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CALRETININ-CONTAINING INTERNEURONS INNERVATE BOTH PRINCIPAL CELLS AND INTERNEURONS IN THE CA1 REGION OF THE HUMAN HIPPOCAMPUS*

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Hippocampal interneurons consist of functionally diverse cell types, most of them target the dendrites or perisomatic region of pyramidal cells with a few exceptions, like the calretinin-containing cells in the rat: they selectively innervate other interneurons. However, no electron microscopic data are available about the synaptic connections of calretinin-immunoreactive neurons in the human hippocampus. We aimed to provide these data to establish whether interneuron-selective interneurons indeed represent an essential feature of hippocampal circuits across distant species. Two types of calretinin-immunostained terminals were found in the CA1 region: one of them presumably derived from the thalamic reuniens nucleus, and established asymmetric synapses on dendrites and spines. The other type originating from local interneurons formed symmetric synapses on both pyramidal and interneuron dendrites. Distribution of postsynaptic targets showed that 26.8% of the targets were CR-positive interneuron dendrites, and 25.2% proved to be proximal pyramidal dendrites. CR-negative interneuron dendrites were also contacted (12.4%). Small caliber postsynaptic dendrites were not classified (28%). Somata were rarely contacted (7.6%). The present data suggest that calretinin-positive boutons do show a preference for other interneurons, but a considerable proportion of the targets are pyramidal cells. We propose that interneuron-selective inhibitory cells exist in the human Ammon's horn, and boutons innervating pyramidal cells derive from another cell type that might not exist in rodents.

Keywords: Calcium binding proteins – interneuron selective cell – electron microscopy – species differences – postsynaptic targets

INTRODUCTION

The population of non-principal cells of the hippocampus consists of several functionally distinct cell types, which can be differentiated by their connectivity and/or their calcium-binding protein (calretinin, calbindin, parvalbumin), neuropeptide or other marker content [2, 3, 12, 15]. The neurochemical marker content correlates

Abbreviations: BSA: bovine serum albumin; CA1, CA2, CA3: Hippocampal subfields according to Lorente de No; CR: Calretinin; DAB: 3,3'-Diamino-benzidine 4 HCl; GABA: Gamma-aminobutyric acid; PB: Phosphate buffer; str.: stratum; TBS: TRIS buffered saline.

*Dedicated to Professor József Hámori on the occasion of his 70th birthday.

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with input-output features, i.e., parvalbumin is present in basket and axo-axonic cells [14, 16, 27], calbindin labels dendritic inhibitory cells [9], and calretinin (CR) were found in interneuron-selective inhibitory cells in rats [10] (for review see [7]). Jacobowitz and Winsky [13] were the first to describe CR-containing non-principal cells in the rat hippocampus. Calretinin, a new member of the calmodulin superfamily, was found predominantly in neurons [28].

Data about the CR-positive interneurons in human and primate hippocampus are sparse [4, 25]. In rodents, CR was found in interneurons controlling other interneurons in the hippocampus [10], therefore they may participate in the inhibitory regulation or synchronization of interneuronal activity. However, the distribution and morphology of CR-positive interneurons in the human hippocampus were found to be different from those of the rat [25]. Therefore, the question emerged, whether their target selectivity and functional role may also represent a species difference. Additionally, CR-positive cells were shown to be vulnerable to ischaemic and epileptic injury both in rats and human [8, 19, 20], therefore knowledge of their role in local circuits would help us understand the mechanisms of these disorders.

In the present study we examined the fine structure of CR-positive interneurons, their inputs and postsynaptic target distribution in the CA1 region of the human hippocampus.

MATERIALS AND METHODS

CR-containing elements were examined in the hippocampus of six control human subjects. Control brain samples were obtained from a 37 years old woman who died by accident, from a 47 and a 48 years old woman and a 51 and a 56 years old man who had cardiac arrest, and a 53 years old man who died by suffocation. None of the control subjects had a record of any neurological disorder. Brains were removed 2–4 hours after death, the dissection was performed in the Semmelweis University, Budapest, and the regulations of the Hungarian Ministry of Health were followed.

After removal, the hippocampal tissue of 4 subjects was immediately dissected into 2 mm thick blocks, and immersed into a fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB, pH = 7.4). Fixative was hourly changed to a fresh solution during constant agitation for 6 hours, and then the blocks were postfixed in the same fixative overnight. Two of the control brains (control numbers 10 and 11) were removed from the skull 2 hours after death and both internal carotid and vertebral arteries were cannulated; the brains were perfused with physiological saline (2 liters in 30 min) followed by a fixative solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB (6 liters in 1.5 hour). The hippocampus was removed after perfusion and cut into 2 mm thick blocks, which were postfixed in the same fixative solution overnight.

Immunocytochemistry

Sixty μm thick Vibratome sections were cut from the blocks, and following washing in phosphate buffer they were immersed in 30% sucrose in 0.1 M PB for 1–2 days, then freeze-thawed three times over liquid nitrogen. Alternate sections were processed for immunostaining for calretinin as follows: sections were transferred to TRIS buffered saline (TBS, pH = 7.4), then endogenous peroxidase activity was blocked by 1% H_2O_2 in TBS for 10 min. Nonspecific immunoglobulin binding of the tissue was blocked by 5% milk powder and 2% bovine serum albumin in TBS. Polyclonal rabbit antiserum against calretinin (1 : 5000) [28] was used for two days at 4 °C. For the visualization of the immunopositive elements, biotinylated anti-rabbit IgG (1 : 200, Vector) was applied as secondary serum followed by avidin-biotinylated horseradish peroxidase complex (ABC, 1 : 200, Vector). The tissue-bound peroxidase was visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) as a chromogene. TBS was used for all the washes (3×10 min between each step) and dilution of the antisera. Sections were then treated with 1% OsO_4 (in PB, for 30 min), dehydrated in ethanol (1% uranyl acetate was added at the 70% ethanol stage for 30 min) and embedded in Durcupan (ACM, Fluka). After light microscopic examination, areas of interest were reembedded and sectioned for electron microscopy. Ultrathin sections were collected on Formvar-coated single slot grids, stained with lead citrate, and examined with a Hitachi 7100 electron microscope.

Electron microscopic analyses of the postsynaptic targets of CR-immunostained axon terminals

The target elements of CR-positive axon terminals were examined in two control brains fixed by perfusion post mortem (control numbers 10 and 11). Blocks containing all layers of CA1 were prepared from CR-immunostained sections. Serial ultrathin sections were cut from the blocks, and CR-containing terminals were analyzed in every 6th section in order to avoid sampling the same synapses. The sections were systematically scanned, and photographs were taken from every CR-positive synaptic terminal found in each section. The distribution of the postsynaptic target elements of CR-immunoreactive terminals was then determined.

RESULTS

Dependence of CR-immunostaining on fixation and post mortem delay

The distribution and morphology of CR-positive elements were examined in ten human control hippocampi. The results revealed that the post mortem delay, age of the subjects, and the fixation procedure greatly influenced the number of stained cells and the quality of their staining. The general distribution and morphology of the cells

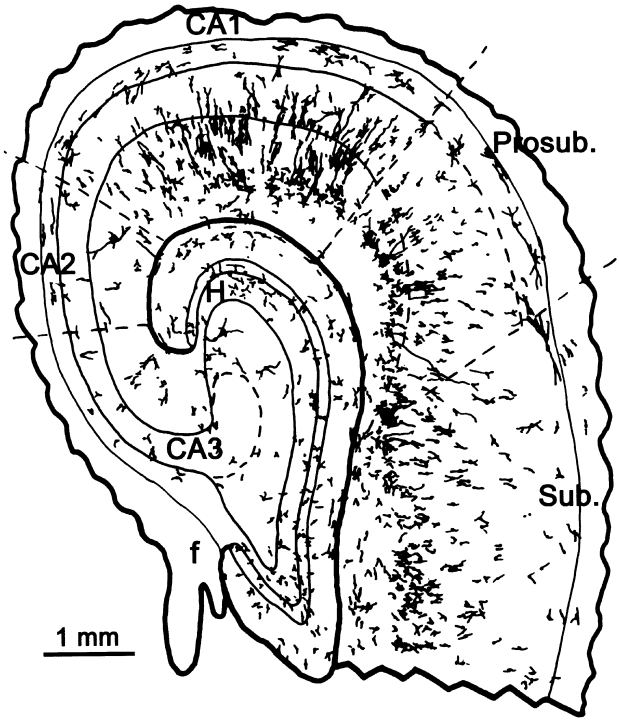


Fig. 1. Camera lucida drawing of CR-immunostained cells in the human hippocampus. CR-positive cells are present in every subfield and layer. The dentate gyrus contains numerous multipolar and fusiform CR-positive cells in every region, hilar cells are the most abundant. There are numerous CR-positive cells in all layers of cornu Ammonis, the CA2 and CA3 regions contain less CR-positive cells than the CA1 subfield. Subfields are separated by broken lines. Abbreviations: CA3, CA2, CA1: hippocampal subfields; f: fimbria; H: hilus; Prosub.: prosubiculum; Sub.: subiculum. Scale bar: 1 mm

were similar in each case, but the number of cells varied among the subjects. The smallest number of cells was found in immersion fixed subjects with long post mortem delays (longer than 6 hours), therefore these control subjects were excluded from the present study. Cell numbers were high in the 2 hours post mortem controls suggesting that the antigenecity was far better retained for CR. We found that the age of the subject also affects the quality and quantity of immunostaining. Subjects older than 60 years usually showed the signs of arteriosclerosis and most antisera resulted in weaker staining and lower number of cells. Therefore we excluded the subjects older than 60. Perfusion fixation resulted in more numerous CR-immunostained profiles and better ultrastructural preservation than immersion fixation, therefore hippocampi removed from two perfusion-fixed brains (2 post mortem delay hours) were used in this study for the electron microscopic examination. For light microscopic observations other four subjects with 2 hours post mortem delay and immersion fixation were also included.

Distribution and morphology of CR-immunopositive elements

Calretinin was found exclusively in non-principal cells in the hippocampus of rats and primata [11, 13, 22, 25, 31]. They form a distinct subpopulation of interneurons having a negligible overlap with other calcium binding protein-containing interneurons in rat and monkey [23, 29, 31].

The distribution of CR-immunoreactive cells is shown in Fig. 1. Hippocampal subfields are labeled according to Lorente de No [17], with modifications suggested by Seress in 1988 [30]. CR-positive cells are present in every region of the human hippocampus [25]. Hilar cells are multipolar or fusiform, the dendrites are largely confined to the hilus, but some enter the stratum moleculare. The cells in stratum moleculare are fusiform and the dendrites that run parallel with the laminar borders are located in the most distal part of stratum moleculare (i.e. close to the fissure or the pia). The dentate gyrus contains numerous multipolar CR-positive interneurons in every layer. A unique CR-positive cell type with a small cell body and only a few short dendrites are also present in all layers (Fig. 2c). Immunostaining for CR also reveals a characteristic axonal network at the top of the granule cell layer-inner third of the molecular layer (Fig. 2c) and in the CA2 region. This characteristic CR-positive fiber band was shown to originate from the supramammillary nucleus in primates and rats [5, 18, 24]. There are numerous CR-positive interneurons in all layers of cornu Ammonis (Figs 1, 2a-b), although their number is not homogeneous among the subfields. The CA2 and CA3 regions contain less CR-positive cells than the CA1 subfield (Figs 1, 2a-b). They can be found mostly in strata pyramidale and radiatum. Their dendrites are smooth or rarely spiny, they are often beaded (Figs 2a-b), run radially in the strata pyramidale and radiatum, and mostly horizontally in the stratum oriens. A special, multipolar cell type (lacking in rodents) is also present in the CA1 region, at the bottom of the stratum radiatum (Fig. 2a).

Long segments of CR-positive dendrites originating from different cells were often attached to each others, as described in rat [11]. Such dendritic braids were observed in all subfields of the human hippocampus in our material (Fig. 3), most frequently in the CA1 area (Figs 3b, c). The vertically running intermingled dendrites are often juxtaposed and run together.

Synaptic input of the CR-immunopositive interneurons

In the present study we focused the electron microscopic analyses to the CA1 area, since the ultrastructural features of CR-immunoreactive cells in the human dentate gyrus have already been described [20].

The general ultrastructural features of CR-positive cells in the CA1 region were similar to that described in monkey [31]. The cytoplasmic rim around the often infolded nucleus was usually thin, but contained numerous mitochondria. The synaptic input of the somata was unusually poor compared to other interneurons. CR-positive dendrites were examined in all layers of the CA1 area, CR-positive terminals

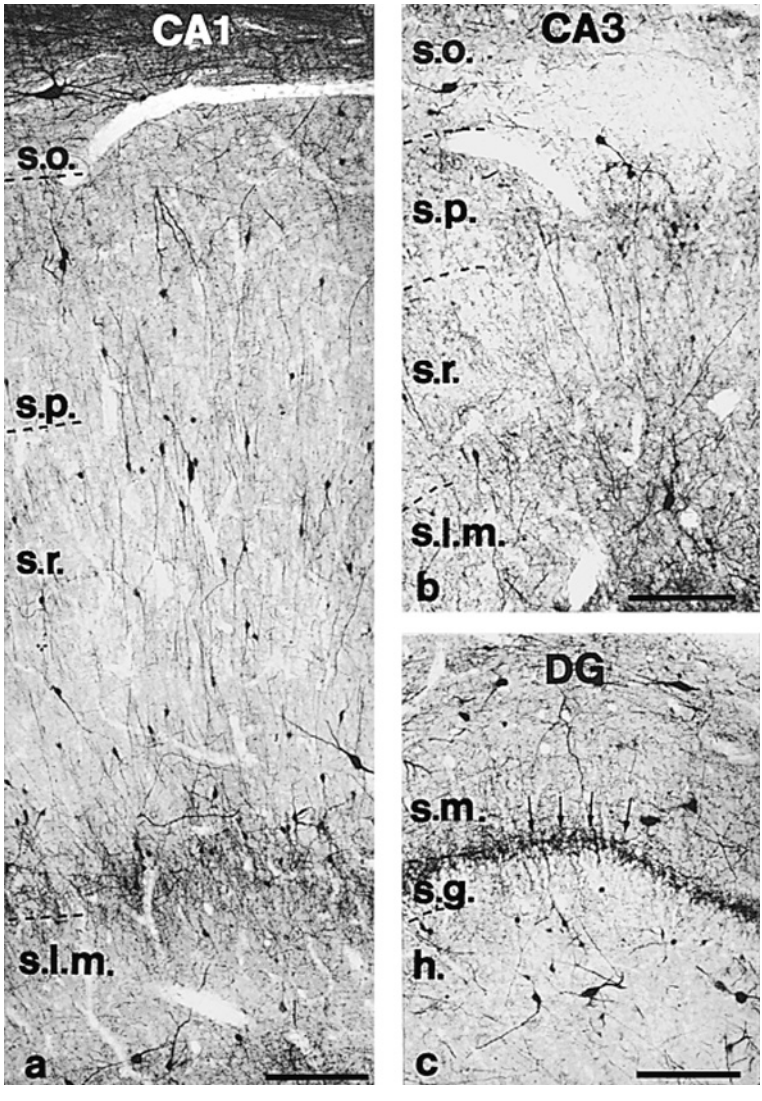


Fig. 2. Light micrographs of sections immunostained for CR. a) Numerous, morphologically heterogeneous CR-positive cells are present in the CA1 region, they are most frequent in the strata radiatum and pyramidale. Their dendrites are smooth or sparsely spiny, run radially in the strata pyramidale and radiatum, and horizontally in the stratum oriens. A multipolar cell type is also present in the CA1 region, at the bottom of the stratum radiatum. b) CA3 region contains less CR-positive cells than the CA1 region, although they are morphologically similar. c) Numerous multipolar and fusiform CR-positive cells are present in every layer of the dentate gyrus, small cells with few, short dendrites are also present. The cells in the stratum moleculare are usually fusiform and the dendrites that run parallel with the layer-borders are located in the most distal part of stratum moleculare. A dense CR-positive axon-bundle is visible at the top of the granule cell layer (arrows). Borders of layers are labeled by broken lines (the stratum lucidum in the CA3 subfield is not labeled); h: hilus; s.g.: stratum granulosum; s.m.: stratum moleculare; s.l.m.: stratum lacunosum-moleculare; s.r.: stratum radiatum; s.p: stratum pyramidale; s.o.: stratum oriens. Scale bars: 200 μm

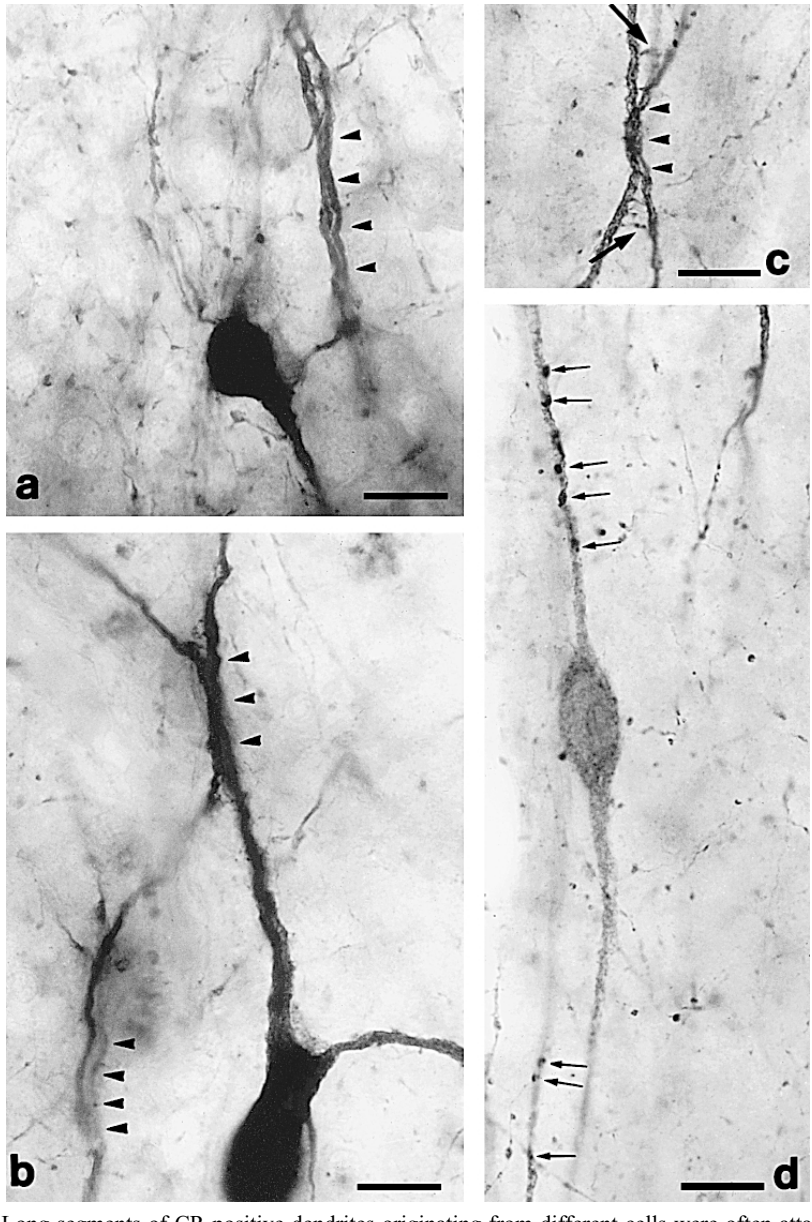


Fig. 3. Long segments of CR-positive dendrites originating from different cells were often attached to each others in all subfields of the human hippocampus. They were observed in the dentate gyrus (a, arrowheads) and most frequently in the CA1 area (b, c), where the vertically running intermingled dendrites run together (arrowheads). Finger-like appendages between the connecting dendrites were often visible (c, arrows). d) Dendrites of CR-positive interneurons often receive multiple contacts from CR-positive axon terminals (arrows). Scale bars: 15 μm

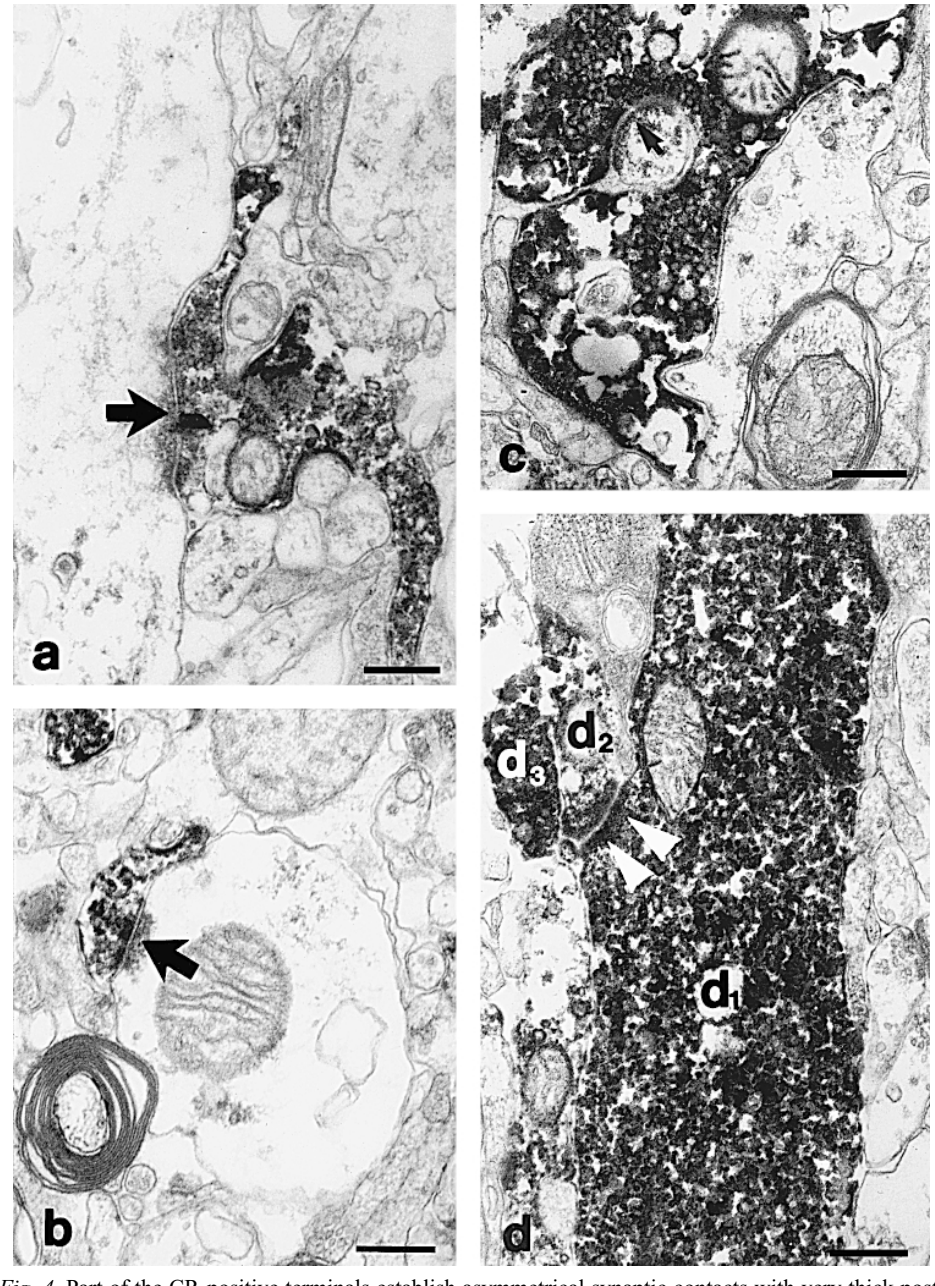


Fig. 4. Part of the CR-positive terminals establish asymmetrical synaptic contacts with very thick post-synaptic density in the strata radiatum and lacunosum-moleculare. They most frequently terminate on dendrites (a, b, arrows), rarely on spines (c, arrow). d) Electron microscopic examination of juxtaposed CR-positive dendrites shows that dendro-dendritic zona adherentia are present between d_1 and d_2 (white arrowheads), the closely attached d_3 is not in connection with them in this section. Scale bars: 0.5 μm

were often seen in close contact with them (Fig. 3d). Their synaptic input was moderate, like in the dentate gyrus [20], and asymmetric synaptic contacts were more frequently seen than symmetric ones. The horizontal dendrites in the stratum oriens and lacunosum-moleculare received predominantly asymmetric contacts.

Juxtaposed dendrites were also analyzed in the electron microscope. Similarly to the rat [11] zona or puncta adherentia were often observed between these dendrites (Fig. 4d). Usually two, but sometimes three or more dendrites were connected. Gap junctions were not seen, which is likely due to suboptimal ultrastructural preservation.

Synaptic targets of CR-positive terminals

The target elements of CR-positive axon terminals were examined in two control brains fixed by perfusion post mortem. All layers of CA1 have been sampled and scanned systematically. Photographs were taken of every CR-positive synaptic terminal in every 6th section, and the distribution of the postsynaptic target elements of the CR-immunoreactive terminals was determined.

In total, 127 terminals were examined in control number 10 and 112 terminals in control number 11. Most of them established symmetric synapses (95 out of 127 and 91 out of 112 in control numbers 10 and 11, respectively), but CR-positive terminals establishing asymmetric contacts with very thick postsynaptic specialization were also found (Figs 4a–c). The vast majority of the asymmetric contacts were found in strata lacunosum-moleculare and radiatum. They usually contacted unstained dendrites (Figs 4a, b), rarely spines (Fig. 4c). Occasionally CR-positive dendrites were also innervated.

Most of the CR-positive boutons forming symmetric contacts were localized in the strata pyramidale and radiatum, stratum oriens contained a moderate number of terminals, and they were rarely seen in the stratum lacunosum-moleculare (3 out of 186 terminals). The targets were usually thick proximal dendrites. At least 28.4% of the postsynaptic profiles proved to be CR-positive (Table 1, Fig. 5a), and thus belonged to CR-positive interneurons. The CR-negative postsynaptic dendrites were classified according to their electron microscopic morphology as pyramidal dendrites, interneuron dendrites or unidentified dendrites. The identification was based on the number and size of the mitochondria (interneurons usually have some larger ones, Fig. 5b), and synaptic input of the given dendritic shaft (pyramidal dendritic shafts receive no asymmetrical synapses in the rat [21]). If it was possible, the dendrite was followed back to the parent cell body. Proximal apical and basal dendrites of pyramidal cells were unequivocally identified in these cases (25.2%, Table 1, Fig. 6). We were unable to determine the type of the small caliber dendrites (Table 1). Part of the dendrites (124%) presumably belonged to CR-negative interneurons (Fig. 5b). Five (control 10) and six (control 11) contacts were found on CR-negative, pyramidal cell bodies (Fig. 7), and one and two in control 10 and 11, respectively, on CR-immunoreactive interneuron somata (Table 1).

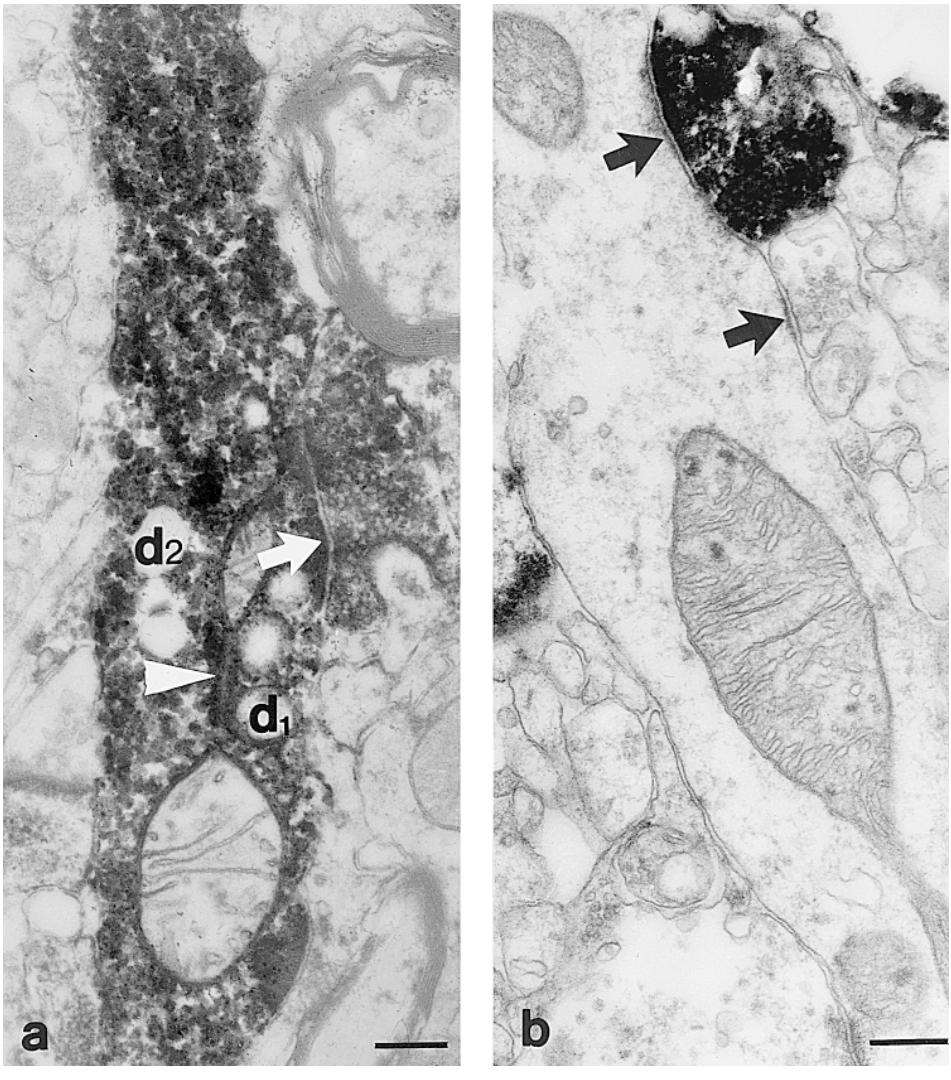
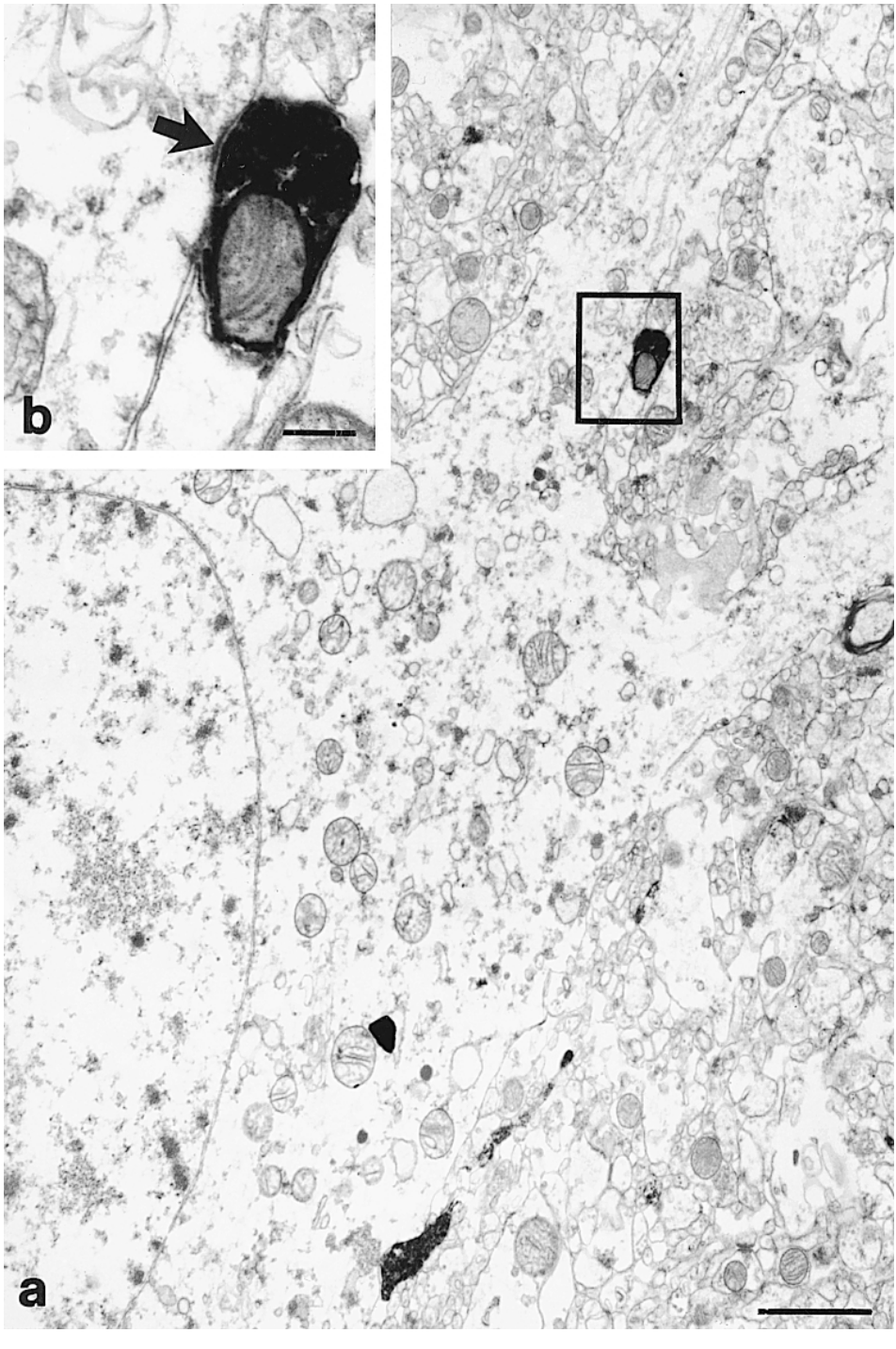


Fig. 5. Terminals of CR-positive interneurons establish symmetric synaptic contacts. They frequently terminate on other CR-positive interneuronal dendrites (a, d_1 dendrite, white arrow). A zona adherentia is present between the two CR-positive dendrites (d_1 and d_2, white arrowhead). Dendrites of CR-negative interneurons also receive synaptic contacts from CR-positive inhibitory terminals (b, arrow). Scale bars: 0.5 µm

Fig. 6. A CR-positive terminal establishing symmetric synapses is present on a proximal basal dendrite of a pyramidal-like CR-negative cell (a). The cell is located in the stratum pyramidale, has no infoldings of its nuclear membrane, and possesses basal and apical dendrites. The framed area showing the synaptic contact (arrow) is seen in 'b' at higher magnification. Scale bars: a) 2.5 µm; b) 0.5 µm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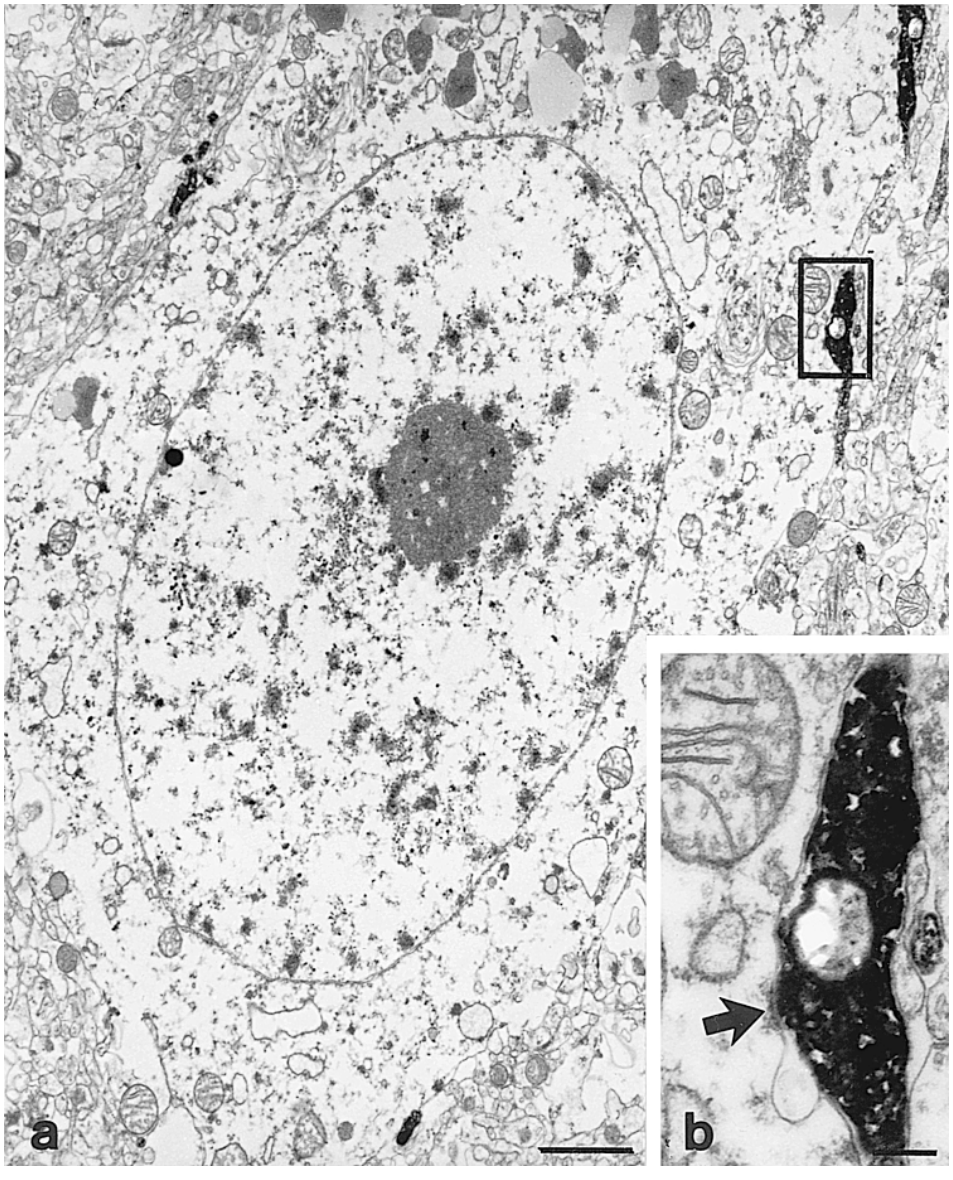

Fig. 7. Low-power electron micrograph of a CR-negative pyramidal-like cell from the stratum pyramidale contacted by a CR-positive bouton (a). The framed area with the symmetric synaptic contact (arrow) between the soma and the bouton is shown in b) at higher magnification. Scale bars: a) 2.5 μm; b) 0.5 μm

Table 1

Postsynaptic target distribution of CR-positive terminals establishing symmetric synaptic contacts in the CA1 region of two control human hippocampi. The number (N) of examined terminals is given in parenthesis. The vast majority of the terminals contacted dendrites, rarely somata. Both principal and non-principal cells were innervated. Unstained dendrites were classified according to their electron microscopic morphology

	Postsynaptic targets	
	Human control number 10 (N = 95)	Human control number 11 (N = 91)
CR-positive soma	1	2
	1.0%	2.2%
Pyramidal soma	5	6
	5.3%	6.6%
CR-positive dendrite	29	21
	30.5%	23.1%
CR-negative interneuron dendrite	11	12
	11.6%	13.2%
Pyramidal dendrite	26	21
	27.4%	23.1%
Unidentified dendrite	23	29
	24.2%	31.9%

DISCUSSION

The number of CR-immunostained profiles in human subjects highly depends on the post mortem delay and the quality of fixation. Post mortem fixation by perfusion (two brains in the present study) provided the best immunostaining both quantitatively and qualitatively (Fig. 1). Significantly less cells were present in the samples with post mortem delays longer than 6 hours. It suggests that CR-positive cells are sensitive for energy-poor conditions as it was described earlier in rats [8], and fixation quality, therefore comparison of a post mortem control human CR-staining with any type of pathological conditions should be taken with precaution.

The CR-positive cells with smooth dendrites appear to form a functionally homogeneous group in rats. Their axons terminate largely on other hippocampal interneurons and a subset colocalizes VIP [1, 10]. They were suggested to play an important role in the synchronization of principal cell activity via disinhibition [7, 10].

CR-immunopositive interneurons are morphologically more heterogeneous in the human hippocampus than in the rat [25]. In rats a spiny CR positive interneuron subgroup with horizontal dendrites is present in the CA3 stratum lucidum, which is absent in human [25]. In human, however, a group of small neurons are present in the hippocampus, which cannot be seen in the rat. Both in rat and human cornu Ammonis smooth, radially oriented cells predominate [10, 25], which frequently run

together and are juxtaposed. In addition, the human CA1 region occasionally contains radially oriented spiny cells and a special multipolar cell type localized at the bottom of stratum radiatum [25].

Possible functional role of the CR-immunopositive interneurons derived from their postsynaptic targets

Two types of CR-positive terminals were found in the CA1 region. One of them established symmetric synapses in all layers, the other formed asymmetric synapses in strata radiatum and lacunosum-moleculare. Since CR was proved to be present in local interneurons [11, 26] which give symmetric synapses [11], this latter type of boutons are unlikely to originate from interneurons, they presumably derive from an extrahippocampal source. Two pathways are known to establish CR-positive asymmetric synapses in the hippocampus: The supramammillary projection, which terminates in the dentate gyrus and in the CA3a–CA2 areas [5, 18, 24], and a projection from the nucleus reuniens thalami terminating in stratum lacunosum-moleculare of the CA1 region in the rat [6, 32, 33]. Since the terminals were largely found in this layer, they presumably originated from the nucleus reuniens thalami.

The terminals establishing symmetric synapses likely belong to local interneurons. The vast majority of them terminated on dendrites, similarly to that found in the monkey [31], therefore the CR-positive interneurons are presumably dendritic inhibitory cells. In rats, they invariably established contacts with other GABAergic interneurons [10]. We were unable to process the sections for postembedding immunogold staining for GABA to determine the transmitter identity of the targets because of the low glutaraldehyde concentration of the fixative solution. However, we examined the ultrastructural features of the postsynaptic targets carefully to identify their type. At least 26% of the postsynaptic targets definitely belonged to interneurons, since they were CR-positive dendrites (Fig. 5a). We found interneuron dendrites among the CR-negative targets as well (Fig. 5b). Part of the targets were identified as pyramidal cell dendrites, most of them were proximal apical or basal shafts, which could be followed back to the parent cells (Fig. 6). Therefore we can conclude that the CR-positive interneurons innervate both principal cells and interneurons, although the latter targets were far more frequent than their relative occurrence in the tissue.

The question emerges, whether the same CR-positive interneuron terminates on both types of dendrites or different types specialize to innervate either interneurons or pyramidal cells. To answer this question an analysis of the axonal arbors of individual filled CR-positive interneurons would be necessary, but such data are not available in the human hippocampus. One may speculate that CR-positive interneurons in CA1 with smooth and juxtaposed dendrites that are morphologically similar to the regular CR-positive cells in the rat CA1 region might have similar connectivity, i.e. they might terminate on interneuron dendrites. However, the other types, the sparsely spiny cells with radial dendrites, or the multipolar cells at the border of the

strata radiatum and lacunosum moleculare, which are not present in rats, might be responsible for the inhibition of the pyramidal cells. In conclusion, we propose that in the human hippocampus the CR-positive interneurons form a functionally heterogeneous group, since they participate in the inhibition of both principal and non-principal cells, representing a clear species difference from rodents. The fact that interneurons with a marked target preference for other interneurons also exist in the human hippocampus suggests that synchronization of inhibition is a basic operational principle of hippocampal circuits across a wide range of species.

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