Environmental Control of the Survival and Differentiation of Dentate Granule Neurons

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Dentate granule cells (DGCs) and their microcircuits have been implicated in hippocampus-dependent memory encoding and epileptogenesis. Little is known about how the proper maturation of DGCs is determined by their intrinsic programs or external factors during development. In order to explore this, we dispersed premature DGCs on living hippocampal slices. Here we report that the survival and network formation of DGCs are supported by local cues present in the dentate gyrus *ex vivo*. The density of surviving DGCs was almost uniform throughout the host slices 12 h after implantation but gradually became heterogenous across substrata, with the cells engrafted onto the stratum granulosum scoring the highest rate of survival. The mossy fiber axons arising from DGCs growing on this substratum were properly guided towards CA3, whereas other misplaced DGCs exhibited heterotopic axon projection. In particular, about half of the axons originating from the hilus were misguided into the molecular layer, which resembles the supragranular mossy fiber sprouting seen in epileptic disorders. These results suggest that local environmental factors influence the cell adhesion, neurite polarization and axon guidance of DGCs.

Keywords: axon guidance, dentate gyrus, green fluorescent protein, hippocampus, mossy fiber, slice overlay assay

Introduction

Dentate granule cells (DGCs), one of the major types of excitatory neurons in the hippocampal formation, are densely packed in the stratum granulosum of the dentate gyrus (for a review, see Amaral and Witter, 1995), which plays roles in learning and memory as well as epileptogenesis. The development of the dentate gyrus is a multistep process (Altman and Bayer, 1990). The first phase is the migration of neuroepithelium-derived cells to the stratum granulosum to form and extend the skeleton of that layer. The second phase is the volumetric increase of the stratum granulosum during the infantile and juvenile periods due to the influx of large numbers of cells produced in the dentate hilus. The third phase is the neurogenesis of DGCs in the subgranular zone. The majority of DGCs, hence, are generated late — largely postnatally (Altman and Das, 1965; Kaplan and Hinds, 1977; Altman and Bayer, 1990). After migration to the stratum granulosum, the post-mitotic DGCs display an extremely polarized morphology; the axons extend toward the CA3 region through the hilus, whereas their dendrites are directed in the opposite direction, towards the molecular layer. The late development and simple morphology of DGCs could offer an ideal opportunity for studying morphogenesis of neurons, yet the mechanisms responsible for the maturation, survival or neuronal polarization are ill-defined.

In this study, we focused on elucidating whether polarization is triggered by external (local) stimuli or an intrinsic program. To what extent is cell location necessary or sufficient for proper neuritogenesis? And what are the consequences of misplacing cells within the dentate gyrus or hippocampus? This last question is of particular importance because epileptic activity causes DGCs to disperse ectopically from the stratum granulosum (Parent *et al.*, 1997; Scharfman *et al.*, 2000) and because these ectopic DGCs potentially contribute to epileptogenesis (Scharfman *et al.*, 2000; Dashtipour *et al.*, 2001).

In an effort to illustrate the cellular dynamics of DGCs, we have applied a 'cell-incorporated' organotypic culture system, which was originally introduced by Förster *et al.* (1998) and Polleux *et al.* (1998, 2000). This method allowed us to elucidate the behavior of DGCs and their axons at the single cell level under experimentally manipulated conditions and thereby clarify the impact of environment on maturation, survival, and axon guidance.

Materials and Methods

Organotypic Cultures of Hippocampal Slices

Hippocampal slice cultures were prepared from 6-day-old Sprague–Dawley rats, as described elsewhere (Ikegaya, 1999), in accordance with the Japanese Pharmacological Society guide for the care and use of laboratory animals. Briefly, brains were aseptically cut into 300 μm thick slices using a DTK-1500 vibratome (Dosaka, Kyoto, Japan). The entorhino-hippocampal cortex was dissected out under stereomicroscopic controls and maintained in 5% CO_2 at 37°C on a tissue culture insert (Millicell; Millipore, Bedford, MA) which was submerged in 1 ml of culture media consisting of 50% minimal essential medium (Invitrogen, Gaithersburg, MD), 25% horse serum (Cell Culture Lab, Cleveland, OH) and 25% Hanks' balanced salt solution. They were maintained for 4 days in culture (pre-culturing) and then underwent transplantation of DGCs expressing green fluorescent protein (GFP(+) DGCs).

Cell-incorporated Slice Cultures

DGCs were prepared from 3-day-old transgenic Sprague–Dawley rats expressing GFP (SLC, Shizuoka, Japan; Kim *et al.*, 2003), as previously described (Ikegaya *et al.*, 2000). Briefly, the hippocampal formation was dissected out and the dentate gyri were carefully isolated. The tissues were treated with trypsin and triturated into a single-cell suspension. The cells were resuspended in media at a density of 1.0×10^4 cells/ml (but 5.0×10^4 cells/ml for Fig. 1) before being plated onto living hippocampal slices that had been pre-cultivated for 4 days. The cell suspension (50–100 μl) was dropped onto each slice with extreme care to ensure that the cells stayed on the slice. The resulting cultures routinely contained >75% DGCs, but harbored a mixture of other neurons and glial cells; DGCs were identifiable by their smaller cell bodies as compared to Ammon's horn pyramidal cells, astrocytes and fibroblasts (Baba *et al.*, 2003). DGCs express calbindin D_{28k} only after they reach maturity (Cameron *et al.*, 1993; Goodman *et al.*, 1993) and ~40% of rat DGCs develop around postnatal day 3 (Schlessinger *et al.*, 1975). In our cultures, ~50% of microtubule-associated protein-2

(MAP-2)-positive neurons in our preparation were immunopositive for calbindin D_{28k} which indicates that many of these neurons were probably DGCs (Baba *et al.*, 2003).

Immunofluorescence Analysis

Four days after the transplantation, cultures were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate-buffered solution and permeabilized with 0.1% Triton X-100. Nonspecific antibody binding was blocked by 60 min incubation with 1% goat serum at room temperature. Tissue was stained overnight at 4°C with primary mouse monoclonal antibody against MAP-2 (1:1000, MAB378; Chemicon, Temecula, CA), S-100 β-subunit (1:1000, S2532; Sigma, St Louis, MO) or polysialylated neural cell adhesion molecule (PSA-NCAM; 1:3000, MAB5324; Chemicon). It was then washed and incubated with anti-mouse IgG Alexa-594 (1:200, A-11032; Molecular Probes, Eugene, OR) or anti-mouse IgG+IgM rhodamine (1:500, 03-18-09; KPL, Gaithersburg, MD), respectively, for 2 h at room temperature. Samples were mounted on coverslips using Vectasheild medium (Vector, Burlingame, CA) and digitally imaged using a Nikon TE300 Eclipse microscope with an MRC-1000 confocal microscope (BioRad, Cambridge, MA).

Bromodeoxyuridine Uptake Assays

Ten micromolar bromodeoxyuridine (BrdU; B-5002, Sigma) was included in the culture medium at the time of cell transplantation. The cultures were kept in the continuous presence of BrdU for 4 days. This concentration of BrdU is able to detect proliferation in organotypic slice cultures (Lavdas *et al.*, 1997). Cultures were fixed 4 days later and processed for BrdU immunocytochemistry using a primary mouse monoclonal BrdU antibody (1:1000, 4°C overnight, No. 1170376; Roche, Indianapolis, IN) and a secondary anti-mouse IgG Alexa-594 (1:200, A-11032; Molecular Probes).

Cell Survival Assays

Unless otherwise specified, the cocultures were fixed with 4% paraformaldehyde 4 days after transplantation and incubated with NeuroTrace fluorescent Nissl (1:100, N-21482; Molecular Probes) for 3 h at room temperature, and the morphology of GFP(+) DGCs was observed with a MRC-1000 confocal imaging system (BioRad). Following digital photography of both Nissl and GFP fluorescence and transmitted-light images, GFP(+) cells were counted in each substratum: CA1 stratum oriens, CA1 stratum pyramidale, CA1 stratum radiatum, CA1 and CA3 stratum lacunosum-moleculare, CA3 stratum oriens, CA3 stratum pyramidale, CA3 stratum lucidum, CA3 stratum radiatum, dentate hilus, stratum granulosum, or dentate molecular layer (see the schematic draw in Fig. 2*B*). The area (in mm^2) of each subarea was obtained with Laser Sharp Processing software (BioRad). The locations of the stratum pyramidale and granulosum were easily identified because of the high density of pyramidal cells and DGCs in those areas, respectively. The stratum oriens was identified as the portion adjacent to the stratum pyramidale. The stratum lucidum was the band-like area (~70 μm of width) that was located adjacent to the stratum pyramidale and slightly transparent under transmitted-light microscopic observations because of the high density of giant mossy fiber synapses. The CA1 stratum radiatum was the region adjacent to the stratum pyramidale, with a width of ~200 μm, while the CA3 stratum radiatum was the region adjacent to the stratum lucidum, the width ranging from 150 to 180 μm. The stratum lacunosum-moleculare was the area adjacent to the stratum radiatum that often appeared to be slightly dark in transmitted-light images. Because the border between CA1 and CA3 was usually ambiguous, we did not separate the stratum lacunosum-moleculare into CA1 and CA3 potions. The cell density (cells/mm^2), defined as the cell number divided by the area of each substratum, was calculated and averaged across experiments.

Scoring of Axon Orientation

Three days after transplantation, the axons of GFP(+) cells were digitally imaged using 20× objectives and analysed using Laser Sharp Processing software (Biorad). Based on their orientation, the axons were scored as being directed upward or downward relative to a tangent line to the curvature of either the stratum granulosum or pyramidale and through the cell soma. This 180° segmentation of orientation was shown to be optimal for detecting significant preference in axon direction in our experimental conditions (Mizuhashi *et al.*, 2001). Differences of measured axon orientation from chance (50%) were judged by χ^2 test.

Results

DGCs were prepared from postnatal day 3 GFP(+) rat pups and dispersed over pre-cultured entorhino-hippocampal slices. After 4 days, we could easily find GFP(+) cells surviving on the host slices (Figs 1*A* and 2*A*). The vast majority of these grafted cells (>95%) were immunopositive for the neuronal marker MAP-2 but not the glial marker S-100 (Fig. 1*A*,*B*) and were also morphologically identifiable as 'neurons' by the fact that they developed characteristic neuronal-like processes: two morphologically distinct types of neurite, i.e. each cell usually had a single thin, long process and several thick, relatively shorter ones. The latter displayed an evident immunoreactivity for MAP-2 and usually bore multiple branches at the proximal segment (Fig. 1*A*). These features are hallmarks of dendrites. On the other hand, the single, thinner process was always immunonegative for MAP-2 (Fig. 1*A*) and, thus, presumably an axon. In addition, 94.5% of 1219 neuron-like GFP(+) cells that were collected from four independent experiments displayed immunoreactivity for PSA-NCAM. Although PSA-NCAM is not a perfect marker of DGCs, it is relatively specific for DGCs, especially at this early stage of development (Seki and Arai, 1997). Most of MAP-2-negative and S-100-positive GFP(+) cells were distributed at the edge of the slices, perhaps because of their ability to migrate (Fig. 1*B*).

Immediately after transplantation, GFP(+) cells were distributed evenly across the surface of the host slice, the average density being ~300–500 cells/mm^2 (Fig. 2*C*). After 4 days, however, the distribution of GFP(+) cells was not uniform across subregions (Fig. 2*A*,*B*). The number of cells adhering to the dentate gyrus was significantly greater than that in the Ammon's horn ($P < 0.05$, Tukey's multiple range test after one-way ANOVA). In particular, the DGCs that were attached onto the host stratum granulosum exhibited the highest survival rate (Fig. 2*B*,*C*). To determine whether the larger number of GFP(+) cells in the dentate area was due to proliferation after the transplantation, we examined whether or not these cells showed BrdU uptake activity. No BrdU-positive GFP(+) neurons were found over the slices (Fig. 1*C*), suggesting that no new GFP(+) neurons were produced in our cultures.

Bioelectrical activity during development may regulate the total number of DGCs (Gould, 1994) and their morphology (Frotscher *et al.*, 2000), but we found no evidence that the survival rates at 4 days post DGC grafting were significantly changed by application of 1 μM tetrodotoxin or 50 μM picrotoxin (Fig. 2*D*), which were applied immediately after the grafting and continuously present for 4 days. Thus, the preferential survival in the dentate gyrus is independent of neuronal activity.

The *in vivo* DGCs project their 'mossy fiber' axons to CA3 pyramidal cells and hilar interneurons (for a review, see Henze *et al.*, 2000). We sought to determine the trajectories of GFP(+) mossy fibers, i.e. MAP-2-negative thin neurites (Figs 1*A* and 3*A*,*B*). For this orientation assay, GFP(+) cells were dispersed at a lower density of 1.0×10^4 cells/ml and observed 3 days later so that single GFP axons could be discriminated. Quantitative analyses indicated that 94.5% of DGCs growing on stratum granulosum gave rise to axons directed toward dentate hilus

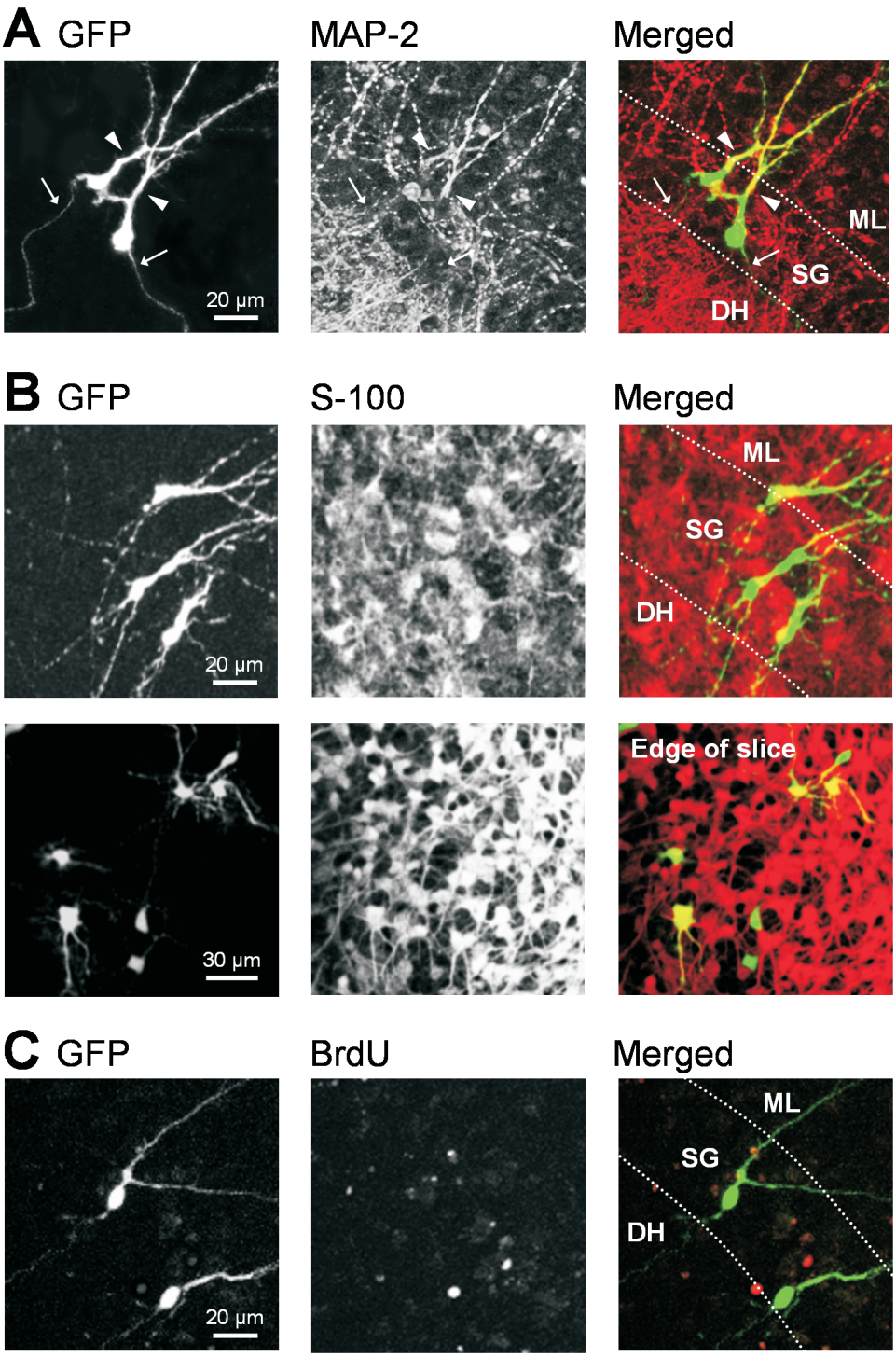

Figure 1. Dispersed GFP(+) neurons survive on organotypic cultures of slices. (*A*) Representative confocal images of GFP(+) DGCs transplanted on a hippocampal slice. Immunolabelling against MAP-2 was performed 3 days after grafting. Superimposition of double-labeled images (Merged) indicated that the thick and relatively short neurites (arrowheads) were MAP-2-positive dendrites. Thus, axons were morphologically identifiable as thin and long neurites (arrows). (*B*) Typical confocal images of GFP signal and S-100 immunosignal in the dentate gyrus (upper row) and at the edge of a host slice (lower row). The S-100 image of the lower row was obtained from the outermost portion of the slice and thus the density of cells in the left half was relatively low. This is not due to uneven lightning. Most GFP(+) cells transplanted on the slices were immunonegative to S-100, while S-100-positive GFP(+) cells were often found at the edge of cultured slices. Also, GFP(+) cells at the edge of slices usually had only short processes. (*C*) Confocal images of GFP signal and BrdU immunostaining in the dentate gyrus. No GFP(+) cells were immunopositive for BrdU. ML, molecular layer; DH, dentate hilus; SG, stratum granulosum.

and the remaining 5.6% directed axons toward the molecular layer (χ^2 test, $n = 163$, $P < 0.01$; Fig. 3*C*). The latter minor fraction might be ascribable to the axons of other neuron types than DGCs. This axon orientation was unaffected by continuous treatment with tetrodotoxin or picrotoxin (Fig. 5*A*). Interestingly, the axons of DGCs growing on CA1 and CA3 stratum

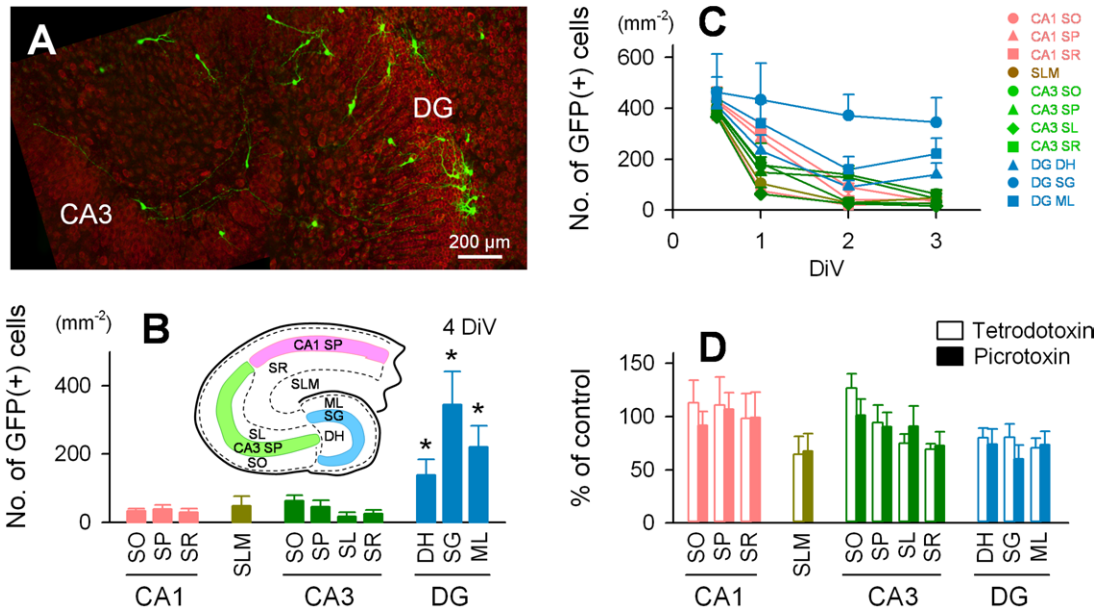

Figure 2. Favored DGC survival on the dentate area in cell-incorporated cultures. (*A*) Confocal image of a cultured entorhino-hippocampal slice on which GFP(+) DGCs survive unevenly. Four days after grafting, the culture was stained with a Nissl method with red-fluorescent probes. (*B*) The density of GFP(+) cells surviving on each hippocampal substratum was measured at day 4 *in vitro* (4 DiV). The subregions are depicted in the inset. The areas CA1 and CA3 include strata oriens (SO), pyramidale (SP), radiatum (SR), lacunosum-moleculare (SLM) and lucidum (SL). The dentate gyrus (DG) includes dentate hilus (DH), stratum granulosum (SG) and molecular layer (ML). The highest density was observed in the dentate gyrus. $^*P < 0.05$ versus any substrata other than three dentate subareas: Tukey's multiple range test after one-way ANOVA. Data are means ± SEM of 10 independent experiments (each 12–16 slices). (*C*) Changes in the density of GFP(+) neurons in each subarea. Note that the initial density was almost equal among the substrata. DiV, days *in vitro*. (*D*) No effect of neural activity on the survival rate of GFP(+) neurons at day 4. Tetrodotoxin (1 μM) or picrotoxin (50 μM) were chronically applied for 4 days following implantation of GFP(+) cells. The ordinate indicates the percentage of the number of surviving cells to that in control (without drugs) in each substratum.

apparently displayed no preference of orientation (χ^2 test, n = 55, $P > 0.1$; Fig. 3*D*), implying that an extracellular signal present in the stratum granulosum promotes polarized orientation of the DGC soma or axon.

We also examined the axon orientation of ectopic GFP(+) DGCs on the dentate gyrus and found that 85.5% of axons originating from DGCs in molecular layer stayed within the molecular layer and stratum granulosum but did not enter the dentate hilus (n = 62; Fig. 4*A*). By contrast, the axons of DGCs in the hilus were oriented towards CA3 in 42.7% of cases, while the remaining 57.3% were directed aberrantly towards the molecular layer (n = 103; Fig. 4*B*). The ratios of axon orientation were unchanged by tetrodotoxin or picrotoxin (Fig. 5).

Finally, we traced the axons of DGCs growing in the stratum lucidum. Most axons (93.8%) grew within the stratum lucidum, of which 53.3% were oriented towards CA1 and the remaining (46.7%) towards the dentate gyrus (n = 32; Fig. 6) – perhaps indicating non-oriented projections of the axons within the stratum lucidum.

Discussion

In spite of much evidence for the distinctive behaviors and potential pathogenic contributions of DGCs, the mechanisms supporting their survival and maturation are almost unknown. To evaluate the dynamic interactions between the intrinsic programs of DGCs and local environmental cues, we have established a cell-incorporated culture system, in which isolated GFP(+) DGCs are plated onto entorhino-hippocampal slices. The GFP signal in the GFP(+) rat line used here was sufficiently robust to allow us to trace the morphology of DGCs and their threadlike axons. Using this coculture system, we have shown for the first time that the survival and axon polarization of DGCs are regulated by local environmental cues.

Although DGCs undergo postnatal neurogenesis that persists into adulthood, the vast majority of DGCs are produced in the first 3 weeks after birth (Amaral and Dent, 1981). Because we prepared GFP(+) cells from P3 rats, our preparation could include post-mitotic DGCs as well as their progenitor cells. However, we did not find BrdU-positive GFP(+) cells in our cell-incorporated cultures. Therefore, the contribution of proliferation of DGC progenitors to our results is minimal.

The DGCs growing on the host stratum granulosum displayed a preference of axon orientation toward dentate hilus, whereas those misplaced onto CA1 or CA3 stratum pyramidale exhibited no preference of orientation even though they were located adjacent to CA3 stratum lucidum, a normal recipient site of mossy fibers. Apparently, the distance between the DGC soma and its usual target has no influence on axon polarization. Therefore, long-range diffusible factors derived from CA3 target cells are unlikely to mediate axon polarization, but, rather, local environmental cues in stratum granulosum are probably responsible for the proper morphogenesis of DGCs.

The same logic holds true for DGC axon guidance. About half of ectopic hilar DGCs did not project their axons to the adjacent target CA3 but instead projected to the further subregion, i.e. the inner molecular layer. This unusual target selection may again emphasize an important role of the soma location. Indeed, the hilus constitutes a normal mossy fiber route; none the less, half of the hilar DGCs were incapable of finding their normal course. One possible explanation for this apparent paradox is contact-dependent axon guidance. Mossy fibers

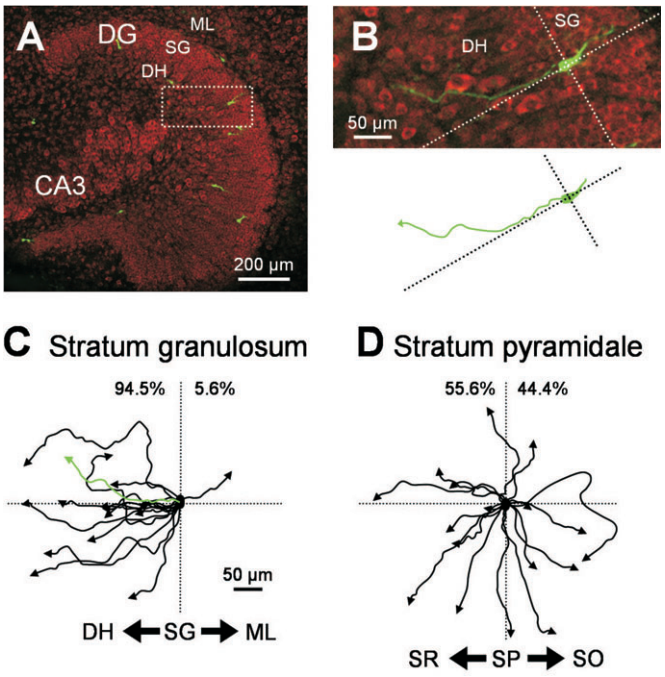

Figure 3. Proper axon orientation of DGCs engrafted on the stratum granulosum. (*A*) Confocal image of GFP(+) DGCs growing on a Nissl-stained slice culture. *B*, Image of the boxed region in *B* was taken at higher magnification (upper). The dotted lines indicate the tangential and perpendicular lines to the stratum granulosum at the soma of the GFP(+) DGC. This neuron was reconstructed with camera lucida drawings as 1-bit resolution (lower). The axon course is indicated by the curved arrow. (*C*, *D*) Three days after grafting, GFP(+) cells bearing axons >50 μm in length, with the somata situated within stratum granulosum (*C*) and CA1 and CA3 stratum pyramidale (*D*), were selected for analyzing their axon morphology. The curved arrows indicate axon traces. The green line is the same axon in *B*. DGCs growing over the stratum granulosum possessed axons oriented toward dentate hilus (94.5%, χ^2 test, $n = 163$, $P < 0.01$), whereas those over the stratum pyramidale exhibited an expected ratio for random orientation (55.6%, χ^2 test, $n = 55$, $P > 0.1$). To discriminate an axon trajectory from others, we illustrated the traces of representative 15 and 17 DGCs that were randomly selected from six different experiments (each experiment involves four to eight slices, each slice involving 1–11 cells). Variation of results between experiments was <5.3% and are statistically negligible. The summary data appear in Figure 5*A*, *B*. DH, dentate hilus; SG, stratum granulosum; ML, dentate molecular layer; SR, stratum radiatum; SP, stratum pyramidale; SO, stratum oriens.

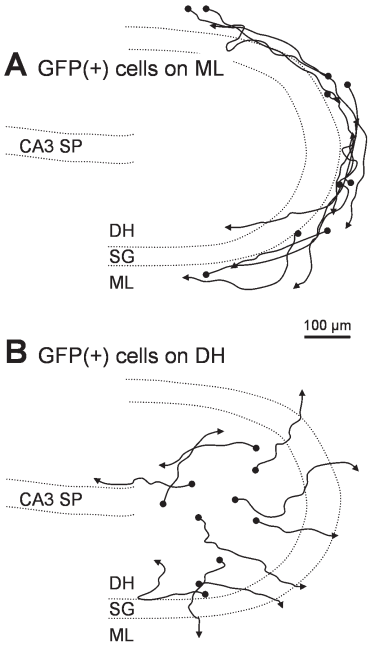

Figure 4. Abnormal axon direction of DGCs growing on molecular layer (*A*) or dentate hilus (*B*) was analyzed 3 days after transplantation. The positions of DGC somata are indicated by the small black circles, and their axon routes are shown as the curved arrows. Fifty-three out of 62 DGCs (85.5%) on molecular layer project the axons within the molecular layer, whereas 55 of 103 hilar DGCs (53.2%) extended their axons toward the molecular layer. The plots indicate representative 10 axons that were randomly selected from five different experiments (each experiment involves three to eight slices, each slice involving two to six cells). Data are summarized in Figure 5*C*, *D*. DH, dentate hilus; SG, stratum granulosum; ML, molecular layer; SP, stratum pyramidale.

appear to form a 'cohesive' bundle (Nowakowski and Davis, 1985; Drakew *et al.*, 2002) and thereby, premature DGCs can trace the axons of previously generated cells (Koyama *et al.*, 2004). If this is the case, when DGCs are deposited on the hilus, they would follow the bundle, but by chance, half of them are guided in the correct direction towards CA3 and the remaining in the wrong direction away from CA3. This explanation can not only account for the results of Figure 4*B*, but also is consistent with the phenomenon seen in Figure 6. Like hilar DGCs, DGCs in stratum lucidum extended their axons towards either CA1 or DG, the ratio of orientation being nearly at the chance level (50%). Interestingly, the data in Figure 6 contrast with our previous study, which showed that the mossy fibers of DGCs growing in CA3 stratum radiatum preferentially extended into stratum lucidum, but soon halted therein without running horizontally within the stratum lucidum (Mizuhashi *et al.*, 2001). In this study, we removed the dentate gyri from the host hippocampal slices 7 days before DGC implantation. This procedure may have led to denervation of previously established mossy fibers, in which case the implanted DGCs could not trace a cohesive bundle. The cohesive bundle hypothesis, therefore, is consistent with all the phenomena we have observed so far.

The malpositioning of DGCs in the present study is quite artificial, but similar ectopia of DGCs is often found in mutant mice and in human diseases. For example, in reeler mice, which are mutant for reelin, a serine protease secreted by Cajal–Retzius cells (Quattrocchi *et al.*, 2002), DGCs are not arranged in a densely packed layer and the width of the granule cell layer becomes greater, a phenomenon termed granule cell dispersion (Stanfield and Cowan, 1979). In this mutant, DGCs are occasionally distributed over the hilar region (Drakew *et al.*, 2002). Similar malformation of the dentate gyrus has been reported in other mutants, e.g. scrambler (Sweet *et al.*, 1996) or mice lacking the following genes: apolipoprotein E receptor 2 (Trommsdorff *et al.*, 1999), mouse disabled1 (Howell *et al.*, 1997), p35 (Wenzel *et al.*, 2001) and very low density lipoprotein receptor (Trommsdorff *et al.*, 1999).

Clinical evidence demonstrates that granule cell dispersion is also observed in temporal lobe epilepsy patients (Houser, 1990; Armstrong, 1993; Rakic and Caviness, 1995; El Bahh *et al.*, 1999). In addition, newly born DGCs are likely to migrate from their site of origin into the hilus and inner molecular layer after pilocarpine-induced status epilepticus (Parent *et al.*, 1997; Scharfman *et al.*, 2000). This abnormal migration of neurons may be attributable to deficiency in reelin expression (Haas *et al.*, 2002; Frotscher *et al.*, 2003). An abnormal feature in epileptic hippocampus is aberrant sprouting of DGC axons, which gives rise to their ectopic innervation into the inner

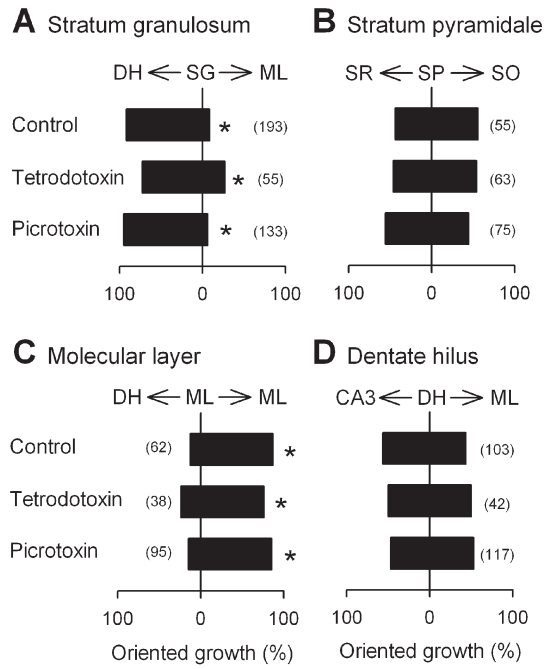

Figure 5. Lack of the effect of neural activity on axon orientation of DGCs growing on the stratum granulosum (*A*), stratum pyramidale (*B*), molecular layer (*C*) and dentate hilus (*D*). DGCs were cultured on hippocampal slices in the presence of 1 μM tetrodotoxin or 50 μM picrotoxin for 3 days after implantation. The numbers in parentheses indicate the number of cells analyzed. No statistical significance was observed in any substrata (χ^2 test, $P > 0.05$). *$P < 0.05$ versus the chance level (50%), χ^2 test. DH, dentate hilus; SG, stratum granulosum; ML, dentate molecular layer; SR, stratum radiatum; SP, stratum pyramidale; SO, stratum oriens.

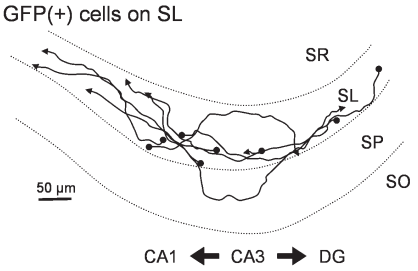

Figure 6. Bidirectional axon extension in stratum lucidum. Axon orientation of granule cells growing on the stratum lucidum was analyzed 3 days after transplantation. The curved arrows indicate axon traces. The plots indicate eight representative axons that were randomly selected from three different experiments. Most of axons grew within stratum lucidum, but showed no statistical preference of orientation toward CA1 or DG (χ^2 test, $n = 32$, $P > 0.1$). SR, stratum radiatum; SL, stratum lucidum; SP, stratum pyramidale; SO, stratum oriens; DG, dentate gyrus.

molecular layer (McNamara, 1994). Although there is a large body of evidence for sprouting of supragranular mossy fibers in the absence of DGC malpositioning (Laurberg and Zimmer, 1981; Frotscher and Zimmer, 1983), some sprouted axons arise from ectopic DGCs (Scharfman *et al.*, 2000). These axons may be related to prolonged epileptogenesis because the hilar DGCs exhibit abnormal, pro-epileptogenic burst firing probably via less inhibitory afferents (Dashtipour *et al.*, 2001). It has not yet been determined, however, whether the aberrant sprouting results from ectopic soma localization or paroxysmal activity itself. To this point, our findings have shown that DGC malpositioning lead to supragranular innervations even in the presence of tetrodotoxin, suggesting that cell misplacement is causal. Interestingly, hilar DGC axons easily grew into the molecular layer whereas supragranular axons failed to grow out of the molecular layer (Fig. 4). Thus, the stratum granulosum seems to behave like a one-way barrier to mossy fiber outgrowth. This biased outgrowth could underlie the pathologic sprouting.

Survival rate of grafted DGCs was particularly great in the stratum granulosum. Although we could not determine whether this subregion produces growth factors supporting DGC survival or chemoattractants for migration or instead merely offers better scaffolding for cell attachment, it is intriguing that the place suitable to survival also plays a crucial role for proper morphogenesis. Proper maturation may be required for subsequent survival and so it is possible that the different behaviors we observed in different locations are merely due to the genesis of different cell types in each location, in spite of the fact that our cultures predominantly contained DGCs.

In summary, the present study was designed to investigate the behavior of DGCs in relation to the hippocampal environment and has shown that survival, cell polarization and axon guidance depend on where the cell bodies are. The cell-incorporated culture system has also been demonstrated to be highly amenable to the direct study of the maturation and network incorporation of individual neurons under specific circumstances.

Notes

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References

Altman J, Bayer SA (1990) Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods. J Comp Neurol 301:365–381.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319–335.

Amaral DG, Dent JA (1981) Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. J Comp Neurol 195:51–86.

Amaral DG, Witter MP (1995) Hippocampal formation. In: The rat nervous system (Paxinos G, ed.), pp. 443–492. San Diego, CA: Academic Press.

Armstrong DD (1993) The neuropathology of temporal lobe epilepsy. J Neuropathol Exp Neurol 52:433–443.

Baba A, Yamada MK, Nishiyama N, Matsuki N, Ikegaya Y (2003) Different Ca^{2+} dynamics between isolated hippocampal pyramidal cells and dentate granule cells. J Neurocytol 31:41–48.

Cameron HA, Woolley CS, Mcewen BS, Gould E (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56:337–344.

Dashtipour K, Tran PH, Okazaki MM, Nadler JV, Ribak CE (2001) Ultrastructural features and synaptic connections of hilar ectopic granule cells in the rat dentate gyrus are different from those of granule cells in the granule cell layer. Brain Res 890:261–271.

Drakew A, Deller T, Heimrich B, Gebhardt C, Del Turco D, Tielsch A, Forster E, Herz J, Frotscher M (2002) Dentate granule cells in

reeler mutants and VLDLR and ApoER2 knockout mice. Exp Neurol 176:12–24

El Bahh B, Lespinet V, Lurton D, Coussemacq M, Le Gal La Salle G, Rougier A (1999) Correlation between granule cell dispersion, mossy fiber sprouting, and hippocampal cell loss in temporal lobe epilepsy. Epilepsia 40:1393–1401.

Förster E, Kaltschmidt C, Deng J, Cremer H, Deller T, Frotscher M (1998) Lamina-specific cell adhesion on living slices of hippocampus. Development 125:3399–3410.

Frotscher M, Zimmer J (1983) Lesion-induced mossy fibers to the molecular layer of the rat fascia dentata: identification of postsynaptic granule cells by the Golgi-EM technique. J Comp Neurol 215:299–311.

Frotscher M, Drakew A, Heimrich B (2000) Role of afferent innervation and neuronal activity in dendritic development and spine maturation of fascia dentata granule cells. Cereb Cortex 10:946–951.

Frotscher M, Haas CA, Forster E (2003) Reelin controls granule cell migration in the dentate gyrus by acting on the radial glial scaffold. Cereb Cortex 13:634–640.

Goodman JH, Wasterlain CG, Massarweh WF, Dean E, Sollas AL, Sloviter RS (1993) Calbindin-D28k immunoreactivity and selective vulnerability to ischemia in the dentate gyrus of the developing rat. Brain Res 606:309–314.

Gould E (1994) The effects of adrenal steroids and excitatory input on neuronal birth and survival. Ann N Y Acad Sci 743:73–92.

Haas CA, Dudeck O, Kirsch M, Huszka C, Kann G, Pollak S, Zentner J, Frotscher M (2002) Role for reelin in the development of granule cell dispersion in temporal lobe epilepsy. J Neurosci 22:5797–5802.

Henze DA, Urban NN, Barrionuevo G (2000) The multifarious hippocampal mossy fiber pathway: a review. Neuroscience 98:407–427.

Houser CR (1990) Granule cell dispersion in the dentate gyrus of humans with temporal lobe epilepsy. Brain Res 535:195–204.

Howell BW, Hawkes R, Soriano P, Cooper JA (1997) Neuronal position in the developing brain is regulated by mouse disabled-1. Nature 389:733–737.

Ikegaya Y (1999) Abnormal targeting of developing hippocampal mossy fibers after epileptiform activities via L-type Ca^{2+} channel activation *in vitro*. J Neurosci 19:802–812.

Ikegaya Y, Nishiyama N, Matsuki N (2000) L-type Ca^{2+} channel blocker inhibits mossy fiber sprouting and cognitive deficits following pilocarpine seizures in immature mice. Neuroscience 98:647–659.

Kaplan MS, Hinds JW (1977) Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092–1094.

Kim J-A, Yamada, MK, Nishiyama N, Matsuki N, Ikegaya Y (2003) Mossy fiber pathfinding in multilayer organotypic cultures of rat hippocampal slices. Cell Mol Neurobiol 23:115–119.

Koyama R, Yamada MK, Nishiyama N, Matsuki N, Ikegaya Y (2004) Developmental switch of axon guidance cues for hippocampal mossy fiber *in vitro*. Dev Biol 267:29–42.

Laurberg S, Zimmer J (1981) Lesion-induced sprouting of hippocampal mossy fiber collaterals to the fascia dentata in developing and adult rats. J Comp Neurol 200:433–459.

Lavdas AA, Blue ME, Lincoln J, Parnavelas JG (1997) Serotonin promotes the differentiation of glutamate neurons in organotypic slice cultures of the developing cerebral cortex. J Neurosci 17:7872–7880.

McNamara JO (1994) Cellular and molecular basis of epilepsy. J Neurosci 14:3413–3425.

Mizuhashi S, Nishiyama N, Matsuki N, Ikegaya Y (2001) Cyclic nucleotide-mediated regulation of hippocampal mossy fiber development: a target-specific guidance. J Neurosci 21:6181–6194.

Nowakowski RS, Davis TL (1985) Dendritic arbors and dendritic excrescences of abnormally positioned neurons in area CA3c of mice carrying the mutation 'hippocampal lamination defect'. J Comp Neurol 239:267–275.

Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17:3727–3238.

Polleux F, Giger RJ, Ginty DD, Kolodkin AL, Ghosh A (1998) Patterning of cortical efferent projections by semaphorin–neuropilin interactions. Science 282:1904–1906.

Polleux F, Morrow T, Ghosh A (2000) Semaphorin 3A is a chemoattractant for cortical apical dendrites. Nature 404:567–573.

Quattrocchi CC, Wannenes F, Persico AM, Ciafre SA, D'Arcangelo G, Farace MG, Keller F (2002) Reelin is a serine protease of the extracellular matrix. J Biol Chem 277:303–309.

Rakic, P and Caviness, VS Jr (1995) Cortical development: view from neurological mutants two decades later. Neuron 14:1101–1104.

Scharfman HE, Goodman JH, Sollas AL (2000) Granule-like neurons at the hilar/ca3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. J Neurosci 20:6144–6158.

Schlessinger AR, Cowan WM, Gottlieb DI (1975) An autoradiographic study of the time of origin and the pattern of granule cell migration in the dentate gyrus of the rat. J Comp Neurol 159:149–175.

Seki T, Arai Y (1997) Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system. Neurosci Res 17:265–290.

Stanfield BB, Cowan WM (1979) The morphology of the hippocampus and dentate gyrus in normal and reeler mice. J Comp Neurol 185:393–422.

Sweet HO, Bronson RT, Johnson KR, Cook SA, Davisson MT (1996) Scrambler, a new neurological mutation of the mouse with abnormalities of neuronal migration. Mamm Genome 7:798–802.

Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, Nimpf J, Hammer RE, Richardson JA, Herz J (1999) Reeler/disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell 97:689–701.

Wenzel HJ, Robbins CA, Tsai LH, Schwartzkroin PA (2001) Abnormal morphological and functional organization of the hippocampus in a p35 mutant model of cortical dysplasia associated with spontaneous seizures. J Neurosci 21:983–998.