Developmental switch in axon guidance modes of hippocampal mossy fibers in vitro

Ryuta Koyama, Maki K. Yamada, Nobuyoshi Nishiyama, Norio Matsuki, and Yuji Ikegaya*

Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan

Received for publication 16 January 2003, revised 7 October 2003, accepted 11 November 2003

Abstract

Hippocampal mossy fibers (MFs), axons of dentate granule cells, run through a narrow strip, called the stratum lucidum, and make synaptic contacts with CA3 pyramidal cells. This stereotyped pathfinding is assumed to require a tightly controlled guidance system, but the responsible mechanisms have not been proven directly. To clarify the cellular basis for the MF pathfinding, microslices of the dentate gyrus (DG) and Ammon’s horn (AH) were topographically arranged in an organotypic explant coculture system. When collagen gels were interposed between DG and AH slices prepared from postnatal day 6 (P6) rats, the MFs passed across this intervening gap and reached CA3 stratum lucidum. Even when the recipient AH was chemically pre-fixed with paraformaldehyde, the axons were still capable of accessing their normal target area only if the DG and AH slices were directly juxtaposed without a collagen bridge. The data imply that diffusible and contact cues are both involved in MF guidance. To determine how these different cues contribute to MF pathfinding during development, a P6 DG slice was apposed simultaneously to two AH slices prepared from P0 and P13 rats. MFs projected normally to both the host slices, whereas they rarely invaded P0 AH when the two hosts were fixed. Early in development, therefore, the MFs are guided mainly by a chemoattractant gradient, and thereafter, they can find their trajectories by a contact factor, probably via fasciculation with pre-established MFs. The present study proposes a dynamic paradigm in CNS axon pathfinding, that is, developmental changes in axon guidance cues. © 2003 Elsevier Inc. All rights reserved.

Keywords: Mossy fiber; Axon guidance; Hippocampus; Dentate gyrus; Chemoattractant; Fasciculation; Development

Introduction

Various diffusible molecules and contact factors have been identified as guidance cues for developing axons (Goodman, 1996; Müller, 1999; Tessier-Lavigne and Goodman, 1996). Diffusible molecules make relatively long-range gradients in the extracellular milieu and thereby attract or repel axonal growth cones (Sato et al., 1994; Tessier-Lavigne, 1994). Experimentally, the contributions of diffusible cues can be demonstrated by the permeability of collagen gels (Heffner et al., 1990; Pini, 1993; Shirasaki et al., 1995; Tamada et al., 1995; Tessier-Lavigne et al., 1988). On the other hand, contact signals, for example, membrane-bound and cell adhesion molecules and extracellular matrix components, serve as short-range cues by contacting with growing axons. Yamamoto et al. (2000a,b) have shown that the lateral geniculate nucleus axons are capable of developing normal arbors even in chemically ‘fixed’ explants of cortical slices, suggesting contact-dependent axon guidance. In spite of these past suggestions, however, our understanding of how diffusible and contact signals are jointly or discretely involved in the formation of identical networks is still rudimentary.

The axons of hippocampal granule cells, that is, mossy fiber (MF), emanate from dentate gyrus (DG) and are projected accurately to the stratum lucidum and oriens of Ammon’s horn (AH), therein forming giant synapses with CA3 pyramidal cells (Henze et al., 2000). The lamina-specific MF trajectories provide a good model for studying CNS axon guidance.

Both diffusible and contact cues have been implicated in regulating MF pathfinding. Sema3F, a diffusible member of the semaphorin family, induces repulsion of MF axons, and mutant mice lacking Sema3F (Sahay et al., 2003) and its receptors neuropilin-2 (Chen et al., 2000) and Plexin-3A (Bagri et al., 2003; Cheng et al., 2001) display aberrant MF development. Netrin-1 is expressed in the CA3 target region...
and may attract MFs (Steup et al., 2000). The excitatory amino acid glutamate also participates in MF network formation via group II metabotropic glutamate receptors (Koyama et al., 2002). Polysialylated neural cell adhesion molecule (PSA-NCAM) is considered to play a direct role in fasciculation of MFs. Enzymatic or genetic removal of PSA-NCAM results in abnormal MF innervation and ectopic synaptogenesis (Cremer et al., 1997, 2000; Muller et al., 1994; Seki and Arai, 1999; Seki and Rutishauser, 1998). Thus, PSA-NCAM acts as a contact-mediated MF guidance cue. The cell adhesion molecule nectin (Mizoguchi et al., 2002) and the extracellular matrix component laminin (Grimpe et al., 2002) may also serve as contact cues. Nonetheless, nothing is known about why a single axon requires these multiple guidance cues or how the MFs exploit them aptly.

To elucidate the functions of these independent guidance systems, that is, secreta-dependent and contact-dependent mechanisms, during MF development, we employed techniques of collagen gel intercalation and tissue fixation in our explant coculture system (Mizuhashi et al., 2001) and found that these mechanisms work together in MF guidance. Moreover, we report the developmental shift in the relative contributions of diffusible and contact guidance cues.

**Materials and methods**

**Organotypic cultures of hippocampal slices**

Hippocampal slice cultures were prepared from wild-type or transgenic Sprague–Dawley rats expressing green fluorescence protein (GFP(+)) rats (SLC, Shizuoka, Japan) (Hakamata et al., 2001; Kim et al., 2003; Okabe et al., 1997). Unless otherwise specified, postnatal day 6 (P6) rats were utilized (Ikegaya, 1999; Mizuhashi et al., 2001) because the MFs develop mainly during the postnatal second week in rodents (Amaral and Dent, 1981). Animals were deeply anesthetized by hypothermia. Their brains were asceptically removed and cut into transverse slices at 300 μm in thickness in aerated, ice-cold Gey’s balanced salt solution supplemented with 25-mM glucose by using a vibratome (DTK-1500; Dosaka, Kyoto, Japan). The entorhino-hippocampal were dissected out under stereomicroscopic controls, and selected slices were cultured using membrane interface techniques (Yamamoto et al., 1989). Briefly, slices were placed on sterile 30-mm-diameter membranes (Millicell-CM, Millipore, Bedford, MA), and transferred into 6-well tissue culture trays. Cultures were fed with 1 ml of culture medium, which consisted of 50% minimal essential medium (Invitrogen, Gaithersburg, MD), 25% horse serum (Cell Culture Lab, Cleveland, OH) and 25% Hanks’ balanced salt solution. The cultures were maintained in a humidified incubator at 37°C in 5% CO₂. The medium was changed every 3.5 days. Experiments were performed after 10–17 days in vitro (DiV).

**Collagen gel preparation**

The tail of an adult Sprague–Dawley rat (SLC) was washed with soap and sterilized with 70% EtOH in a 100-ml dish for 20 min. The skin was removed with scissors, and the remaining part was cut into four fragments and immersed in saline. Then, the tendons were pulled out with forceps and transferred into a 35-mm dish filled with saline. After removal of saline by putting the tendons on a filter paper, their total weight was measured. The tendons were washed with distilled water and transferred to a 200-ml bottle with magnet, and 0.1% acetic acid was added to make 1% solution. To dissolve collagen, the solution was stirred at 4°C for 3 days and centrifuged at 10,000 × g for 30 min. The supernatant was stored at 4°C.

**Collagen gel-sandwiched cocultures**

An acute entorhino-hippocampal slice was prepared as described above, and DG and AH slices were carefully separated by a small curved scalpel (Mizuhashi et al., 2001). Collagen gels, consisting of 80% collagen solution, 10% 10×DMEM/F12 (Sigma, St. Louis, MO), and 10% alkaline solution (230 mM NaOH and 140 mM NaHCO₃), were interposed between the DG and AH slices using small forceps with caution. In the experiments of Fig. 1, DG slices of P6 GFP(+) rats and AH slices of wild-type littermates were cocultured. A DG slice was grafted near the CA3 region (a normal position) or the CA1 region of an AH slice (an upside-down position) (Figs. 1A, B). Slices and collagen gels were placed as close to each other as possible, preferably without a visible intervening gap.

**NeuroTrace fluorescent Nissl staining**

Cultures were washed three times with phosphate-buffered saline (PBS) for 5 min at room temperature and fixed with 4% paraformaldehyde (PFA) at 4°C for 60 min. After being washed three times with PBS for each 15 min, they were permeabilized with 0.1% Triton-X for 60 min, washed again with PBS for 10 min, and then incubated with NeuroTrace fluorescent Nissl (1:30 dilution) (Molecular Probes, Eugene, OR) for 40 min in a dark room at room temperature. The incubation was terminated by 10-min wash with 0.1% Triton-X, followed by PBS rinse for 2 h at room temperature, and the Nissl-stained slices were observed with a MRC-1000 laser scanning confocal system (BioRad, Cambridge, MA).

**Cocultures of fresh DG slices and chemically fixed AH slices**

Isolated AH slices were fixed with 4% PFA for 60 min at room temperature. They were rinsed six times with PBS for each 10 min so that PFA was completely washed out. The fixed slices were cocultured with fresh DG slices. Unless otherwise specified, the dentate hilus (CA4) of the DG grafts...
was placed facing the CA3c of the AH slice, and they were placed as close to each other as possible.

**Timm staining**

After rinse with PBS, slices were immersed in 0.4% sodium sulfide solution at 4 °C for 15 min, and fixed with 10% (v/v) formaldehyde for 15 min. After PBS wash, they were dehydrated with 70% and 96% ethanol twice for 30 min and then dried. To perform silver sulfide staining, the slices were incubated with citrate-buffered solution containing 20% Arabic gum, 2.1% AgNO₃, and 0.085% hydroquinone in a dark room at 26 °C for 50 min. The slices were washed with distilled water at the end of the reaction. To quantify MF terminals, the images were digitized with a FinePix S1Pro CCD camera (Fuji Photo Film, Tokyo Japan) equipped with an ECLIPSE TE300 microscope (Nikon, Tokyo, Japan). Average pixel intensities were estimated by acquiring intensity values (8 bit resolution) in each five different areas (5 × 400 μm²) within CA3 stratum lucidum and radiatum and dentate hilus. Timm grain intensity was determined by (values of stratum lucidum / values of stratum radiatum) / (values of dentate hilus – values of stratum radiatum) × 100.

**Iontophoretic Dil labeling**

Slices were fixed with PBS containing 4% PFA for 24 h. A glass micropipette (1 MΩ resistance) filled with the fluorescent carbocyanine dye 1,1'-diocctadecyl 3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) (0.5% in ethanol) was inserted into dentate hilus, and a single positive pulse (100 V, 10 s) was applied through the pipette. After 10 days of incubation in the same fixative at room temperature, the labeled MFs were observed by using the confocal imaging system MRC-1000 (BioRad) with a 20× objective (Nikon).

**Polysialic acid deletion by N-glycopeptidase F treatment**

Sixteen isolated AH slices were fixed with 4% PFA for 60 min at room temperature. They were rinsed six times with PBS for each 10 min so that PFA was completely washed out. The AH slices were treated with 0.05 mU/μl glycopeptidase F (Peptide: N-glycosidase F) (Takara, Tokyo, Japan) at 26 °C for 50 min. They were again rinsed six times with PBS for each 10 min. Eight of 16 AH slices were stained overnight at 4°C with primary mouse monoclonal antibody against PSA-NCAM (1:1000, MAB5324) (Chemicon, Temecula, CA), washed, and incubated with anti-mouse IgA+IgG+IgM FITC (1:500) (Sigma) for 2 h at room temperature. The remaining eight AH slices were cocultured with fresh DG slices for 10 DIV and then MFs were labeled by DiI. The samples were imaged with confocal system MRC-1000 (BioRad) with a 20× objective (Nikon).

**Assessment of cell death**

Cell death was assessed by uptake of propidium iodide (PI) (Molecular Probes). PI is a polar compound that only enters cells with damaged membranes and emits red fluorescence after binding to nucleic acids (Macklis and Madison, 1990). At DiV 5, the dye was added to culture medium at a final concentration of 10 μg/ml, and the cultures were...
kept at 37°C for 24 h. PI fluorescence images were obtained with the confocal system MRC-1000 (BioRad).

Astrocyte-conditioned medium

Glia cells were cultured in Eagle’s medium (Nissui Pharmaceuticals, Tokyo, Japan) containing 30 mM glucose, 2 mM glutamine, 1 mM pyruvate, and 10% fetal bovine serum (Sanko Jun-yaku, Tokyo, Japan). Astrocyte-conditioned medium was prepared from cultures of cortical astrocytes. Neonatal Sprague–Dawley rats (SLC) were deeply anesthetized with ether, and the cerebral cortex was dissected out and cut into pieces. After incubation with 0.25% trypsin (Difco, Detroit, MI) and 0.01% deoxyribonuclease I (DNase I) (Sigma) at 37°C for 40 min, the tissue was centrifuged at 1200 rpm for 5 min, and the pellet was resuspended in Eagle’s medium. The cells were mechanically dissociated by being passed 5–12 times through a 28-gauge needle and subsequently used for neuron culture as astrocyte-conditioned medium.

Dispersed culture of dentate granule cells

Unless otherwise specified, neurons were cultivated in Neurobasal (Life Technologies, Gaithersburg, MD) supplemented with 73 µg/ml l-glutamine and 2% B-27 supplement (Life Technologies). Six-day-old Sprague–Dawley rat pups (SLC) were deeply anesthetized with ether, the formatio hippocampalis was immediately removed, and the DG was isolated with extreme care before dissociation so that cultures contained neurons predominantly from this part of the hippocampal formation. Briefly, after isolation of the formatio hippocampalis, the subicular complex was removed along the sulcus hippocampi, and then the remaining part of the formatio hippocampalis was divided into two parts, that is, the DG and the AH. The DG was cut into pieces and treated with trypsin and DNase I at 37°C for 30 min. The incubation was terminated by addition of heat-inactivated horse serum (Cell Culture Lab). The tissue fragments were centrifuged at 1200 rpm for 5 min, the supernatant was removed, and the pellet was suspended in a mixture of 50% Neurobasal/B-27 and 50% astrocyte-conditioned medium. The suspension was gently triturated until visibly dispersed, followed by being filtered through nylon nets. We were able to obtain approximately 5.0 × 10^5 granule cells from one pup. The cells were plated at a density of 5.0 × 10^3 cells/cm^2 onto 13-mm culture dishes (Falcon) coated with either poly-L-lysine alone (Sigma) and cultivated at 37°C in a humidified 5% CO_2 and 95% air atmosphere. Collagen gels were prepared as described above. To prevent proliferation of glial cells, the culture medium was changed to the conditioned medium-free Neurobasal/B-27 medium or slice culture-conditioned medium supplemented with 2 µM cytosine a-D-arabinofuranoside (Sigma) 24 h after the plating. After 48 h, cultures were fixed with 4% PFA for 60 min at room temperature and subjected to immunostaining.

Slice culture-conditioned medium

Entorhino-hippocampal slices were prepared as described above. CA1 and CA3 regions were carefully separated by a small curved scalpel, and were cultured in the same way as entorhino-hippocampal slice cultures. Each eight slices from either CA1 or CA3 region were placed on sterile 30-mm-diameter membranes (Millicell-CM). The medium was changed at 3.5 DIV. At 7 DIV, culture media from either CA1 slice cultures (CA1-conditioned medium) or CA3 slice cultures (CA3-conditioned medium) were taken and immediately used as a medium for dispersed granule cells.

Immunofluorescence analysis and axon length measurement

Cultures were fixed and permeabilized with 0.1% Triton X-100. Nonspecific antibody binding was blocked by 60-min incubation with 1% goat serum at room temperature. They were stained overnight at 4°C with primary mouse monoclonal antibody against tau-1 (1:2000, MAB3420) (Chemicon) and rabbit polyclonal antibody against microtubules-associated protein-2 (MAP-2) (1:1000, AB5622) (Chemicon), and then incubated with anti-mouse IgG Alexa-488 (1:400) (A-11001, Molecular Probes), anti-rabbit IgG Alexa-546 (1:400) (A-11046, Molecular Probes) and rhodamine phalloidin (1:40) (R-415, Molecular Probes) for 2 h at room temperature. Samples were mounted on cover slips using a Vectashield medium (Vector, Burlingame, CA) and visualized with an AQUACOSMOS system (Hamamatsu Photonics, Hamamatsu, Japan). We defined the longest, tau-1-positive, and MAP-2-negative neurite as an axon and measured its length for analysis.

Results

Evidence for diffusible guidance cues for MFs

To address the possible involvement of diffusible factors in MF axon guidance, collagen gels (100 µm thickness) were interposed between DG and AH slices prepared from P6 GFP(+) rats and wild-type littermates, respectively (Fig. 1A). At DiV 10, the MF tract was detected as GFP signal in the cocultures that were counterstained with red fluorescent Nissl. Confocal observation revealed that massive MFs passed across the intervening collagen gels and reached
CA3 stratum lucidum and partly stratum oriens of the host AH slice (Figs. 1C, E, G), suggesting that diffusible factors guide MFs to the proper target area. We next grafted a GFP(+) DG microslice to an upside-down AH slice, in which case the DG was located ectopically facing the CA1 region of the host (Fig. 1B). Even after 10 DIV, no MFs were found in the collagen gels or the host (Figs. 1D, F, H).

To determine whether no MF entry into the CA1 region is caused by chemorepellents or merely due to a lack of chemoattractants, a GFP(+) DG microslice was directly attached to an upside-down AH slice without a collagen gel bridge (Fig. 2A). The CA1 did not allow the MFs to invade the host slice (Fig. 2D). This is in marked contrast with cocultures with cerebral cortex slices, in which case, the MFs were capable of invading cortical slices if no collagen gels were intervened (Fig. 2B). These data imply the existence of repellents in the CA1 region, but not in the cortex. Unlike the CA3 (Figs. 1G, 7), the cortex did not induce preferential MF ingrowth toward a particular area, and therefore, it does not appear to involve chemoattractants. Indeed, when collagen gels were sandwiched between GFP(+) DG and cortical slices, the MFs did not enter the gels (Fig. 2C). Collagen gels are likely to act as a physical hazard against the axon ingrowth, and MF axons may hence be unable to go into the gel unless chemoattractants exist in the host. Taken together, the cortex seems to be neutral in terms of MF guidance, which again suggests the presence of active MF guidance systems in the CA3 and CA1 substrata.

However, there still remains the possibility that the systems work simply by regulating MF elongation, rather than by determining MF pathfinding. To address this possibility, we isolated dentate granule cells in culture (Fig. 3A) and sought to determine whether or not diffusible molecules released from CA3 and CA1 tissues affect the extension rate of their axons. The cultured neurons were treated with CA3-conditioned or CA1-conditioned media (see Materials and
methods), but the mean length of their axons was unaffected (Fig. 3B). Thus, there appear to be no soluble factors that promote or prevent MF elongation.

Based on these observations, we concluded that the CA3 region produces chemoattractants for MFs whereas the CA1 region releases chemorepellents against MFs or contains nondiffusible molecules that repel MFs and/or prevent MF outgrowth.

To assess the effective range of the CA3 chemoattractants, we sandwiched collagen gels at 20–600 μm in thickness between DG and AH slices of P6 wild-type rats (Fig. 4). Because MF synaptic terminals contain a high concentration of Zn²⁺ (Henze et al., 2000; Ueno et al., 2002), their spatial distribution can be reliably assessed by Timm’s silver sulfide method, a histochemical technique, even in organotypic hippocampal cultures (Ikegaya, 1999; Ikegaya et al., 1997, 1998; Mizuhashi et al., 2001).

At DiV 10, the cocultures with collagen gels were subjected to Timm staining for quantification of MF synapses. Timm signal was detected predominantly in stratum lucidum and dentate hilus of intact slices (Fig. 4A). The MFs are known to run through the stratum oriens as well as lucidum (see Fig. 1G), yet the infrapyramidal Timm signal was faint (Fig. 4A and see also Ikegaya, 1999; Mizuhashi et al., 2001), probably due to a lower density of MF synapses in this subarea (Henze et al., 2000). In slices with collagen gels, the signal in stratum lucidum declined in proportion to the gap length (Figs. 4B, C). At >200 μm in thickness, the signal intensity was almost equivalent to the background level in AH slices that were maintained without DG grafts for 7 days in culture (Fig. 4C). Because these isolated AH slices did not receive MF innervation, the data indicate that MFs could not extend over >200 μm in collagen gels, suggesting that the diffusible factor forms a gradient in collagen gels and thereby attracts MFs towards CA3.

Evidence for contact guidance cues for MFs

Chemically fixed tissues cannot synthesize or secrete diffusible factors. Yamagata and Sanes (1995) have reported that lamina-specific arborization of retinal axons is formed even in chemically fixed tissues of the optic tectum, indicating that the branching is independent of diffusible factors released from the retinorecipient laminae. Likewise, Yamamoto et al. (2000a,b) have shown that thalamocortical axons develop their branches in PFA-fixed cortical slices, also suggesting that contact-dependent mechanisms are enough for the arborization. Thus, the technique of host fixation is useful in evaluating the involvement of diffusible factors. To further confirm that MFs are guided by diffusible cues, we employed the fixation strategy in our collagen gel cocultures (Fig. 5).

Acute AH slices were immersed in 4% PFA for 1 h at room temperature and then intensively washed. The fixed AH was juxtaposed to a fresh microslice of DG with intervening collagen gels (100 μm thickness) and main-
tained under normal culture conditions for 10 DiV. MFs were iontophoretically labeled with the fluorescent neurotracer DiI (Koyama et al., 2002, and see also Fig. 6A). No MFs were found to pass through collagen gels (Fig. 5A), again suggesting a requirement of diffusible factors derived from CA3. We next examined another pattern of coculture, in which a naive DG slice was apposed directly to a fixed AH slice without collagen gels (Fig. 5B). Interestingly, Dil-labeled MFs invaded the fixed host slice and normally grew into the CA3 stratum lucidum. The data imply that ascertainment factors, presumably present in the stratum lucidum, can guide the MFs. Taken together, MFs utilized at least two independent guidance mechanisms, that is, secretory-mediated and contact-mediated mechanisms.

Several nondiffusible molecules have been implicated in regulating MF development and synaptogenesis, including limbic system-associated membrane protein (Pimenta et al., 1995), PSA-NCAM (Cremer et al., 1997, 2000; Muller et al., 1994; Seki and Arai, 1999; Seki and Rutishauser, 1998), nectin/afadin (Mizoguchi et al., 2002), laminin γ1 (Grimpe et al., 2002), and proteolytic processes by tissue plasminogen activator (Baranes et al., 1998; Salles and Strickland, 2002; Wu et al., 2000). Although the present study alone cannot determine which of them is most responsible for MF growth, we attached importance to the histological characteristics of MFs, that is, MFs fasciculate with each other (Henze et al., 2000), and hypothesized that newly forming MFs can find their trajectories by fasciculating with pre-established MFs. This idea is consistent with our previous finding that no MFs invade a fixed AH slice when the DG is explanted ectopically to the exterior edge of host CA3 stratum oriens; this topographic dislocation forced the MFs to cross the alveus to reach their proper target area without tracing pre-established MF trajectories (Mizuhashi et al., 2001).

To address our hypothesis, we established a new series of coculture experiments by using a combination of chemical fixation and MF denervation (Fig. 6). If the contact-dependent MF outgrowth is mediated by fasciculation, the denervation of existent MFs is expected to hinder the subsequent ingrowth of novice MFs. We already confirmed that 7-DiV cultivation of isolated AH slices is enough for deafferentation of the MFs (Fig. 4C, open squares). In an intact slice, Dil-labeled MFs normally elongated into CA3 stratum lucidum and oriens (Fig. 6A). The normal pattern of MF innervation developed when a DG slice was grafted to an isolated AH slice, that is, a MF-denervated slice (Fig. 6B), in which case diffusible factors were still active. To exclude the contribution of the diffusible cues, a MF-denervated AH slice was fixed with PFA and then cocultured with a fresh DG slice. In this case, MFs failed to enter the host (Fig. 6C). As comparable controls, MF-containing AH slices were also prepared; a whole entorhino-hippocampal slice was cultivated for 7 DiV and fixed with PFA. When the AH was microdissected from this slice and cocultured with a fresh DG slice, the MFs projected normally to CA3 stratum lucidum and oriens (Fig. 6D).

Fig. 5. Contact factors alone can guide MFs to stratum lucidum. (A, B) A naïve DG slice of P6 rats was transplanted to PFA-fixed AH slices with (A) or without (B) 100-µm-thick collagen gels as shown in the left schematic diagrams. At DiV 10, the co-cultures were again fixed with PFA, and Dil was iontophoretically injected into the dentate hilus (DH) to visualize the MFs. Confocal Dil images were taken from the boxed regions of the diagrams, containing stratum radiatum (SR), lucidum (SL), pyramidale (SP), and oriens (SO) of CA3. The area between the solid lines in A indicates the sandwiched collagen gels. The solid line in B indicates the boundary between cocultures. The MFs did not innervate the host AH slice through the interposed gels, whereas Dil-labeled MFs (arrow) crossed over the border between the cocultures and reached their proper target stratum lucidum. (C) A PFA-fixed AH slice was treated with N-glycosidase F and juxtaposed to a fresh DG. No MFs entered the host AH slice, suggesting that the contact-mediated guidance of MFs depends on N-linked glycoprotein. Similar results were obtained in every such experiment conducted (each 16 cocultures from four independent experiments). (D) Confocal images of anti-PSA NCAM immunostaining of slices treated with (right) or without (left) N-glycosidase F. No apparent signal of MFs was observed in the stratum lucidum of N-glycopeptidase-treated slices.
Quasi-quantification of Dil-labeled fibers revealed that the MFs growing into denervated (Fig. 6B) or fixed slices (Fig. 6D) were fewer in amount than in normal slices (Table 1) (though the data of denervated slices were not statistically significant). Unfortunately, we could not further quantify the MFs because Timm staining, a method more quantitative than Dil labeling, was invalid for the fixed tissues, resulting in unexpected black-lacquering throughout fixed tissues (data not shown).

The only difference between Figs. 6C and D is whether extant MFs were present or absent in the host AH. To further clarify the correlation of pre-established and newly developing MFs, we tried to separately visualize these fibers (Fig. 7). At DiV 0, the DG of a wild-type entorhino-hippocampal slice was replaced with GFP(+) DG. The coculture was maintained for 7 DiV to allow innervation of GFP(+) MFs. Then, the AH, which was assumed to contain intact MFs, was dissected out. When a fresh DG explant was grafted, the MFs ran normally through stratum lucidum and oriens of the fixed AH. Experiments were repeated with 16–32 different cocultures (4–11 independent experiments), producing similar results.
wild-type DG graft. After another 10 DiV, DiI was injected into the dentate hilus of the grafts to label newly developed MFs (Fig. 7A). The pre-established MFs originating from the GFP(+) DG explant were well preserved in their appropriate position, that is, stratum lucidum, in the fixed AH for 17 DiV (Fig. 7B). The DiI-labeled MFs arising from the retrofitted DG traveled within the same stratum lucidum and did not go out of the GFP-positive MF orbit (Figs. 7C, D). Thus, the pre-existing MFs may behave like a railway to be traced by subsequent MFs via fasciculation.

Axon fasciculation is often achieved by glycoprotein-mediated cell adhesion. One molecular support for MF fasciculation is PSA-NCAM. We therefore examined the effect of deglycosylation on MF outgrowth in chemically fixed slices. After PFA fixation, an AH slice was treated at 37°C for 10 h with 50 µU/ml N-peptidase F, which is reported to specifically cleave N-glycans (GlcNAc-Asn bonds) in glycopeptides or glycoproteins (Cole et al., 1988; Green et al., 1988; Plummer et al., 1984; Zipser and Cole, 1991). Slices treated with the enzyme displayed no apparent immunosignal for PSA-NCAM in stratum lucidum (Fig. 5D). They were cocultured with fresh DG slices, and MFs were labeled with DiI at DiV 10. The fibers failed to enter the deglycosylated slices (Fig. 5C), which suggests a role of N-linked glycoprotein in contact-mediated guidance.

Developmental switch of guidance cues for MFs

Our results imply a role of axon fasciculation in MF pathfinding, but it remains unclear how the pioneer MFs find their pathway in the absence of pre-existing MFs. The massive formation of MFs begins at around 4 days of age and continues until the postnatal second week in rats.
(Amaral and Dent, 1981). Thus, very few MFs are formed in hippocampal slices prepared from P0 rats, while the MFs are almost mature at P13. A P6 DG slice was grafted to two symmetrically aligned AH slices, each of which was prepared from a P0 or P13 rat. DiI-labeled MFs projected normally to stratum lucidum in both the AH slices (Fig. 8A). Quasi-quantification of DiI fluorescence in the host stratum lucidum revealed a tendency of fewer MFs in P0 AH than in P13 AH (Table 1). This difference may be due to a lack of contact cues in P0 AH or a different amount of diffusible cues between P0 and P13. To eliminate the contribution of the diffusible factors, therefore, the host AH were both fixed with PFA and then cocultured with a fresh P6 DG slice. In this case, MFs extended into the P13 AH, but failed to invade the P0 AH (Fig. 8B, Table 1), which suggests that P0 MFs were guided solely by soluble molecules.

It is still possible, however, that levels of contact cues might be low at P0 so that the PFA fixation might disallow their efficacy. To confirm that diffusible cues actually work in P0 AH, we sandwiched collagen gels between a DG slice and an AH slice prepared from a P0, P6, or P13 rat. This experimental procedure did not include tissue fixation, and hence, the quantitative Timm method was available for analysis (Fig. 9). The thickness of collagen gels was adjusted to 50 μm, so that we could obtain strong Timm signals (see Fig. 4C). Even in the presence of collagen gels, the signals were always evident in the stratum lucidum of P0, P6, and P13 AH slices though they were weaker as compared to cocultures without gels (Fig. 9). The data imply that soluble cues are continuously active from the earliest stage of MF development. Interestingly, however, the degree of collagen gel-induced decrease in Timm signals was higher in P13 AH (Fig. 9), which may indicate that contact cue-mediated mechanism is dominant at later stages.

Although some reports may indicate that slices prepared from older animals are less healthy in culture, we are able to maintain P13 slices until 10 DiV without apparent cell loss (Fig. 10). Moreover, in all experiments except for DiI observations, we monitored the cell viability as assessed by PI uptake and found that neither collagen gel-sandwiched cultures nor cocultures with fixed slices induced neuronal death (data not shown). Therefore, we can exclude the possibility that the feeble ability of P13 slices to guide MFs by diffusible factors were due to the possible less viability of older tissues in culture.

Taken together, our results support the hypothesis that the MFs primarily sense soluble molecules for pathfinding at P0 while at P13, they are also guided by contact cues. This developmental switch of guidance cues may be determined by the degree of established MFs.

Fig. 8. Developmental switch of MF guidance cues from secreta-dependent to contact-dependent mechanisms. As shown in the insets, a fresh P6 DG slice was placed immediately adjacent to symmetrically arranged P0 and P13 AH slices without (A) or with PFA fixation (B). The MFs were labeled by Dil injection into dentate hilus (DH) at DiV 10, and confocal images containing stratum radiatum (SR), lucidum (SL), pyramidale (SP), and oriens (SO) of two AH slices were taken in the boxed areas in the insets. The MFs projected to the stratum lucidum of both fresh AH slices (arrows), whereas they extended into fixed P13 AH (arrow) but not into fixed P0 AH. Experiments were repeated with each 16 different cocultures (4–6 independent experiments), producing the same results.
Discussion

The lamina-specific MF formation provides an admirable opportunity to investigate CNS axon guidance because under normal conditions, MFs project accurately to the stratum lucidum and oriens after once converging in the dentate hilus. The highly stereotyped trajectories are not only retained in organotypic cultures but can also be reestablished after axotomy (Dailey et al., 1994; Ikegaya, 1999; Ikegaya et al., 1997, 1998; Kim et al., 2003; Mizuhashi et al., 2001; Nguyen et al., 1996; Zimmer and Gähwiler, 1987). Several studies have independently implicated the involvement of diffusible and contact factors in MF development and regeneration (Chen et al., 2000; Cremer et al., 1997; Grimpe et al., 2002; Koyama et al., 2002; Mizoguchi et al., 2002; Müller et al., 1994; Pimenta et al., 1995; Seki and Rutishauser, 1998). However, nothing is known about how these different guidance systems work in MF guidance. We have suggested that diffusible and contact cues can simultaneously, but independently, guide the MFs, and that their dominance shifts depending on development; early in postnatal development, the MFs are steered primarily by diffusible cues, but contact cues also participate at later stages.

Possible involvement of chemoattractants in lamina-specific MF innervation

Our collagen gel assay undoubtedly indicated the existence of soluble molecules. Because Timm intensity of stratum lucidum was decreased as the thickness of collagen gels was increased, the cue seems to act as an attractant, rather than a repellent, by establishing a concentration gradient. Importantly, we cannot exclude some possible experimental artifacts, that is, the experimental procedures or culture conditions might allow for abnormal diffusion of molecules that normally serve as substrate-bound molecules or are loosely attached to the extracellular matrix. For example, in vitro studies suggest that netrin is a diffusible
guidance cue, but under in vivo conditions, it seems to form a complex with its receptor Frazzled, thereby acting as a contact-dependent cue (Hiramoto et al., 2000). This possibility, however, does not disclaim our conclusion that specific guidance molecules for the MFs actually exist in the CA3, regardless of whether or not they are originally diffusible. We further believe that the molecules work as an attractant because MFs were preferentially guided into CA3 stratum lucidum whereas they displayed no preference of direction in cortical slices. Identification of the responsible molecule(s) is underway in our laboratory.

In cocultures with GFP(+) DG microslices, migrating cells were commonly found (Fig. 1A and see also Kim et al., 2003). In the present study, we did not quantify this type of migration. Such migrated cells appear unlikely to provide physical scaffolding for contact-dependent guidance because MFs could not elongate into fixed AH slices when collagen gels were intercalated (Fig. 5A).

Contact-dependent MF outgrowth

The fact that MFs are capable of growing into chemically fixed AH slices indicates that guidance cues, essential for contact-dependent outgrowth, are retained after PFA fixation and sufficient to establish precise MF networks. The denervation of MFs completely abolished the contact-mediated outgrowth, suggesting a critical requirement of the MFs themselves for MF guidance. Considering that the MFs are highly fasciculated axons (Henze et al., 2000), it is plausible that the fasciculation mediates contact-dependent MF guidance. At the same time, it is important to note that there is still another possibility, that is, MF denervation might induce secondary changes in the recipient CA3, thereby retarding normal MF growth. It is our impression that this possibility appears less feasible because MF guidance was also disturbed by treatment with N-glycopeptidase F.

The fasciculation of MFs is mediated, at least in part, by PSA-NCAM, an N-linked glycoprotein. NCAM is a member of the immunoglobulin superfamily and plays a role in cell–cell interactions via homophilic and heterophilic mechanisms. The adhesive capability of PSA-NCAM is kept intact even after PFA fixation (Yamamoto et al., 2000a). A prominent site of expression of PSA-NCAM is premature MFs, and its removal causes severe defasciculation and ectopic synaptogenesis of the MFs (Cremer et al., 1997, 2000; Muller et al., 1994; Seki and Arai, 1999; Seki and Rutishauser, 1998). Taken together with our result of N-glycosidase digestion, PSA-NCAM is a potent candidate for contact guidance cues.

Once the network is precisely formed, fasciculation-mediated guidance is probably the most reliable mechanisms because it can abolish the risk of allopatric projections by subsequently developing axons. We consider that fasciculation is also beneficial for rapid growth of the developing axons. The speed of MF reelongation after transection is as fast as tens of micrometers per hour (Dailey et al., 1994; Ikegaya et al., 2002). The fasciculation system may ensure such rapid MF outgrowth.

Developmental regulation of guidance cues for MFs

We have shown that there exist two different types of guidance cues to navigate the direction of MF growth. Interestingly, either of the mechanisms is sufficient to develop MF networks, being apparently functional redundancy. Such parallel pathways might help to compensate for a contingent deficiency of either cue. We do not believe, however, that either system is dispensable. Indeed, fewer MFs could be established in fixed or denervated AH slices than in intact AH tissues (see Fig. 6), suggesting that the cooperation of diffusible and contact factors is required for complete MF development. These two factors may be assigned discrete roles in MF network formation.

In this respect, it is intriguing to find that the MFs did not extend into chemically fixed AH slices of P0 rats but did into P13 AH. The findings suggest that secreta-mediated guidance is dominant during the initial stage of MF development. At P0, very few MFs develop yet (Amaral and Dent, 1981), and hence the shortage of established MFs may limit contact-dependent guidance. According to diffusible cues, the pioneer MFs lay a track. Once the MF track is established, later arriving MFs can trace it. The present study proposes, therefore, a novel paradigm in CNS axon guidance, that is, a developmental shift in the weight of two guidance modes.

Accumulating evidence indicates that the MFs are generated throughout adult life because of ongoing neurogenesis of DG granule cells (Altman and Das, 1965; Kaplan and Hinds, 1977) and also undergo continuous turnover over a period of weeks (Gould et al., 2001). Seki and Rutishauser (1998) demonstrated that neonatal exposure to PSA-specific endoneuraminidase induced a transient deficit of PSA-NCAM, causing ectopic MF outgrowth. In spite of the continuous MF replacement, surprisingly, this neonatal aberration is maintained until adulthood. Likewise, Pimenta et al. (1995) reported that functional blocking of limbic system-associated membrane protein in early postnatal rats’ results in aberrant MF growth, which is also retained until adulthood. Thus, the MF ectopia seems irreversible. Similar phenomena are observed in epilepsy. In epileptic hippocampus, the MFs display high-order structural plasticity, that is, aberrant sprouting into dentate molecular layer and CA3 stratum oriens (McNamara, 1994; Represa and Ben-Ari, 1992; Sutula et al., 1989), which may cause prolonged epileptogenesis by forming recurrent excitatory circuits. This pathological network reorganization also appears irrecoverable (Ikegaya et al., 2000). We consider that the irreversibility of ectopic MFs is accounted for by contact-dependent MF guidance; fasciculation with heterotropic MFs may misguide the axons of newly born granule cells. If this is the case, the pathogenic recurrent circuits could be recovered if we could prevent the fasciculation without affecting the secreta-mediated mechanisms. Therefore, our findings are not only of
fundamental importance to understand the mechanisms for CNS axon guidance but may also provide a basis for novel therapeutic targets against epilepsy-associated brain injury.

Acknowledgments

We are grateful to Dr. M. Okabe (Research Institute for Microbial Diseases, Osaka University, Suita, Japan) for providing GFP-transgenic rats and thank Dr. A. Tamada (Division of Behavior and Neurobiology, National Institute for Basic Biology, Okazaki, Japan) for his technical support of the collagen gel method. This work was supported in part by Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by the Research Grant for Longevity Science (13-2) from the Ministry of Health, Labor and Welfare of Japan.

References


