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Research report

Restoration of mossy fiber projection in slice co-cultures of dislocated dentate gyrus and degranulated hippocampus

Jean-Luc Gaiarsa [a,*], Bernd Heimrich [b]

[a] *INSERM U29, Hopital de Port-Royal, 123 Bd. de Port-Royal, 75014 Paris, France*
[b] *Institute of Anatomy, Univ. Freiburg, POB 111, 79001 Freiburg, Germany*

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Abstract

Regional specificity of the mossy fiber projection is a well described feature of hippocampal intrinsic connectivity. Possible mechanisms involved in the formation of this specific projection include attraction molecules localized in the target area or repulsive cues preventing from ingrowth in non-target areas. To test this hypothesis, using organotypic co-cultures of dentate gyrus and irradiated degranulated hippocampal slices, we have disrupted the pathway normally taken by mossy fibers. The dentate gyrus explant was ectopically placed facing the alveus/stratum oriens of the irradiated hippocampal slice forcing the mossy fibers to cross the stratum oriens to reach their target area. Extensive plexuses of labeled mossy fibers were observed in the hilus and adjacent pyramidal cell layer of non-irradiated dentate gyrus explants. A few mossy fibers crossed the border between the co-cultures and reached their specific termination area in the irradiated hippocampus where they formed characteristic multiple synaptic contacts on their target cells. In addition to mossy fibers, numerous thin and varicose non-mossy fibers invade all parts of the co-cultured hippocampus establishing symmetric as well as asymmetric synapses. From these data we assume that mossy fiber axons emerging from dislocated non-irradiated dentate gyrus explants find their normal termination zone in the co-cultured degranulated hippocampal slice even if they are forced to run an unusual pathway. These results support the idea that an attraction signal arising from the target area is involved in the formation of this specific projection.

Keywords: Static culture; γ-Irradiation; Biocytin tracing; Electron microscopy; Synaptogenesis

1. Introduction

The mossy fiber pathway of the hippocampal formation provides a well-known example of neuronal target specificity. These fibers originating from the granule cells of the fascia dentata are the main intrinsic excitatory inputs on the CA3 region. The mossy fibers are restricted not only to specific target region but also to specific portions of the target cells i.e. the proximal dendritic segments [3,5]. In these layers the mossy fiber terminals form the characteristic giant presynaptic boutons and establish multiple synaptic contacts with target cells [3,5,10,11].

It has been suggested that the formation of laminated projections in the hippocampus is determined by the sequential ingrowth of various afferent fiber systems [4] i.e. the earlier arriving projections to the CA3 pyramidal neurons terminate on the more distal part of their dendrites, while the mossy fibers, which develop postnatally [3], terminate on the proximal dendritic segments. However, recent organotypic co-culture experiments have shown that, at least for the entorhinal input which also exhibits a remarkable layer specific termination in the hippocampus, recognition signals rather than temporal events are involved [13]. Therefore, other mechanisms may underlies the formation of specific mossy fibers distribution. One possibility is that molecules localized in target area (the stratum lucidum) selectively influence the growth of mossy fibers. Alternatively, repellent forces exerted by non-target areas such as the strata oriens and radiatum may canalize mossy fiber ingrowth into the appropriate target region.

To address this issue, we used organotypic co-cultures to disrupt the pathway normally taken by mossy

* Corresponding author. Fax: (33) (1) 46 34 16 56.

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fibers. Slice co-cultures of different brain areas provide a useful model to reconstruct extrinsic pathways [7,24,36,37], and also give the possibility to analyse some of the factors which may be involved in axonal pathfinding and target specificity [13,23]. In hippocampal slice cultures the main cell types survive retaining most of their morphological characteristics [8,12,14,19,33] and the mossy fiber system develops in a way which is reminiscent of the in vivo situation [29,38]. Using co-cultures of dentate gyrus and slices of CA1 or CA3 regions, Zimmer and Gähwiler [39] have demonstrated that Timm-stained fibers cross the cut and project only to their appropriate target. However, since the study involved the use of 7-day-old rats, the authors could not exclude priming processes due to mossy fiber projections that were already formed in the CA3 region at that stage of explantation. To prevent the influence of priming, we have used co-cultures of irradiated hippocampal slices of newborn rats and non-irradiated dentate gyrus slices of 5-day-old rats. Irradiation of the hippocampus at birth destroys the neuroblasts giving raise to granule cells resulting in a dramatic reduction of mossy fiber projection [1,16,26,34]. The irradiated CA3 neurons are therefore largely deprived of mossy inputs which may result in the appearance of free synaptic sites. In addition, in the study of Zimmer and Gähwiler [39] the dentate gyrus explants and slices of hippocampal CA3 region were oriented correctly with respect to the hippocampal layers. In the present study, in order to investigate the factor(s) involved in the formation of specific mossy fiber projection (attraction molecules or repellent forces from non target areas), the dentate gyrus explant was ectopically positioned apposing the alveus/stratum oriens of the co-cultured irradiated hippocampal slice. In these conditions, the mossy fibers had to cross the stratum oriens to reach their target area.

The results show that mossy fibers as well as thin varicose fibers entered the irradiated hippocampal slices. However, in contrast to the diffusely distributed thin varicose fibers, the mossy fibers terminals were found only in the broadened pyramidal layer where they establish the characteristics multiple synaptic contacts on thorny excrescences. These results support the idea that chemotropic factors from target area are involved in the formation of the specific mossy fiber projections.

2. Materials and methods

2.1. Irradiation

Newborn rats were irradiated (600 rad) at the day of birth (P0) by a cobalt bomb (Centre d'Etudes Nucléaires, Fontenay aux Roses). γ-Rays were collimated using a lead plate to irradiate only one hemisphere of the brain.

2.2. Co-cultures

We used interphase cultures as described by Stoppini and colleagues [32]. Briefly, for preparing dentate gyrus slices, hippocampi of 5-day-old rat pups were dissected under steril conditions and cut perpendicularly to their longitudinal axis into 400-μm-thick slices. Sections were stored in cold (4° C) minimal essential medium (MEM) and the dentate gyrus/hilus were separated from the hippocampus proper with a razor blade. Only, slices of dentate gyrus exhibiting an obviously undamaged granule cell layer were selected. Irradiated rats were sacrificed one day after treatment. The hippocampi of irradiated hemispheres were prepared as described above. A slice of dentate gyrus and a slice of irradiated hippocampus were placed within 1 mm of each other (Fig. 1) on moistened translucent membranes of tissue culture inserts (Millicell-CM, Millipore). These inserts were transferred into 6-Multiwell plates each filled with 0.8 ml of medium. The medium consists of 25% heat inactivated horse serum, 50% MEM and 25% Hanks' balanced salt solution, enriched with glutamine to a final concentration of 2 mM and adjusted to pH 7.3. The medium was replaced three times per week. Co-cultures were incubated for 14–16 days. The soluble tracer biocytin (Sigma) was used to label the developed dentato–hippocampal pathway. Small crystals of biocytin were placed on the surface of the non-irradiated dentate gyrus/hilus culture with a micropipette and incubated for 2 h to allow for the biocytin uptake. Thereafter biocytin was removed from the co-cultures by extensive washing with medium. The cultures were incubated for additional 48 h to allow the anterograde transport of the tracer. After fixation (1–2 h) of the tissue in 4% paraformaldehyde, 0.08% glutaraldehyde and 15% saturated picric acid in 0.1 M phosphate buffer (PB) cultures were rinsed in PB and vibratome-cut in 40 μm sections. Selected sections were incubated for 3 h in ABC-Elite standard (1:50, Vector laboratories, Burlingame, CA) and washed again in PB. Biocytin-labeled fibers were visualized using diaminobenzidine (DAB) as a chromogen with a cobalt/nickel intensification protocol (for details see Heimrich and Frotscher [19]). The DAB reaction was monitored and stopped by several rinses in cold PB. For light microscopy, co-cultures were counterstained with Cresyl violet. Part of the vibratome-cut hippocampal target cultures showing ingrown labeled fibers were osmicated in 1% OsO_4 in PB for 10–20 min, dehydrated, flat-embedded and photographed. Ultrathin-sections were mounted on Formvar coated single slot-grids, stained with 5% lead citrate and 2% uranyl

acetate, and analyzed under a Zeiss 110 electron microscope.

3. Results

3.1. General aspect of the co-cultures

Co-cultures of irradiated degranulated hippocampus and untreated dentate gyrus slices survived well during an extended period of time (14–16 days). In the dentate gyrus/hilus slices from non-irradiated 5-day-old rats, the granule cell layer had a characteristic U-shaped appearance and the hilar portion of the pyramidal layer was well preserved (Fig. 2a). Cresyl violet staining revealed the presence of hilar neurons in the untreated dentate gyrus/hilus explant (Fig. 2a,b). In the irradiated hippocampal cultures, the pyramidal cell layers were well preserved, showing a widening of the cell layers, a normal occurrence under these conditions (Fig. 2a). As expected from treatment with γ-ray, there was no visible granule cell layer in the irradiated culture (Fig. 2a), while hilar neurons were preserved.

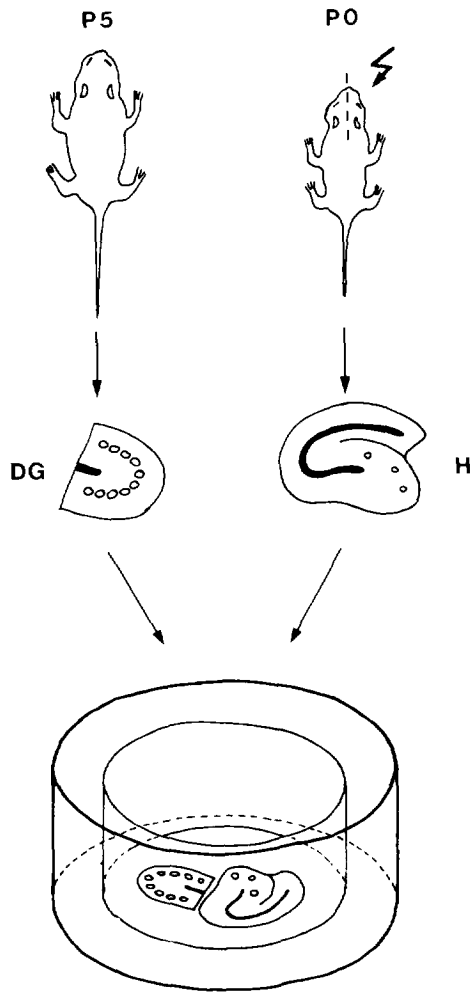

Fig. 1. Schematic drawing of the experimental procedure. Hippocampal slices from 5-day-old rat pups (P5) were prepared and the dentate gyrus/hilus was separated from the hippocampus proper. Rats were irradiated at birth (P0) and hippocampal slices were prepared one day after irradiation. A slice of dentate gyrus/hilus was positioned facing the CA3 alveus/stratum oriens of the irradiated hippocampal slice at a distance of about 1 mm.

3.2. Biocytin-labeled fiber ingrowth in the target-irradiated hippocampal slices

Numerous granule cells and hilar neurons were stained by biocytin in the dentate gyrus/hilus explants (Fig. 2). The granule cells were densely packed and extended long dendrites into the molecular layer (Fig. 2b). Biocytin-labeled axons, optically identified as mossy fiber axons because of the presence of giant varicosities [3,5], were found throughout the hilus of dentate gyrus/hilus slice cultures, 'sandwiching' the hilar portion of the pyramidal layer thereby giving rise to characteristic giant mossy fiber terminals (Fig. 2b). A few mossy fiber-like axons crossed the border between the co-cultured slices and penetrated into the irradiated hippocampal slices. In the irradiated target hippocampal slices, the mossy fibers terminated only in the pyramidal layer of the CA3 region (Fig. 3a,b) where they formed characteristic giant terminals (Fig. 3b). In addition to the mossy fiber axons, numerous thin and varicose fibers were found in the dentate gyrus/hilus slice cultures. These fibers also crossed the border between the two co-cultures to reach the target-irradiated slices. In contrast to the restricted distribution of the mossy fibers to the regio inferior, these axonal processes were distributed over the entire co-cultured hippocampus with a prominent projection found in the dentate gyrus of the irradiated hippocampal slices (Fig. 2a).

3.3. Synapses formation in the target-irradiated hippocampal slices

Electron microscopic analysis of the axonal profiles in various regions of the hippocampal cultures revealed the presence of several types of biocytin-labeled presynaptic terminals. The biocytin-labeled fibers established mainly symmetric contacts on the dendritic shafts of hippocampal neurons (Fig. 4a,b,c) and asymmetric contacts on spines (Fig. 4d) in all regions of the co-cultured hippocampus. In contrast, labeled mossy fiber-like terminals were detected only in the widened pyramidal cell layer of irradiated hippocampal CA3 region. These boutons displayed the typical ultrastructural characteristics of mossy fiber boutons as seen in situ. As described in vivo, they had a complex shape and established multiple asymmetric synapses with complex invaginated dendritic spines (Fig. 5a,b,c). Synaptic con-

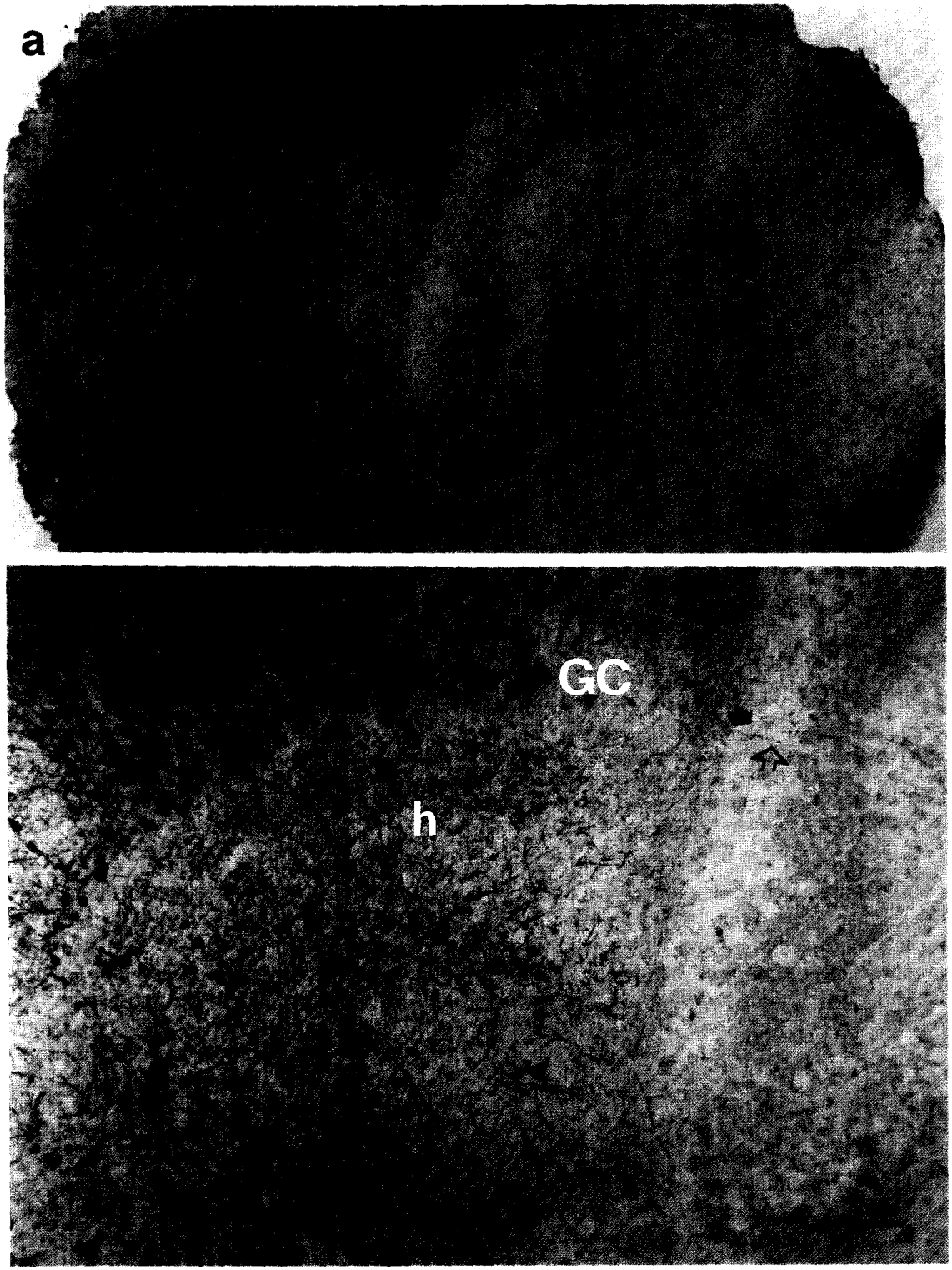

Fig. 2. Co-culture of dentate gyrus/hilus (dg) and irradiated hippocampal (ih) slices incubated for 16 days, labeled with biocytin, and counterstained with Cresyl violet. A: the organotypic appearance is evident in both slices despite a widening of the cell layers. The granule cell layer in the hippocampal slice is no longer present. In the dentate gyrus/hilus explant, labeled biocytin granule cells are densely packed and extend dendrites into the molecular layer. Labeled fibers originating from the dentate gyrus/hilus explant were found in the irradiated hippocampal co-culture (open arrow). Scale bar: 200 μm. B: at higher magnification, labeled mossy fiber boutons (arrows) were found in the hilus and in the vicinity of the hilar portion of the pyramidal layer in the dentate gyrus/hilus explant. The open arrow points to an individual fiber that had crossed the border between the two co-cultivated slices. GC, granule cell layer; h, hilus; scale bar: 100 μm.

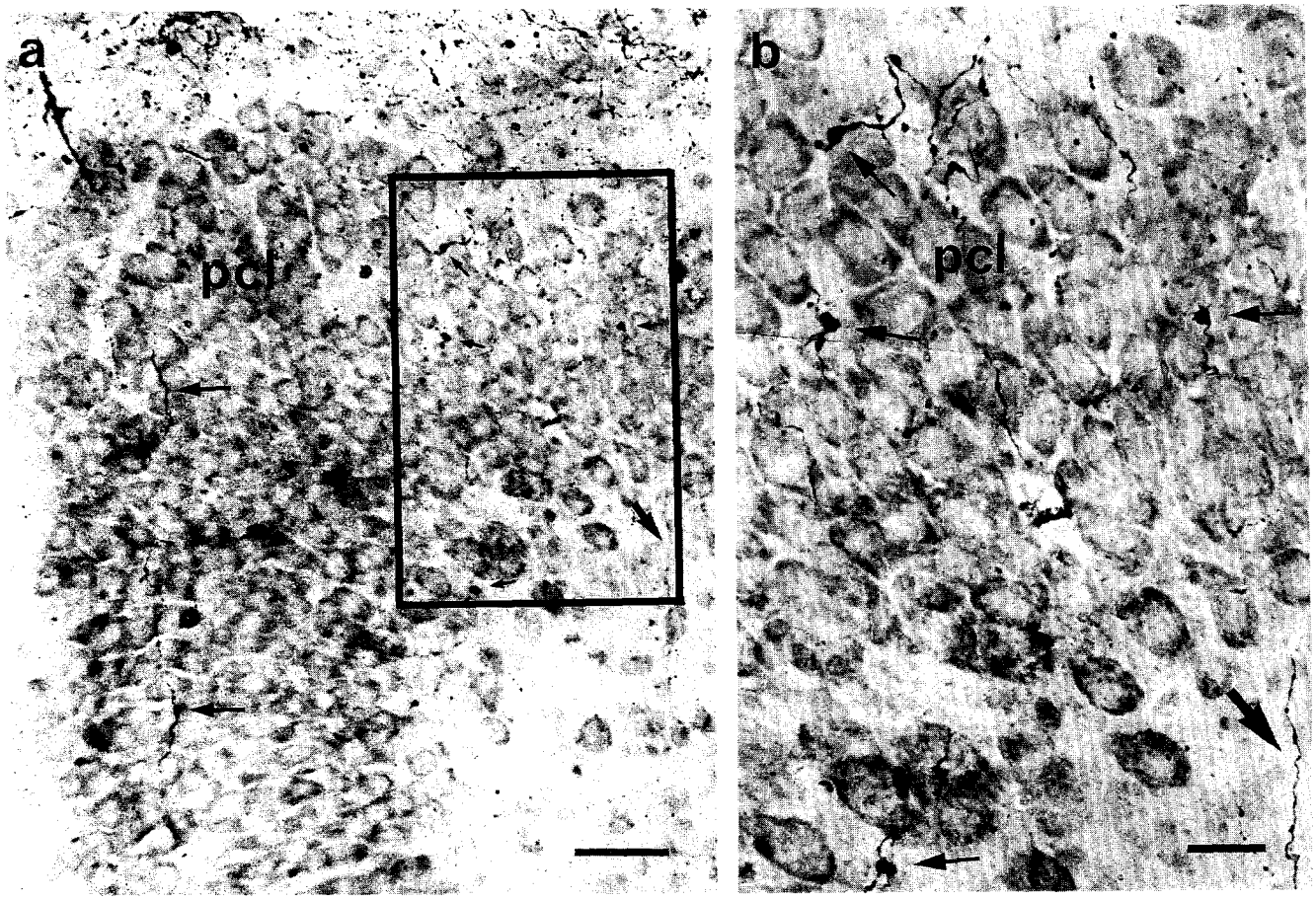

Fig. 3. Biocytin-labeled fibers in the irradiated hippocampal target slice. A: biocytin-labeled varicose fibers (large arrow) pass through the pyramidal cell layers of the irradiated hippocampus. In the CA2 region (boxed area) small arrows point to giant mossy fiber boutons in the cell layer. Scale bar: 50 μm. B: this light micrograph corresponds to the boxed area in A shown at a higher magnification. Mossy fiber axons forming en passant synapses were found between pyramidal neurons (arrow). Bold arrow marks a varicose fiber in the stratum radiatum of the co-cultured hippocampus. pcl, pyramidal cell layer; scale bar: 20 μm.

tacts were also found on dendritic shafts (Fig. 5a and c) indicating that synaptogenesis and mossy fiber maturation still occur after 2 weeks in vitro. Unlabeled mossy fiber boutons were also often seen beside the labeled mossy fiber terminals and synapsed onto the same neuron (Fig. 5a,c).

4. Discussion

In the present study we have examined the formation of dentato–hippocampal projections in organotypic slice co-cultures of non-irradiated dentate gyrus/hilus and irradiated degranulated hippocampus. As found in previous studies [8,12,14,19] the main cell types survived. In the dentate gyrus/hilus cultures dissected from 5-day-old rat pups, the granule cells layer of the fascia dentata retained its U-shaped structure and the pyramidal cell of the hilar portion of the regio inferior were densely packed. In addition the hilar neurons of the dentate gyrus/hilus explants were well preserved. In the irradiated hippocampal co-cultured slices, the pyramidal cell layer broadened during incubation period.

In several co-culture experiments of hippocampus and its afferent systems (entorhinal cortex, commissural pathway, cholinergic septo–hippocampal projections) it has been shown that the afferent fibers correctly reconstruct projections [13,20,23] and exhibit normal functional properties as as illustrated by Gähwiler and Brown [15] on the cholinergic septo–hippocampal pathway. In the present study the fiber pathways that developed in vitro also shared remarkable similarities to those that have been described in vivo.

4.1. Non-mossy fiber ingrowth

Among the fibers that invaded the co-cultured irradiated hippocampus, thin and varicose fibers were found in all hippocampal regions where they established symmetric and asymmetric contacts. Since these fiber terminals did not exhibit the characteristic fea-

tures of mossy fibers, they probably did not originate from granule cells of the dentate gyrus explant. However, it is possible that these fibers did indeed arise from granule cells, but since they did not synapse with the appropriate target area, they did not develop the characteristic features of mossy fiber terminals. This hypothesis is unlikely for the following reasons: (1) immature as well as mature mossy fiber terminals always establish asymmetric synaptic contacts [3,10,11,17] while in our experiments the thin and varicose fibers mainly establish asymmetric synapses; (2) in kindled rats, mossy fibers sprout in the stratum oriens of the CA3 region and in the stratum moleculare of the dentate gyrus [25,27]. Even in these non-target areas, the mossy fiber terminals still develop their characteristic morphology [25,27]; (3) the small non-mossy fiber terminals were also observed in the CA3 pyramidal region, the target area of the mossy fibers, and where characteristic mossy fiber terminals were found. These diffusely distributed fibers are more likely to originate from biocytin labeled hilar neurons of the dentate gyrus explant (Fig. 2b). Indeed, in vivo immunocytochemical studies have shown that the majority of hilar neurons are GAD-positive [30]. These cells may therefore provide the numerous symmetric dendritic shaft contacts observed in various regions of the co-cultured irradiated hippocampal slice. In vivo, the majority of hilar neurons form the associational and commissural pathways projecting to the contralateral and ipsilateral dentate gyrus respectively [6,30,31,35]. In our co-cultures, the thin varicose fibers were found mainly in the dentate gyrus, but also in the hippocampus proper of

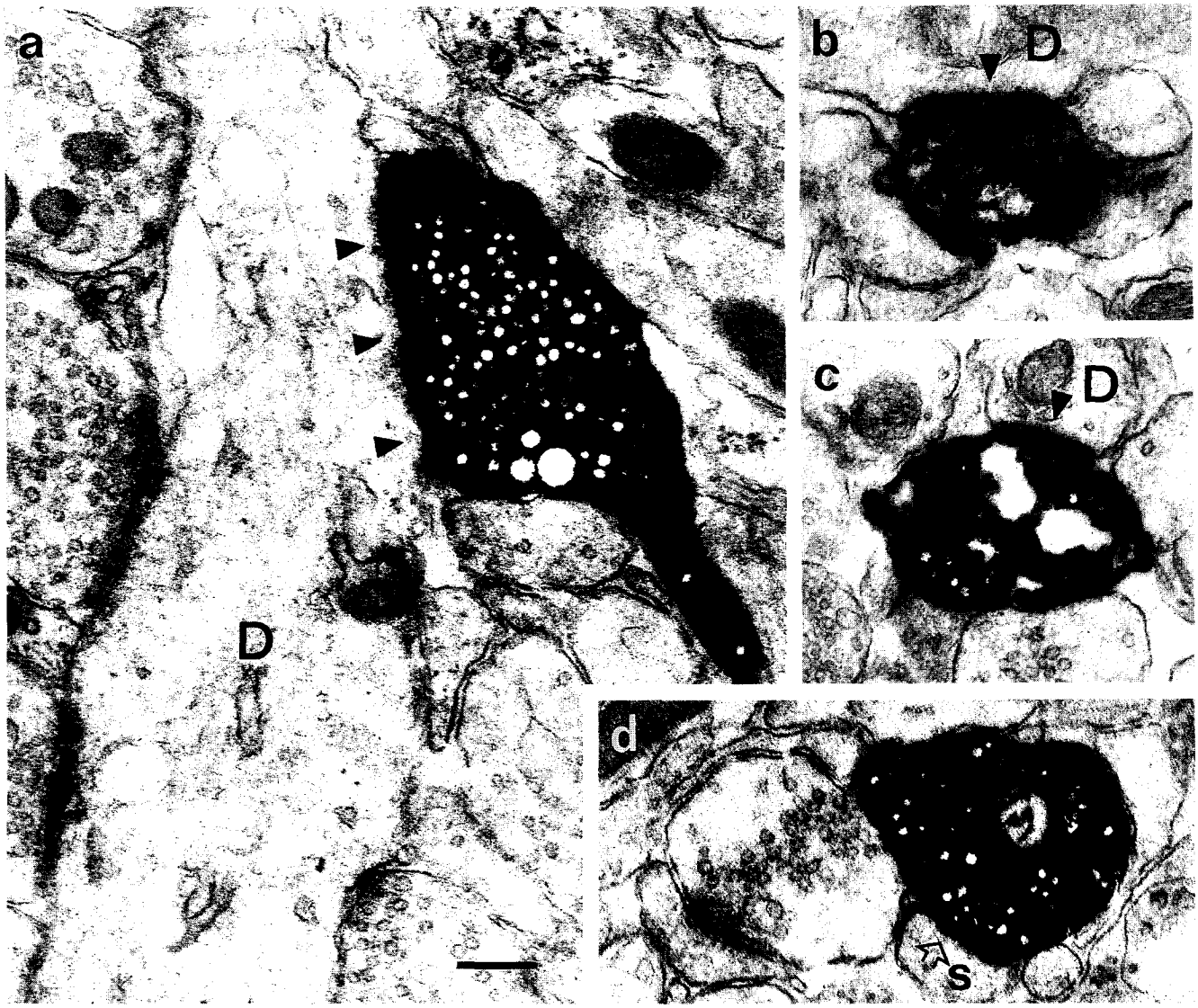

Fig. 4. Electron micrographs of biocytin-labeled non-mossy fiber terminals in various regions of the irradiated hippocampal target culture. The presynaptic terminals densely filled with vesicles establish symmetric contacts with dendritic shafts (arrowheads in a, b and c) and asymmetric contacts (open arrow in d) on dendritic spine. D, dendrite; S, spine; scale bar: 0.25 μm.

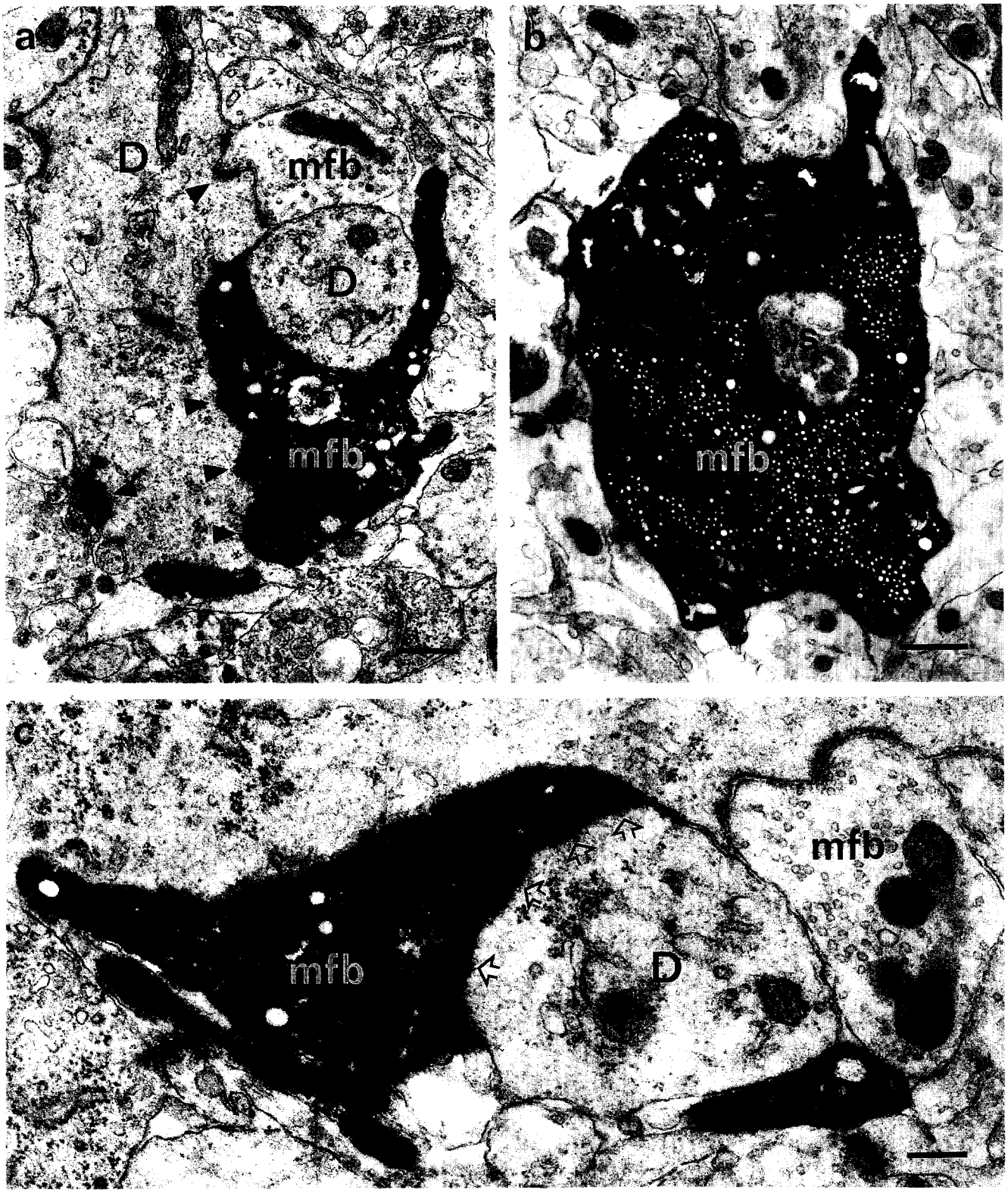

Fig. 5. Electron micrographs of the mossy fiber boutons in the CA3 pyramidal layer of the co-culture. A: large biocytin-labeled as well as unlabeled terminals (mfb) with a characteristics of mossy fiber terminals establish multiple asymmetric contacts on spine like protrusions (arrowheads). scale bar: 0.5 μm. B: a typical biocytin-labeled mossy fiber terminal, with numerous mitochondria and synaptic vesicles, completely engulfs a dendritic spine (S). Scale bar: 0.5 μm. C: a labeled bouton forms desmosome like contacts or asymmetric synaptic contacts (open arrows) with dendritic shaft (D). Scale bar: 0.25 μm.

irradiated hippocampal slices, suggesting that some of these fibers projected abnormally due to the lack of some of the target cells i.e. the granule cells. In addition to symmetric contacts, few terminals establish asymmetric contacts on dendritic spines. These terminals may originate from mossy cells, a characteristic cell type in the hilus [2] which has been reported to represent a population of excitatory neurons [28], or even from pyramidal neurons of the hilar portion of the co-cultured dentate gyrus/hilus slice.

4.2. Mossy fiber ingrowth

Biocytin labeled axons, optically identified as mossy fibers because of the presence of giant varicosities [3,5], also crossed the border between the two cultures and entered the target culture. However, in contrast to the diffusely distributed thin and varicose axonal processes, the biocytin-labeled mossy fiber axons were restricted to the CA3 region of the co-cultured irradiated hippocampal slices. In this region, labeled mossy fibers terminated mainly in the pyramidal layer. Some labeled mossy fibers arising from the co-cultured dentate gyrus were also observed in the strata oriens and radiatum of the irradiated hippocampal slices, probably resulting from the widening of the CA3 pyramidal layer.

Therefore, as already reported [39], the regional specificity of the mossy fiber projection is well preserved in co-culture experiments. However, in the present study, based on the electron microscopic analysis of the mossy fiber-like structures, we further show that the mossy fibers not only invade their appropriate target area, but also form the characteristic giant boutons which establish multiple synaptic contacts with thorny excrescences [3,5], thus restoring these specific connections in vitro. In addition, we demonstrate that the target specificity of the mossy fiber system does not depend on the growth pathway of the ingrowing axons. Besides these labeled axon terminals originating exclusively from the dentate explant, we often found unlabeled mossy fiber boutons that established synaptic contacts on the same dendrites of CA3 pyramidal neurons. These unlabeled boutons may result from incomplete tracing, or more reasonably, they may emerge from intrinsic granule cells of the irradiated hippocampus that survived the irradiation [16,26].

4.3. Conclusion

Several studies provide evidence that guidance to specific targets is achieved by spatially distributed attraction markers [7,13,18,23] or by repulsion cues that prevent the ingrowth [9,21,22]. From our results, it is obvious that as in vivo [34], the ectopical position of the dentate gyrus explant does not influence the formation of the specific mossy fiber projection in vitro, suggesting that chemotropic attraction from the target area rather than repellent action from non-target areas plays a role in the development of this projection.

The development of the mossy fiber pattern has been demonstrated in vitro by Zimmer and Gähwiler [39] using co-cultures of dentate gyrus and regio inferior of hippocampus which had been correctly orientated. In this study the investigators used 7-day-old rats. Since part of the mossy fibers are already present in the CA3 region at postnatal day 7, a priming process may take place, thus aiding mossy fiber ingrowth from the dentate gyrus. To prevent this priming, we have used rats subjected to γ-ray irradiations at birth a manipulation known to induce dramatic losses of granule cells [16,26,34] thus preventing the formation of the mossy fiber projection. In addition, in our experimental approach, the dentate gyrus was positioned facing the alveus/stratum oriens of the irradiated hippocampal slices forcing the mossy fibers to cross the stratum oriens in order to reach their appropriate target zone. Even under these conditions, the mossy fibers arising from the dislocated dentate gyrus explant terminated on their normal target cells and formed characteristic synapses. This observation suggests that stratum oriens does not exhibit repulsion properties that prevent the ingrowth of the mossy fibers. In the present study we cannot completely exclude that some mossy fibers grew into non-target areas during incubation, and due to the presence of inhibitory factors the mossy fibers terminals were not stabilized. If this occured, the mossy fibers would not be present in the non-target areas after 14–16 days of incubation. This possibility seems unlikely however since in kindled rats as well as in kainate treated rats mossy fibers sprout in non-target areas where they establish stable synaptic contacts [25,27].

In our co-culture experiments, the mossy fibers were forced to cross the stratum oriens to reach their appropriate target zone. In addition, the mossy fibers did not invade the stratum radiatum, but established synaptic contact within the CA3 pyramidal layer. These observations strongly support the idea that an attraction signal arising from the target cues is involved in the formation of this specific fiber projection.

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