

# Differential Expression of Synaptophysin and Synaptoporin During Pre- and Postnatal Development of the Rat Hippocampal Network

D. Grabs<sup>1</sup>, M. Bergmann<sup>1</sup>, Th. Schuster<sup>1,4</sup>, P. A. Fox<sup>1</sup>, M. Bricht<sup>2</sup> and M. Gratzl<sup>3</sup>

<sup>1</sup>Institut für Anatomie (Charité), Humboldt-Universität Berlin, D-10098 Berlin, Germany

<sup>2</sup>Naturwissenschaftliches und Medizinisches Institut an der Universität Tübingen, D-72762 Reutlingen, Germany

<sup>3</sup>Abteilung Anatomie und Zellbiologie, Universität Ulm, D-89069 Ulm, Germany

<sup>4</sup>Present address: Abteilung Neurobiologie, ETH Zürich, CH-8093 Zürich, Switzerland

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## Abstract

The closely related synaptic vesicle membrane proteins synaptophysin and synaptoporin are abundant in the hippocampal formation of the adult rat. But the prenatal hippocampal formation contains only synaptophysin, which is first detected at embryonic day 17 (E17) in perikarya and axons of the pyramidal neurons. At E21 synaptophysin immunoreactivity extends into the apical dendrites of these cells and in newly formed terminals contacting these dendrites. The transient presence of synaptophysin in axons and dendrites suggests a functional involvement of synaptophysin in fibre outgrowth of developing pyramidal neurons. Synaptoporin expression parallels the formation of dentate granule cell synaptic contacts with pyramidal neurons: the amount of hippocampal synaptoporin, determined in immunoblots and by synaptoporin immunostaining of developing mossy fibre terminals, increases during the first postnatal week. Moreover, in the adult, synaptoporin is found exclusively in the mossy fibre terminals present in the hilar region of the dentate gyrus and the regio inferior of the cornu ammonis. In contrast, synaptophysin is present in all synaptic fields of the hippocampal formation, including the mossy fibre terminals, where it colocalizes with synaptoporin in the same boutons. Our data indicate that granule neuron terminals differ from all other terminals of the hippocampal formation by the presence of both synaptoporin and synaptophysin. This difference, observed in the earliest synaptic contacts in the postnatal hippocampus and persisting into adult life, suggests distinct functions of synaptoporin in these nerve terminals.

## Introduction

The rat hippocampal formation provides an ideal model to analyse the composition of the synaptic vesicle membranes because the hippocampal cytoarchitecture, synaptic connections and neurotransmitters are well understood. For example, the axons of dentate granule neurons (mossy fibres) traverse the hilus of the dentate gyrus, form axonal collaterals and terminate as *en passant* synapses on hilar interneurons (for review see Amaral, 1978). Specifically, they terminate as large boutons on dendrites apical and basal to the cell bodies of cornu ammonis 4 (CA4) pyramidal neurons as well as the proximal part of the apical dendrites of CA3 pyramidal neurons (Blackstad and Kjaerheim, 1961). In addition, dentate granule neurons form smaller synapses with non-pyramidal interneurons in the CA3 region (Frotscher, 1985). Glutamate is the principal excitatory transmitter in the hippocampal formation involved in signalling between granule and pyramidal neurons (Crawford and Connor, 1973). Glutamate excites granule neurons via perforant path axons

and conveys signals between the pyramidal neurons of CA3 and CA1 via Schaffer collaterals.

Synaptic vesicles contain characteristic proteins, including synaptophysin and synaptoporin (Jahn and De Camilli, 1991; Jahn and Südhof, 1994). Synaptophysin and synaptoporin are members of the putative channel protein family present in the membranes of synaptic vesicles (for review see Betz, 1990). These proteins are characterized by four transmembrane domains and N- and C-terminals exposed to the cytoplasmic side (Johnston *et al.*, 1989b; Knaus *et al.*, 1990). Until recently, the composition of synaptic vesicle membranes was believed to be similar. Evidence now available reveals that synaptophysin and synaptoporin are differentially expressed in subpopulations of rat central nervous system neurons (Marquèze-Pouey *et al.*, 1991; Bergmann *et al.*, 1993; Ovtcharoff *et al.*, 1993).

The hippocampus contains large amounts of synaptophysin and synaptoporin. In the present study we focused on the differential

expression of synaptophysin and synaptoporin in adult and developing hippocampal neurons.

## Materials and methods

Adult and pregnant rats (Sprague–Dawley) were purchased from Charles River (Sulzfeld, Germany). The animals were anaesthetized with 4% chloral hydrate (1 ml/100 g body weight). Embryonic brains were obtained from pregnant rats on days 17 and 21 (E17, E21; day of insemination = day 0). Brains were removed from newborn (postnatal day 0, P0), young postnatal (P3, P5 and P8) and adult rats and processed as follows.

Tissues from different rat brain areas were frozen in liquid nitrogen. The samples were homogenized and proteins (10 µg per lane for the detection of synaptophysin and 20 µg for synaptoporin) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Laemmli, 1970) and transferred to nitrocellulose (Towbin *et al.*, 1979) as described previously in detail (Lahr *et al.*, 1990; Bergmann *et al.*, 1991). For the detection of antigen in blots, polyclonal rabbit antisera directed against rat synaptophysin (kindly provided by R. Jahn, New Haven, CT) or synaptoporin (kindly provided by H. Betz, Frankfurt/Main, Germany) were used. The antisera were diluted 1:1000 (anti-synaptophysin) or 1:200 (anti-synaptoporin) in PBS containing 0.5% bovine serum albumin and 0.05% sodium azide. Antigen/antibody complexes were visualized using the avidin–biotin–peroxidase complex (ABC) technique (Hsu *et al.*, 1981). For quantification the optical density of the immunoreactive bands was measured in the linear range of detection (Schilling and Gratzl, 1988).

For immunohistochemistry, paraffin sections (6 µm) of Bouin-fixed brains were mounted on gelatine-coated slides. In addition, vibratome sections (20 µm) fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) were used for the investigation of the developing hippocampus as well as for double immunostaining. Sections were incubated with the same antibodies as those used for immunoblotting. For double immunofluorescence studies, monoclonal mouse synaptophysin (1:50; Progen, Heidelberg, Germany) and polyclonal synaptoporin antiserum (see above) were used. Details of the subsequently applied ABC technique have been described previously (Bergmann *et al.*, 1993; Ovtcharoff *et al.*, 1993). Fluorescein-labelled rat IgG and Texas red-labelled avidin, which binds to biotinylated mouse IgG [Camon, Wiesbaden, Germany] were used for immunofluorescence. Double immunofluorescence was examined with a confocal laser scanning microscope (Zeiss LSM 410) and a high-resolution objective (Zeiss Plan-Apochromat 63×/1.4 oil) in two separate detection channels (excitation 488 nm/emission 515–545 nm; ex 543/em >590 nm). Controls incubated with 2% (vol/vol) normal goat and/or normal horse serum in phosphate-buffered saline or with normal rabbit serum (1:1000) instead of the first antibody were negative.

For ultrastructural studies rats were perfused through the ascending aorta with a fixative containing 2% paraformaldehyde, 0.5% glutaraldehyde, 1% acrolein, 2.5% dimethyl sulphoxide (vol/vol) and 9 mM CaCl<sub>2</sub> in 0.1 M cacodylate buffer (pH 7.2). Brains were removed, postfixed in the same fixative for 4 h and placed in phosphate-buffered saline overnight. Sections (20 µm) were cut on a vibratome and processed for pre-embedding staining employing the ABC technique (Bergmann *et al.*, 1993; Ovtcharoff *et al.*, 1993). Briefly, sections were incubated with the first antibodies for 48 h, and second antibodies as well as the detection system were applied for 2 h. Specimens were postfixed in 1% OsO<sub>4</sub> for 45 min, dehydrated and embedded in Epon. Ultrathin sections were stained with 4% uranyl acetate and 0.2% lead citrate.

## Results

### *Distribution of synaptophysin and synaptoporin in the rat central nervous system*

Western blots revealed a strong synaptophysin-immunoreactive band of 38 kDa in extracts of the hippocampal formation and the neocortex. Fainter bands were detected in the olfactory bulb, the cerebellum and the spinal cord (Fig. 1A).

Synaptoporin, migrating slightly faster (37 kDa), was highest in the hippocampal formation and the neocortex. A faint band was detected in the olfactory bulb. In contrast, in the cerebellum and the spinal cord no immunoreactive bands for synaptoporin were seen (Fig. 1B).

In the hippocampal formation synaptoporin increased during the first postnatal week (Fig. 1C). Quantification of synaptoporin in immunoblots carried out with samples of the developing hippocampal formation showed 27.7% (SD 6.6%; *n* = 5) of the adult level on P3 and 44.5% (SD 4.2%; *n* = 5) on P5 (Fig. 1D).

### *Expression of synaptophysin and synaptoporin in the hippocampus during development*

Synaptophysin immunoreactivity was first detected on E17 in perikarya of pyramidal cells, in fibres between their cell bodies (Fig. 2A) and in the fimbria hippocampi (Fig. 2B). At E21 synaptophysin immunoreactivity was found in the apical dendrites of pyramidal cells (Fig. 2C). Moreover, punctate staining was found in all terminal fields of the hippocampal formation (Fig. 2D), including the developing stratum lucidum. In summary, observations revealed that during prenatal development synaptophysin appears first in pyramidal cell bodies and their axons and subsequently in apical dendrites of pyramidal cells and the newly formed terminals of the hippocampal formation.

All hippocampal terminal fields showed punctate synaptophysin immunoreactivity in postnatal and adult rats (see also Fig. 5A). The developing dentate gyrus granule cells of postnatal rats (up to day 8) exhibited perikaryal staining (not shown), while the axons and dendrites of postnatal pyramidal cells were immunonegative.

Immunostaining first detected synaptoporin at P3 in the terminal field of the hippocampal granule neurons in the stratum lucidum of CA3 (Fig. 3A). Thereafter, synaptoporin immunoreactivity increased between P5 (not shown) and P8 (Fig. 3B). In the hilar region of the dentate gyrus, as well as in the suprapyramidal and infrapyramidal layer of CA4, immunoreactivity for synaptoporin was also first observed at P3 and was more intense at P8 (not shown). No immunostaining for synaptoporin was detected in the hippocampal formation in the prenatal period and at P0. Taken together, synaptoporin immunoreactivity was only observed postnatally in terminal fields of developing granule neurons.

### *Expression of synaptophysin and synaptoporin in the adult hippocampal formation*

Immunostaining for synaptoporin in the adult was restricted to the hilar region of the dentate gyrus, the supra- and infrapyramidal layers in CA4 and the stratum lucidum of CA3 (Fig. 4A). At the boundary between CA3 and CA2/1 an abrupt decrement in synaptoporin immunoreactivity was evident (Fig. 4A). The localization of synaptoporin immunoreactivity is consistent with the synaptic field of the mossy fibre system.

Ultrastructural observations revealed numerous synaptoporin-immunostained synaptic vesicles in the giant mossy fibre boutons

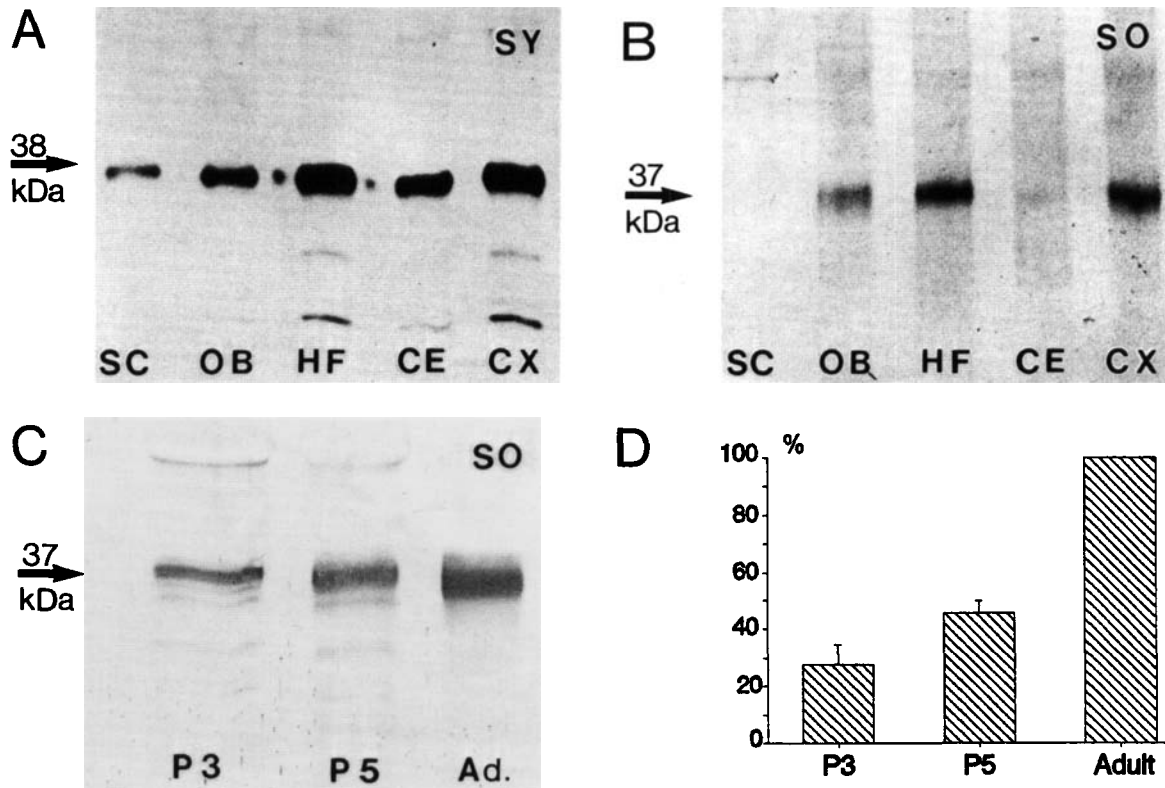


FIG. 1. Distribution of synaptophysin and synaptoporin in different parts of the central nervous system. (A) Immunoblots revealed large amounts of synaptophysin (SY) in the hippocampal formation (HF) and the neocortex (CX) and less in the spinal cord (SC), the olfactory bulb (OB) and the cerebellum (CE). (B) Synaptoporin (SO) was most abundant in the hippocampal formation and the neocortex, but less in the olfactory bulb. No immunoreactive bands were observed in samples of the spinal cord and the cerebellum. (C) The amounts of synaptoporin increased during hippocampal development between postnatal day 3 (P3), P5 and adult. (D) Quantification of the intensities of the bands revealed  $27.7 \pm 6.6\%$  (SD,  $n = 5$ ) and  $44.5 \pm 4.2\%$  ( $n = 5$ ) of the adult level at P3 and P5 respectively. Arrows point to the immunoreactive synaptoporin and synaptophysin bands and give their molecular masses determined in sodium dodecylsulphate-polyacrylamide gel electrophoresis. For details and evaluation of the linearity see Materials and methods.

(Fig. 4B). Some terminals with unlabelled synaptic vesicles were found in the vicinity of heavily stained terminals (Fig. 4B). Light and electron microscopic observations revealed that mossy fibre terminals also contain synaptophysin; for example, synaptophysin-immunostained mossy fibre boutons were found in CA3 (Fig. 5A). The distribution of synaptoporin in the same section was also documented with confocal laser microscopy (Fig. 5B). Most mossy fibre terminals contained both synaptophysin and synaptoporin (Fig. 5C), whereas synaptophysin was present in smaller terminals in the stratum radiatum and other layers of the same region (Fig. 5A, C).

## Discussion

Synaptic vesicle membrane proteins participate in the storage of neurotransmitters, in the docking, fusion and recycling of synaptic vesicles. The functions of carriers and the electrochemical proton gradient during accumulation of neurotransmitters in synaptic vesicles are evident. Recent observations on the effect of clostridial neurotoxins and complexes formed between synaptic vesicle membrane proteins and other components of the nerve terminal suggest that synaptobrevin and synaptotagmin are essential elements of the exocytosis machinery of synaptic vesicles (O'Connor *et al.*, 1993; Söllner *et al.*, 1993). However, the precise roles of these two proteins in regulated neurotransmission remain to be defined. The most abundant synaptic vesicle protein, synaptophysin, and its congener synaptoporin have

been proposed to participate as oligomers in the formation of the pore-initiating membrane fusion (Thomas *et al.*, 1988; Knaus *et al.*, 1990).

Studies on the developmental expression of synaptophysin and synaptoporin in the central nervous system revealed that these proteins are present not only in mature terminals but also in outgrowing axons (Bergmann *et al.*, 1991, 1993; Scarfone *et al.*, 1991; Ovtcharoff *et al.*, 1993; this study) and, unexpectedly, in the cytoplasmic bridge connecting retinal photoreceptor cell bodies with the rod component (Schmied and Holtzman, 1989), in dendrites of the continuously regenerating olfactory receptor neurons (Bergmann *et al.*, 1993) and, transiently, in dendrites of developing hippocampal pyramidal neurons (this study). While synaptic vesicles in outgrowing axons could serve as a reservoir for subsequent synaptogenesis, synaptic vesicle membrane antigens in developing dendrites are unlikely to contribute to synaptic organelles. Thus it is possible that synaptophysin exists in dendritic subcellular structures different from synaptic vesicles. In a variety of cells, evidence has been provided for this notion: for example, in PC12 cells synaptophysin is present in the early endosomal compartment (Linstedt and Kelly, 1991b; Régner-Vigouroux *et al.*, 1991; Lah and Burry, 1993); synaptophysin and synaptoporin associate with early endosomes in transfected non-neuronal cells (Johnston *et al.*, 1989a; Cameron *et al.*, 1991; Feany *et al.*, 1993; Fykse *et al.*, 1993); and synaptophysin (but not other synaptic vesicle membrane proteins) colocalizes in the perikaryal-dendritic region with the

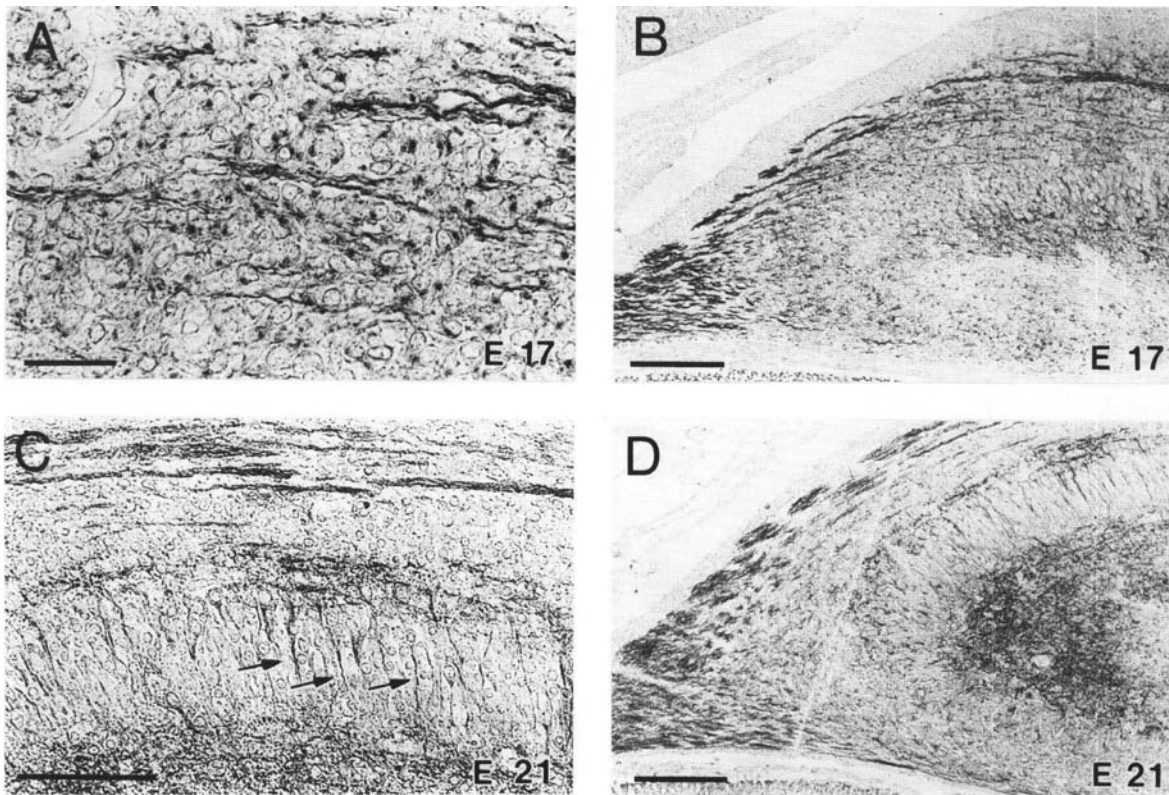


FIG. 2. Localization of synaptophysin in the developing hippocampal formation. (A) Perikarya of pyramidal cells and fibres between their cell bodies exhibited immunostaining at E17. (B) Additionally, fibres of the fimbria hippocampi were heavily stained at this stage. (C) At E21 immunoreactive dendrites (arrows) were found between the pyramidal neurons and in the developing stratum radiatum. (D) Punctate staining was detected in all synaptic fields of the hippocampal formation, including the developing stratum lucidum. Also, fibres in the fimbria hippocampi were immunopositive. Scale bars: A, 25 μm; B–D, 100 μm.

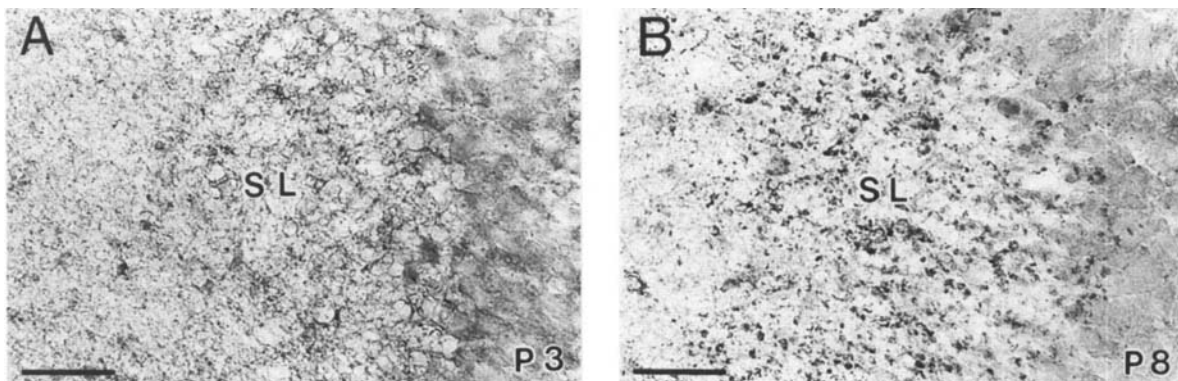


FIG. 3. Developmental expression of synaptoporin in the hippocampal formation. (A) First punctate synaptoporin immunostaining in the stratum lucidum (SL) of cornu ammonis 3 was detected at P3. (B) Immunoreactivity increased during the first postnatal week. At P8 heavily stained mossy fibre boutons were found in the stratum lucidum (SL). A and B are vibratome sections. Scale bars, 50 μm.

transferrin receptor, a marker for early endosomes in cultured hippocampal neurons (Mundigl *et al.*, 1993). Thus it appears that synaptophysin participates in endosomal membrane recycling; moreover, its function during endocytosis in transfected fibroblasts is functionally linked to the cytoplasmic carboxy-terminal tail of synaptophysin (Linstedt and Kelly, 1991a). Taken together, the available data suggest that synaptophysin participates both in regulated transmitter release by mature terminals and in constitutive pathways

related to the function of endosomes. In developing neurons membrane recycling is a prominent process during outgrowth of axons and dendrites and in mature synapses it is coupled to exocytosis. It is tempting to speculate that synaptophysin participates in membrane recycling during membrane biogenesis of dendrites and axons as well as in exocytosis.

The terminals of the granule neurons, the mossy fibre boutons, differ from all other terminals in the hippocampal formation, which

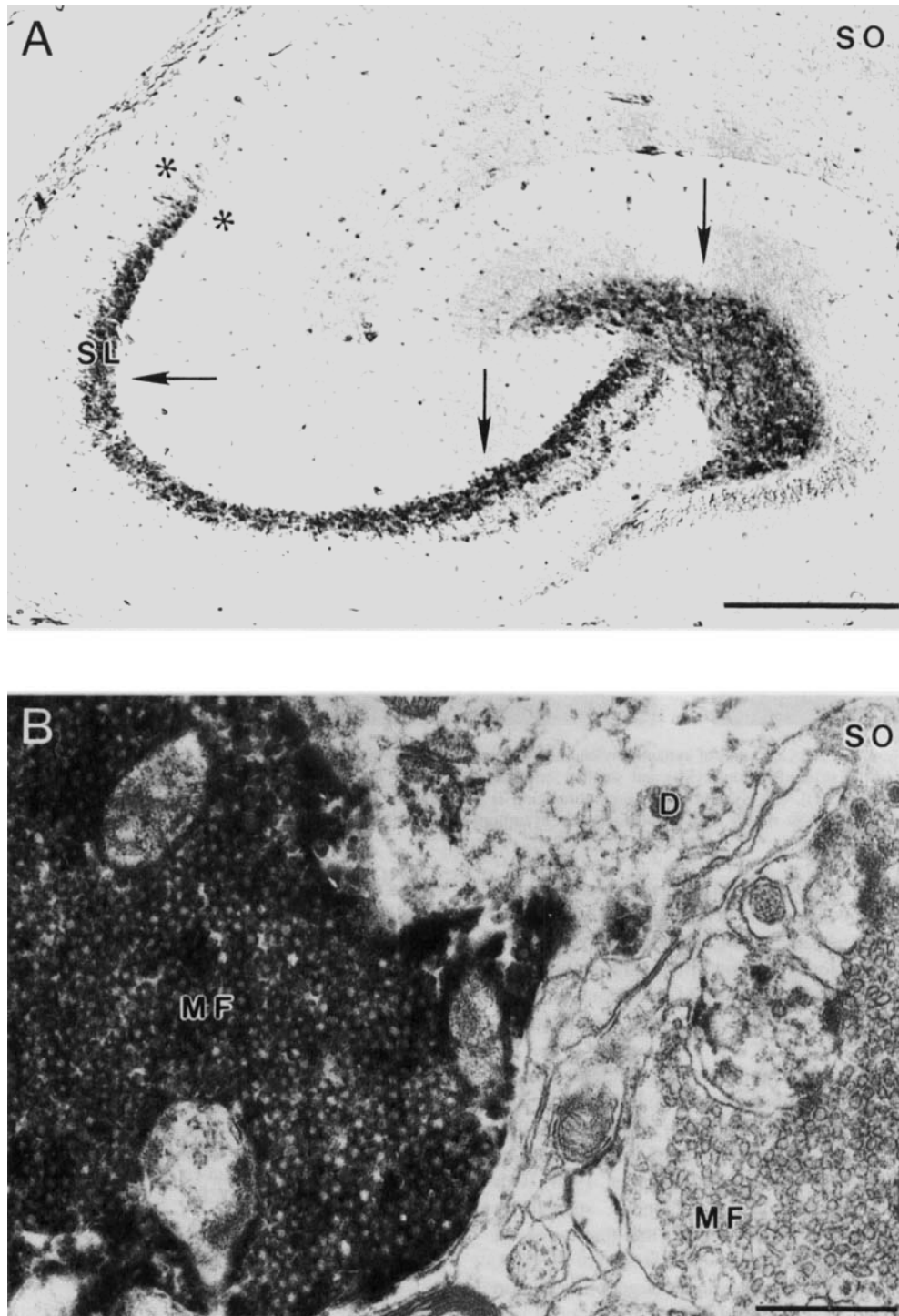


FIG. 4. Distribution and ultrastructural localization of synaptoporin in the adult hippocampal formation. (A) In the adult hippocampal formation synaptoporin (SO) is restricted to the terminal fields of the mossy fibre system (arrows). The hilus fasciae dentatae as well as the regio inferior of the cornu ammonis are heavily stained. In CA4, unstained cell bodies of pyramidal neurons separate synaptoporin-immunoreactive supra- and infra/intrapyrarnidal mossy fibre boutons. Also the stratum lucidum (SL) of the CA3 region is densely labelled. The boundary to regions CA2/1 is marked with asterisks. No synaptoporin immunoreactivity was found in CA2/1 region. Scale bar, 400  $\mu\text{m}$ . (B) Synaptoporin-immunostained and unstained mossy fibre boutons (MF) contact the same pyramidal neuron dendrite (D) in the CA4 region. Scale bar, 0.5  $\mu\text{m}$ .

only contain synaptophysin (this study), by the presence of both synaptophysin and synaptoporin (Marqu ze-Pouey *et al.*, 1991; Fykse *et al.*, 1993; this study). These synaptic vesicle membrane proteins

differ mainly in the cytoplasmic carboxy-terminal tail (Knaus *et al.*, 1990). Thus the putative function of these proteins during endocytosis, which is linked to this part of the molecule (Linstedt and Kelly,

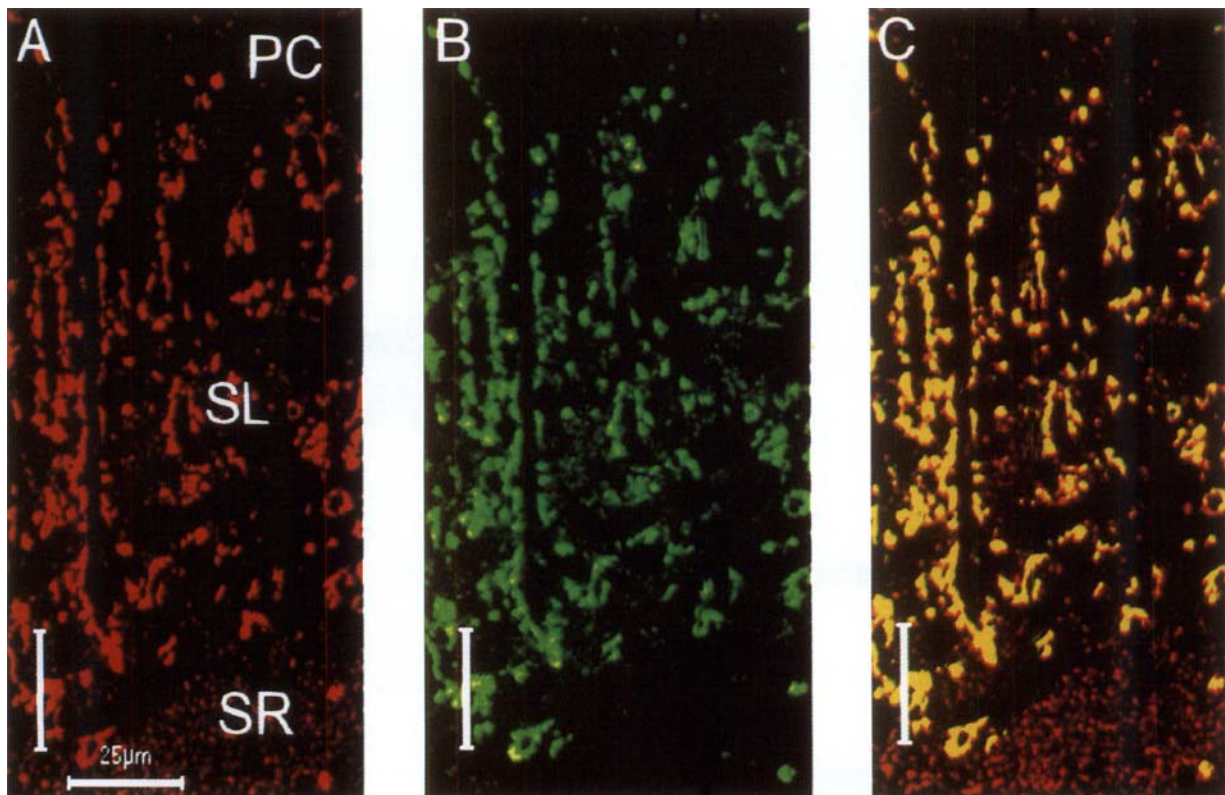


FIG. 5. Differential expression and colocalization of synaptophysin and synaptoporin in the hippocampal formation. (A) Synaptophysin immunostaining revealed large mossy fibre boutons in the stratum lucidum (SL) and smaller terminals in all other fields of the hippocampal formation [e.g. in the pyramidal cell (PC) layer and the stratum radiatum (SR)]. (B) Synaptoporin immunoreaction is restricted to the mossy fibre boutons in the stratum lucidum (SL). (C) Double immunofluorescence indicated that most mossy fibre boutons contain synaptoporin in addition to synaptophysin (yellow). Scale bar, 25  $\mu$ m.

1991a), is likely to be modulated in these terminals in a very specific way. Since synaptoporin is already present in the mossy fibre terminals during the first postnatal week and in the adult, it is a necessary component during early synaptogenesis and in the adult circuitry. In this respect hippocampal granule neurons differ from striatal neurons, which synthesize first synaptophysin and later synaptoporin (Ovtscharoff *et al.*, 1993).

The time course of maturation of hippocampal granule neurons is well known (Bayer, 1980). Mossy fibre terminals can be specifically stained with a cytochemical reaction that localizes zinc (Haug, 1967); this technique reveals the first axon sproutings of these neurons at P1 (Zimmer, 1978) and their boutons at P3 (Zimmer, 1978). Our observations indicate that hippocampal synaptoporin specifically increases in the mossy fibre terminals during the first week of postnatal life. SNAP-25 (synaptosomal-associated protein of 25 kDa), a further component of the fusion machinery and a target of botulinum A toxin (Blasi *et al.*, 1993; Schiavo *et al.*, 1993), is incorporated into the terminals by the third week (Oyler *et al.*, 1991). Functional maturation of granule cells parallels the formation of synaptic contacts since weak synaptic field potentials were found at P5 and their synapses revealed characteristic electrophysiological features at P10 (Bliss *et al.*, 1974). This implies that synaptogenesis of the hippocampal granule neurons, storage of zinc, expression of synaptoporin and the onset of function of the mossy fibres are closely correlated.

Zinc, an important endogenous modulator of glutamate receptors (Westbrook and Mayer, 1987; Hollmann *et al.*, 1993) and localized in synaptic vesicles of mossy fibre boutons (Dyck *et al.*, 1993), disappears after long-term potentiation (Sloviter, 1985). This suggests

a close relationship between synaptoporin-positive vesicle membranes and the co-release of zinc (Slomianka, 1992) and glutamate (Assaf and Chung, 1984; Howell *et al.*, 1984) in signalling between dentate granule neurons and pyramidal neurons in the adult.

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#### Abbreviations

ABC	avidin-biotin-peroxidase complex
E	embryonic day
CA1	hippocampal subfield cornu ammonis 1
CA2	hippocampal subfield cornu ammonis 2
CA3	hippocampal subfield cornu ammonis 3
CA4	hippocampal subfield cornu ammonis 4
LTP	long-term potentiation
P	postnatal day
PC12	phaeochromocytoma cell line PC12

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