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Rapid Communication

Mossy Fiber Pathfinding in Multilayer Organotypic Cultures of Rat Hippocampal Slices

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SUMMARY

1. Using a novel technique of organotypic cultures, in which two hippocampal slices were cocultured in a bilayer style, we found that the mossy fibers arising from the dentate gyrus grafted onto another dentate tissue grew along the CA3 stratum lucidum of the host hippocampal slice. The same transplantation of a CA1 microslice failed to form a network with the host hippocampus.

2. Thus, the type of grafted neurons is important to determine whether they can form an appropriate network after transplantation.

KEY WORDS: mossy fiber; hippocampus; dentate gyrus; granule cell; CA1; green fluorescent protein; axon guidance; explant; nerve transplantation.

The hippocampal formation contains a one-way circuitry in order of the perforant paths onto dentate granule cells, the mossy fibers onto CA3 pyramidal cells and then the Schaffer collaterals onto CA1 pyramidal cells, which is well known as the entorhino-dentato-hippocampal trisynaptic pathway (Amaral and Witter, 1995). To date, however, it remains unclear how this orderly arranged network is precisely formed during development. Using organotypic cultures of entorhino-hippocampal slices, our previous study indicated that ectopically grafted dentate gyri extended the mossy fibers into the host CA3 region but not into the CA1 region (Mizuhashi *et al.*, 2001), which suggests that an attractant for the mossy fibers is present in the CA3 region, ensuring a target-specific guidance. In this explant coculture system, however, the mossy fibers were forced to get across the alveus hippocampi before invading the

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host slices, and thus they were often erroneously guided along the alvear edge of the host slices.

In the present study, therefore, we developed a bilayer explant coculture system, in which a microslice of the dentate gyrus is transplanted onto another whole hippocampal slice so that the explant mossy fibers can reach their proper target area without crossing the alveus. This new technique also allowed us to investigate the cell-type-specific effect on graft-host network formation.

Organotypic hippocampal cultures were prepared from 6-day-old Sprague Dawley wild-type rats or green fluorescent protein (GFP)-expressing transgenic rats (Okabe *et al.*, 1997), in accordance with the Japanese Pharmacological Society guide for the care and use of laboratory animals. The experiment procedures used have been described elsewhere (Ikegaya, 1999). Animals were deeply anesthetized by hypothermia, and their brains were horizontally cut into 300-μm-thick slices. The entorhino-hippocampi were dissected out by using a small, curved scalpel. The CA1 region and the fascia dentata were isolated from the slices of GFP-expressing transgenic rats. Either of the microslices (about 500×500 μm) was laid on the upper blade of the stratum granulosum of the wild-type slices with extreme care and cultivated using the membrane interface techniques (Fig. 1(A)). Cultures were fed with 1 mL of culture medium consisting of 50% minimal essential medium (Life Technologies, Grand Island, NY), 25% horse serum, and 25% Hank's balanced salt solution (Cell Culture Lab, Cleveland, OH), and maintained at 37°C in a humidified 5% CO_2 and 95% air atmosphere. After 3 days in culture, they were fixed with 4% paraformaldehyde at 4°C for 120 min and then incubated with 0.1% Triton-X and NeuroTrace fluorescent Nissl (1:30 dilution) (Molecular Probes, Eugene, OR) for 40 min in the dark at room temperature. GFP- and Nissl-fluorescence images were obtained with the confocal imaging system BioRad MRC-1000 (BioRad Microscience Division, Cambridge, MA), equipped with an inverted microscope (ECLIPSE TE300, Nikon, Tokyo, Japan).

To distinguish grafted tissues from host slices, the grafts and the hosts were prepared from GFP-expressing transgenic rats and wild-type littermates, respectively (Fig. 1(A)). Three days after transplantation of a small part of GFP-positive dentate gyrus onto a wild-type entorhino-hippocampal slice, the GFP signal was evident in the CA3 stratum lucidum of the host slice in all 21 cases tested (Fig. 1(B) and (C)), indicating that the mossy fibers arose from the graft invaded the host slice and elongated along their proper pathway. Z-series-scan imaging revealed that the GFP-positive mossy fibers ran on the surface of the host slice (Fig. 1(B)). When a microdissected tissue of CA1 was grafted on the dentate area of the host, no GFP signal was detected outside the graft in all 11 cases tested (Fig. 1(D)), suggesting that the heterotopic grafting cannot make a connection with host CA3 neurons.

In the present study, utilization of GFP-transgenic rats enabled us to establish a novel type of organotypic cultures, i.e., a bilayer coculture system. In this coculture system, the grafted dentate gyri project neurites along the mossy fiber trajectories in the host slices. The results suggest that the grafted dentate neurons retain the ability to find their proper target and also that the bilayer cocultures maintain intrinsic guidance cues essential for lamina-specific mossy fiber development, resembling standard organotypic cultures (Dailey *et al.*, 1994; Koyama *et al.*, 2002; Ikegaya *et al.*,

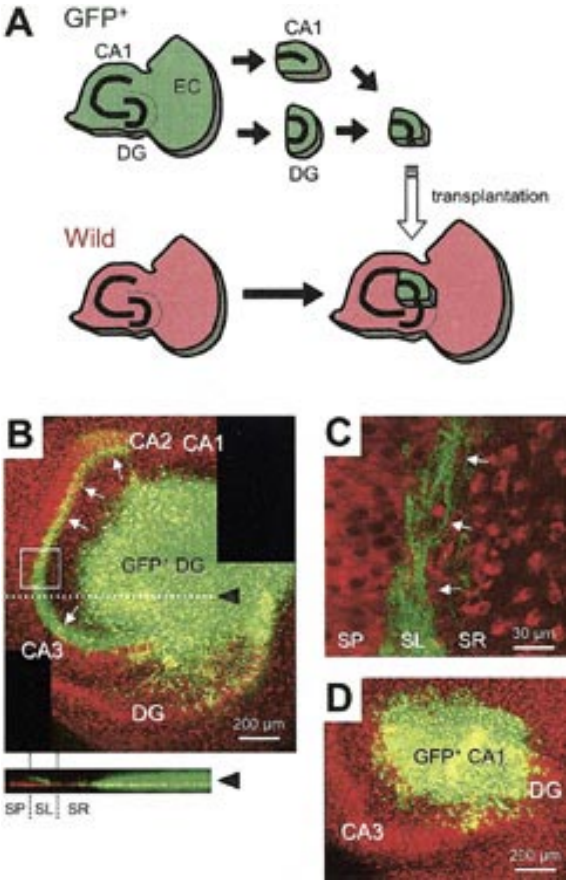

Fig. 1. Cell-type-specific neurite extension into host hippocampal slices. (A) Schematic draws of bilayer organotypic cultures of hippocampal slices. A microslice of the CA1 region or the dentate gyrus prepared from a GFP-expressing transgenic rat (GFP^+) was laid on the upper part of the dentate gyrus of a wild-type slice and cultivated for 3 days. (B) Confocal image of a slice cocultured with GFP-expressing dentate gyrus. The coculture was stained by Nissl method with red-fluorescent probes. The arrows indicate GFP-expressing mossy fibers. The inset shows a Z-series-scan image taken at the dotted line. (C) Image of the boxed region in (B) was taken at higher magnification with a thin confocal section. (D) Confocal image of a slice cocultured with a GFP-expressing CA1 stump. Each experiment was repeated at least four times (each 21 or 11 slices), producing the same results. DG: dentate gyrus, SP: stratum pyramidale, SL: stratum lucidum, SR: stratum radiatum.

in press; Mizuhashi *et al.*, 2001; Zimmer and Gahwiler, 1987). Hence, this coculture system is highly amenable for direct study of topographic mossy fiber growth.

Because synaptic terminals of mossy fibers contain a high concentration of Zn^{2+} (Henze *et al.*, 2000; Ueno *et al.*, 2002), their spatial distribution can be reliably

assessed by Timm's silver sulfide method, a histochemical technique, in organotypic hippocampal cultures (Ikegaya, 1999; Mizuhashi *et al.*, 2001). Thus, most of past studies on axon guidance in the hippocampus have focused on the mossy fibers, but the behavior of CA1 axons remained unclear so far because of no available histological techniques to specifically detect axon terminals of CA1 neurons. Using GFP-expressing explants, we have shown for the first time that the grafted CA1 tissues did not extend neurites into the host slices. Even if the CA1 stump was grafted onto the dentate gyrus, CA1 pyramidal cells did not behave like granule cells. Thus, no ability of CA1 neurons to innervate CA3 is due to their intrinsic properties independent of the soma location or other environmental factors such as surrounding glial cells and the vascular system, suggesting a donor (projecting cell)-dependent formation of the dentato-ammonic pathway. The CA1 pyramidal cells are probably insensitive for guidance signals of the mossy fibers. We previously demonstrated that the mossy fibers did not grow into the CA1 region even when the dentate graft was apposed very closely facing the CA1 region, suggesting a recipient (target cell)-dependent formation of the mossy fiber network (Mizuhashi *et al.*, 2001). Taken together, development of the dentato-ammonic circuit is highly cell-type-specific, requiring a reciprocal recognition of donors and recipients.

The bilayer coculture system is also a potential model of nerve transplantation in neurosurgery. Nerve transplantation has recently attracted much attention in clinical studies, and various cells are being proposed as candidates for transplantation into the injured nervous system (Brundin *et al.*, 2000; Fischer, 2000; Tresco, 2000). However, our data imply that improvident implanting results in an invalid function of the ectopically grafted neurons, giving caution for an optimistic view on nerve transplantation. The present work highlights the necessity to put the right neurons in the right post, and therefore, the bilayer coculture system will also provide an opportunity to evaluate the graft-host compatibility.

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