antibodies used in this study were fluorophore conjugated. Specifications and dilutions of the secondary antibodies are shown in Table 2.

Double immunostaining for VACHT and GAD

The free floating sections were blocked with 3% normal goat serum (NGS) for 1 hour and then incubated with the anti-GAD antibody diluted in 3% NGS for 24 hours in 4°C. After multiple rinses in TBS, sections were incubated (2–3 hours, room temperature) with the secondary antibody conjugated with the

Table 1. Specifications and dilutions of the primary antibodies

Antibodies	Manufacturers	Dilution
Goat anti-VACHT (<i>polyclonal</i>)	Chemicon	1:1000
Rabbit anti-GAD (<i>67 kD; polyclonal</i>)	Chemicon	1:600
Rabbit anti-CALRETININ (<i>polyclonal</i>)	Chemicon	1:1000
Mouse anti-PARVALBUMIN (<i>monoclonal</i>)	Sigma	1:500
Mouse anti-CALBINDIN-D (<i>28 kD; monoclonal</i>)	Sigma	1:100

Table 2. Specifications and dilutions of the secondary antibodies

Antibodies	Manufacturers	Dilution
Cy TM 3-conjugated Donkey anti-Goat	Jackson ImmunoResearch	1:1000
Cy TM 5-conjugated Donkey anti-Rabbit	Jackson ImmunoResearch	1:600
FITC-conjugated Donkey anti-Rabbit	Jackson ImmunoResearch	1:1000
Cy TM 5-conjugated Donkey anti-Mouse	Jackson ImmunoResearch	1:500
FITC-conjugated Donkey anti-Mouse	Jackson ImmunoResearch	1:100

FITC or Cy5. After repeated block with 3% NGS containing 0.2% Triton X-100, the sections were incubated with the anti-VACHT antibody diluted in 3% NGS for 24 hours in 4°C and then, after multiple rinses in PBS, with secondary antibody conjugated with the Cy3 (2–3 hours, room temperature).

Double immunostaining for VACHT and calcium binding proteins

The free floating sections were blocked with 3% NGS containing 0.4% Triton X-100 for 1 hour and then incubated with the mixture of the anti-VACHT antibody together with anti-calretinin, or anti-parvalbumin, or anti-calbindin D28k diluted in 3% NGS for 48 hours in 4°C. After multiple rinses in PBS, sections were incubated (2–3 hours, room temperature) with the mixture of the appropriate secondary antibodies conjugated with the FITC, Cy3 or Cy5.

The staining and its specificity were performed according to the procedure described by Wouterlood et al. [44].

The histological sections were studied under the MicroRadiance AR-2 (Bio Rad, UK) confocal laser-scanning microscope equipped with an Argon laser producing dichromatic light at 488 and 514 nm. The 488-nm line of this laser was applied to excite the fluorescein isothiocyanate (FITC), using a dichroic beam splitter FT 505 and an emission long-pass filter LP 515. The 514-nm line of this laser was applied to excite Cy3, using an excitation filter 514 and an emission long-pass filter E570LP. The 650-nm line of Red Diode was applied to excite Cy5, using an emission long-pass filter HQ660LP. For 3D reconstruction the image analysis program LaserSharp 2000 v. 2.0 (Bio-Rad; UK) was used.

RESULTS

VACHT immunoreactivity

VACHT immunoreactivity was observed in all layers of the hippocampus proper and dentate gyrus, with higher concentrations in the vicinity of principal cell layers (pyramidal and granular; Fig. 1–4). VACHT-immunopositive elements in the neuropil comprised fibres and puncta (which were considered to be axon terminals), which frequently formed “basket” structures that often surround the cells immunonegative for GAD and calcium binding proteins (Fig. 1–4). In any hippocampal region VACHT-positive neurones were observed.

Cholinergic innervation of GAD-positive cells

GAD-positive cells were scattered throughout all layers in the hippocampal areas. The distribution patterns of these neurones were similar to those reported previously using various GABAergic markers [14,20,30,36]. GAD-positive cells were multipolar or horizontal (Fig. 1); some of them were bipolar. GAD-positive puncta were particularly numerous in the pyramidal layer of hippocampus proper and granular layer of dentate gyrus, where they formed peri-

cellular baskets around the unstained cell bodies of pyramidal and granule cells (Fig. 1).

The three-dimensional reconstruction of double immunolabelling sections with anti-VACHT and anti-GAD antibodies showed that significant cholinergic endings were found on GAD-positive cells in the hippocampus (Fig. 1).

Cholinergic innervation of calbindin D28k-positive cells

The general pattern of CB-immunoreactivity was similar to that described in several previous studies [37,38]. The strongest immunoreactivity showed granule cells of the dentate gyrus (Fig. 1A) and pyramidal neurones of the hippocampus proper (in CA1 and CA2 sectors; Fig. 1B). Also nonprincipal cells (mainly in the molecular layer of the dentate gyrus and in the stratum oriens as well as in the stratum radiatum of the hippocampus proper) displayed immunoreactivity for calbindin D28k.

The three-dimensional reconstruction of double immunolabelling sections with anti-VACHT and anti-calbindin-D28k antibodies showed that relatively numerous calbindin D28k-positive, hippocampal neurones, both principal and nonprincipal, possessed many cholinergic endings (Fig. 2).

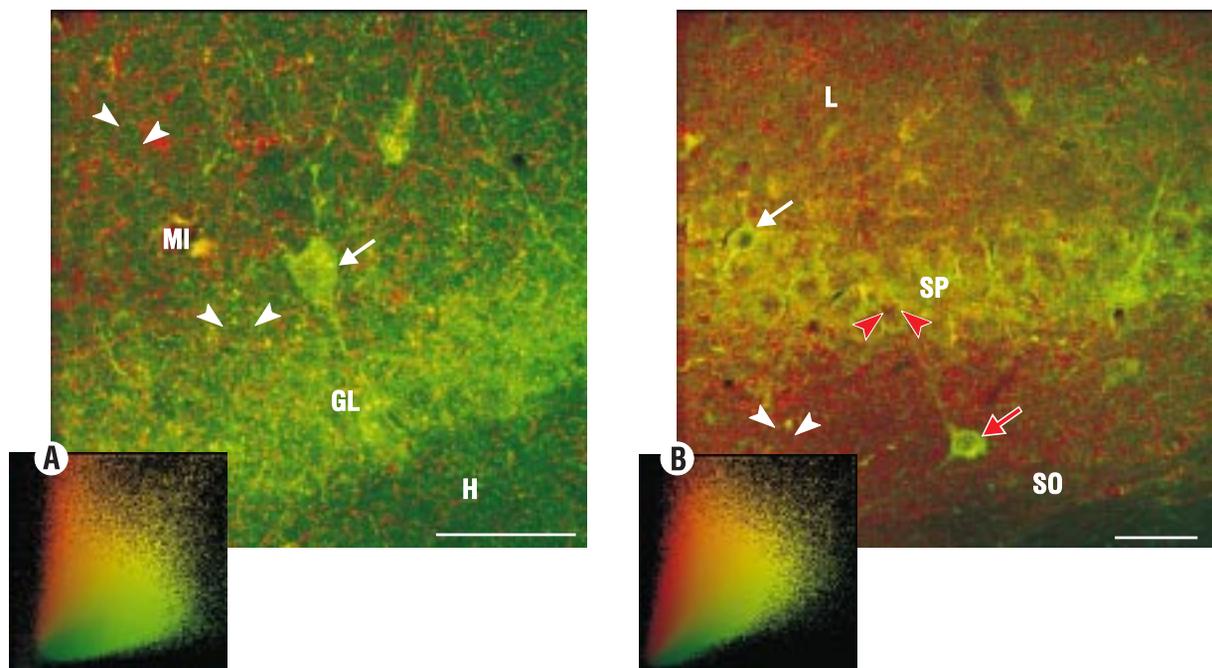


Figure 1. GAD-positive cells (green) and VACHT-positive fibres and terminals (red) in dentate gyrus (A) and hippocampus proper (B). Multipolar (white arrow) and horizontal (red arrow) GAD-positive cells. GAD-positive puncta form pericellular basket around the unstained cell bodies of pyramidal and granule cells (red arrowhead). White arrowheads show VACHT-positive puncta, which formed “basket” structures surrounding the cells immunonegative for GAD. MI — molecular layer, GL — granule cell layer, H — hilus, L — stratum lucidum, SO — stratum oriens, SP — stratum pyramidale. Scale bar — 50 μ m.

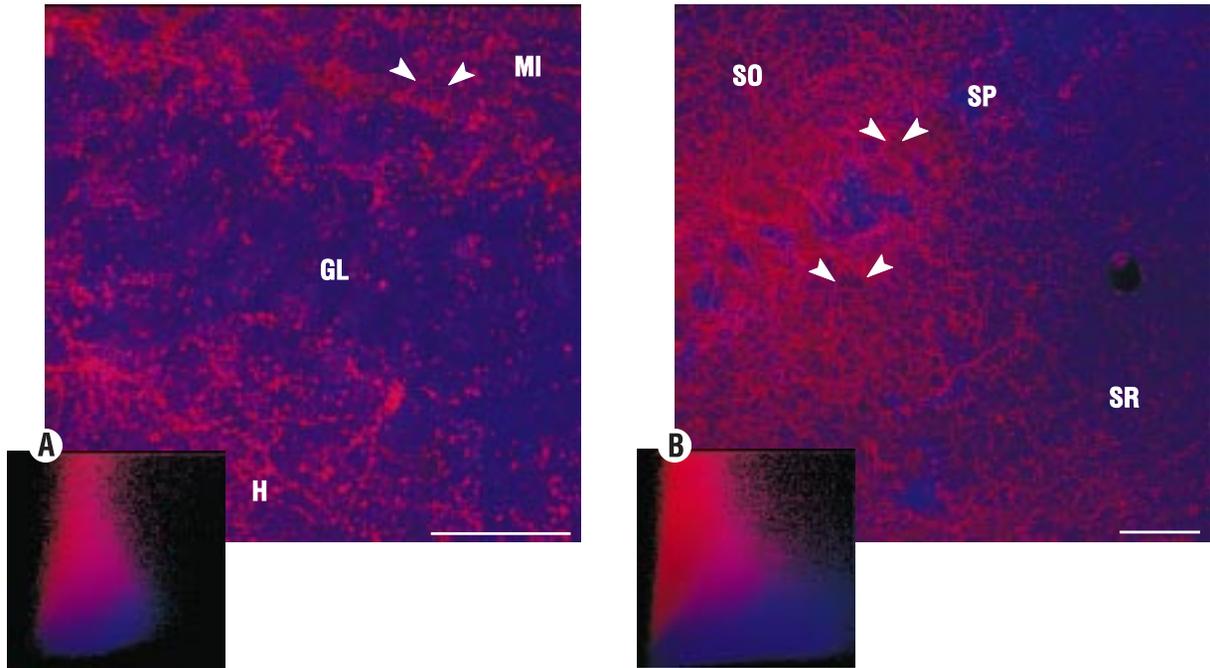


Figure 2. Calbindin D28k-positive cells (*blue*) and VAcHT-positive fibres and terminals (*red*) in dentate gyrus (A) and hippocampus proper (B). White arrowheads show VAcHT-positive puncta, which formed “basket” structures surrounding the cells immunonegative for CB. *MI* — molecular layer, *GL* — granule cell layer, *H* — hilus, *SO* — stratum oriens, *SP* — stratum pyramidale, *SR* — stratum radiatum. Scale bar — 50 μ m.

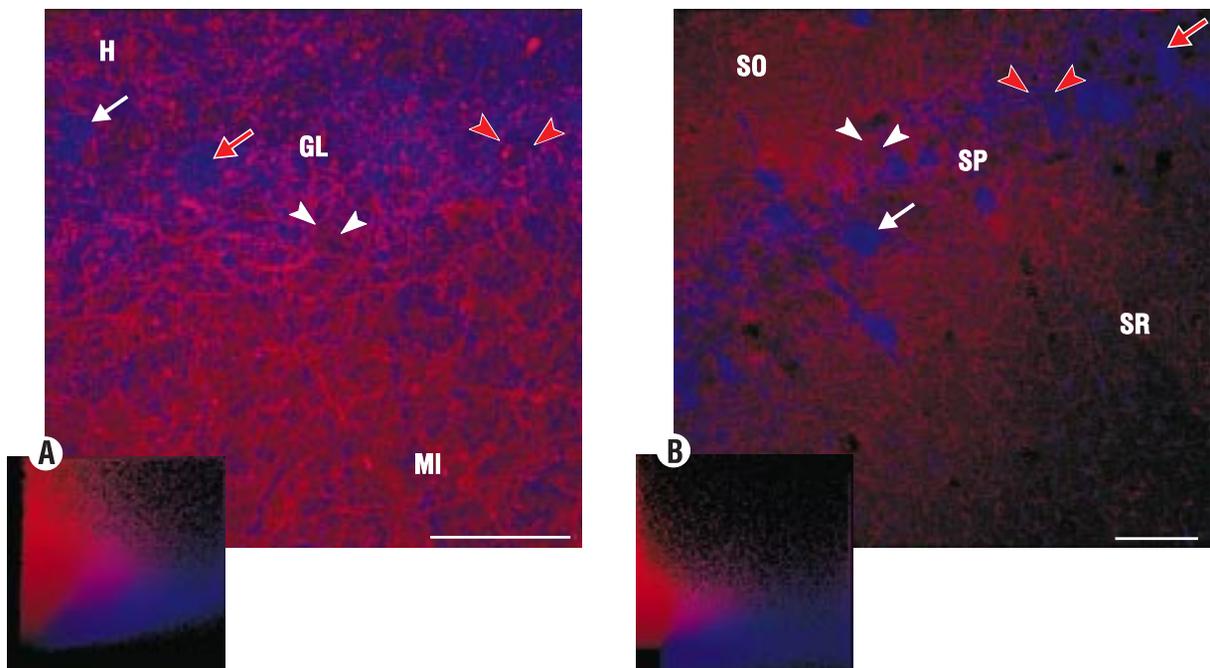


Figure 3. Parvalbumin-positive cells (*blue*) and VAcHT-positive fibres and terminals (*red*) in dentate gyrus (A) and hippocampus proper (B). Multipolar (*white arrow*) and bipolar (*red arrow*) PV-positive cells. PV-positive puncta form pericellular basket around the unstained cell bodies of pyramidal and granule cells (red arrowhead). White arrowheads show VAcHT-positive puncta, which formed “basket” structures surrounding the cells immunonegative for PV. *MI* — molecular layer, *GL* — granule cell layer, *H* — hilus, *SO* — stratum oriens, *SP* — stratum pyramidale, *SR* — stratum radiatum. Scale bar — 50 μ m.

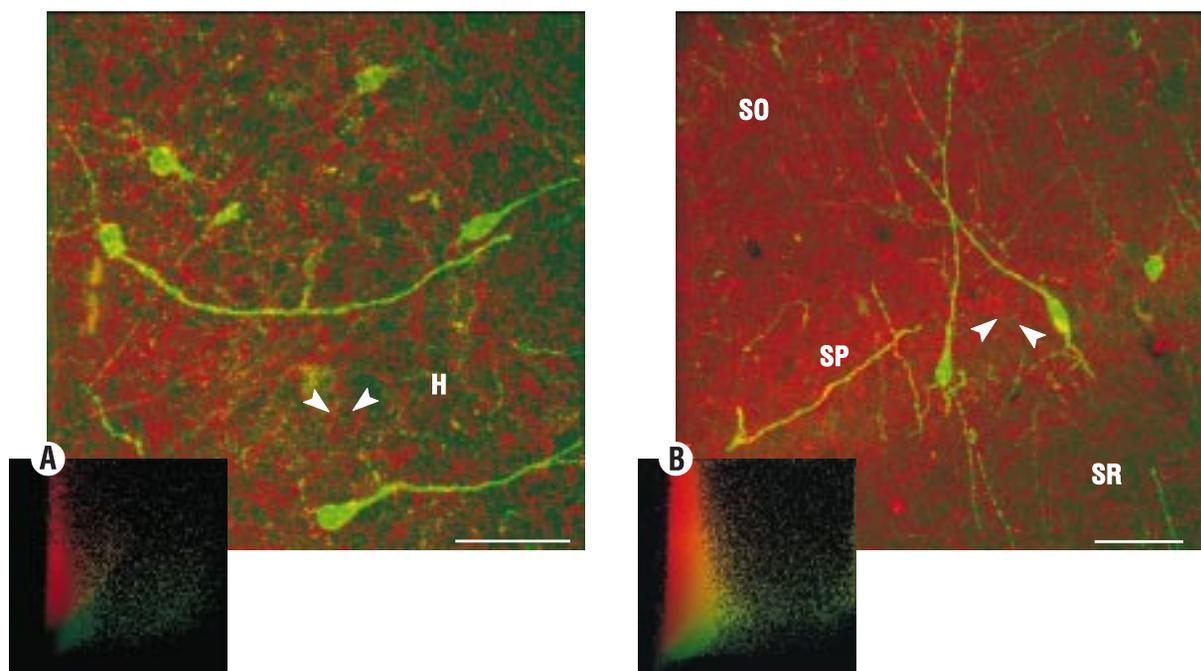


Figure 4. Calretinin-positive cells (*green*) and VAcHT-positive fibres and terminals (*red*) in dentate gyrus (A) and hippocampus proper (B). White arrowheads show VAcHT-positive puncta, which formed “basket” structures surrounding the cells immunonegative for PV. *H* — hilus, *SO* — stratum oriens, *SP* — stratum pyramidale, *SR* — stratum radiatum. Scale bar — 50 μm .

Cholinergic innervation of parvalbumin-positive cells

Distribution of immunoreactivity for parvalbumin in cell bodies, dendrites, and puncta in the hippocampus was similar to that previously described [4,32,33]. In the hippocampus proper, PV-immunoreactivity was observed in CA1 as well as CA2 and CA3 sectors. PV-positive cells were found mainly in the strata oriens and pyramidale. They were variable in size and shape, multipolar or bipolar (Fig. 3). In the dentate gyrus, PV-positive neurones were mainly observed in the granular layer and in regions directly bordering it (Fig. 3A). Most of them were fusiform, pyramidal or horizontal basket cells (Fig. 3). The densest plexus formed by PV-positive terminals occurred in the principal cells layers (pyramidal and granular; Fig. 3).

The three-dimensional reconstruction of double immunolabelling sections with anti-VAcHT and anti-parvalbumin antibodies proved that cholinergic innervations of PV-positive cells in the hippocampus proper and dentate gyrus showed some differences. It seems that parvalbumin-positive neurones in the dentate gyrus receive somewhat more intensive cholinergic innervations than cells in the hippocampus proper.

Cholinergic innervation of calretinin positive cells

The distribution and morphology of calretinin-positive neurones were similar to those described earlier [15,27]. Immunoreactivity of calretinin was detected in different non-pyramidal cells, which were present in all layers and regions of the hippocampus and dentate gyrus. The greatest number of calretinin-positive cell bodies and fibres were observed in the hilus of dentate gyrus (Fig. 4A) and CA3 region of hippocampus proper (Fig. 4B). The three-dimensional reconstruction of double immunolabelling sections with anti-VAcHT and anti-calretinin antibody showed that calretinin-positive cells form considerably fewer synaptic contacts with cholinergic terminals than PV- and CB-positive hippocampal neurones (Fig. 4).

DISCUSSION

The results of the present study demonstrate that neurones containing parvalbumin, calbindin-D28k, calretinin and glutamic acid decarboxylase in the hippocampus of the rat establish synaptic contacts with cholinergic afferents. Leránth and Frotscher [25], Frotscher [10] and Frotscher et al. [13] affirmed that cholinergic (choline acetyltransferase positive) fibres reach three major cell types in the hippocampus

— granule, pyramidal and GABAergic nonprincipal neurones. However, the authors did not observe any predilection of cholinergic afferents to the target cells. The cholinergic terminals establish synapses with cell bodies, proximal as well as peripheral dendritic shafts, spine heads and spine necks of target neurones [9]. It appears that the cholinergic fibres, with the exception of axon initial segments, are ready to accept all available elements as postsynaptic partners [10]. Cholinergic terminals form both symmetric and asymmetric contacts. Even though traditionally symmetric synapses are interpreted as inhibitory, no inhibitory effects of acetylcholine on hippocampal neurone have been reported [10].

The γ -aminobutyric acid is considered as the major inhibitory transmitter present in local circuit neurones in the hippocampus [22]. Hippocampal GABAergic cells are involved in feed-forward inhibition, recurrent inhibition and disinhibition of pyramidal and granular cells [7,12,31,43]. GAD-immunoreactive boutons establish symmetric synaptic contacts on the perikarya, dendrites and axon initial segments of pyramidal and granular neurones [34,39]. GABAergic fibres terminate also on the GABAergic cells [12,35], which indicates inhibition of interneurones resulting in a disinhibitory effect on pyramidal cells [13]. This phenomenon may be an important feature of information processing [31]. It is interesting that acetylcholine has also been reported to cause disinhibition [23]. The existence of rich cholinergic innervations of the GABAergic cells suggests that septohippocampal cholinergic system may control or have an assisting role towards intrinsic inhibitory system of the hippocampus. It seems that cholinergic afferents can influence principal cells indirectly (through GABAergic system) and directly by cholinergic fibres. The slow depolarisation of these neurones, associated with an enhancement of their excitability, is the physiological effect of acetylcholine on the principal cells [5,28].

GABAergic cells of the hippocampus are known to be morphologically heterogeneous and to contain different kinds of calcium binding protein: calbindin-D28k, parvalbumin or calretinin [3,17,29,32,37]. According to Miettinen et al. [27] in the hippocampus of rat, non-pyramidal cells containing CB, PV and CR form a distinct, largely non-overlapping group. The existence of diverse subpopulations of GABAergic cells suggests a strong functional specialisation among the different inhibitory interneurones [17]. Correlation of the anatomical, neurochemical and electrophysiological data suggests that

PV-containing cells may be responsible for the perisomatic, GABA-A receptor mediated inhibition, and may participate in both feed-forward and feed-back circuits, whereas the CB-containing neurones would be involved in feed-forward, GABA-B receptor mediated inhibition in distal dendritic tree [17]. Calbindin-D28k is also present in principal cells. Regions of the hippocampus containing these neurones innervate those areas where principal cells lack this calcium binding protein [37]. Calretinin-immunoreactive neurones presumably receive their main synaptic input through the perforant path [41,42] and may exert feed-forward inhibition on the dendrites of principal cells which may also receive input from the perforant pathway. Therefore, this neuronal population may influence the susceptibility of dendrites of hippocampal principal neurones (both granule and pyramidal) to neocortical input in a distinct dendritic zone at some distance from the somata of parent neurones [29]. Gulyás et al. [16] described a type of CR-positive cell in the hippocampus that is specifically associated with the mossy fibre system, and which therefore receives afferent input exclusively from granule cells. These CR-positive neurones may have a specialised functional role such as synchronising the activity of CA3 pyramidal cells [16]. Recently, Gulyás et al. [15] found that spine-free CR-containing GABAergic interneurones form symmetrical synaptic contacts exclusively on GABAergic dendrites. The unique connectivity of calretinin-containing cells may enable them to play a crucial role in generation of synchronous, rhythmic hippocampal activity by controlling other interneurones terminating on different dendritic and somatic compartments of principal cells. Our results suggest that populations of cells containing distinct calcium binding proteins also differ in regard to cholinergic innervations. It seems that calretinin-positive cells receive less cholinergic input than neurones containing PV and CB. This may be connected with the fact that in the hippocampus, structures containing calretinin not only belong to the intrinsic GABAergic system but have also been identified as part of the hypothalamo-hippocampal system [29] originated from supramammillary nucleus, which is involved in the generation of hippocampal theta rhythm and might be able to filter the signal flow in the hippocampal formation [29]. It seems probable that cholinergic system affects rather hippocampal cells and their fibres than extrinsic elements present in this structure but originated from other brain structures (such as supramammillary CR-positive fibres men-

tioned above). On the other hand, it seems probable that CR-positive interneurons remain under control of some other than the cholinergic system (for example septohippocampal GABAergic fibres specifically terminate only on inhibitory hippocampal interneurons [6]).

In conclusion, the range of cholinergic afferentations, diversity of target structures, and variety of synaptic contacts indicate that cholinergic system plays a very important role in the control of hippocampal functions.

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