Reconstitution of the hippocampal mossy fiber and associational-commissural pathways in a novel dissociated cell culture system

(dentate gyrus/CA3 region/presynaptic terminals/spines)

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Contributed by Eric R. Kandel, December 20, 1995

ABSTRACT Synapses of the hippocampal mossy fiber pathway exhibit several characteristic features, including a unique form of long-term potentiation that does not require activation of the N-methyl-D-aspartate receptor by glutamate, a complex postsynaptic architecture, and sprouting in response to seizures. However, these connections have proven difficult to study in hippocampal slices because of their relative paucity (<0.4%) compared to commissural-collateral synapses. To overcome this problem, we have developed a novel dissociated cell culture system in which we have enriched mossy fiber synapses by increasing the ratio of granule-to- pyramidal cells. As in vivo, mossy fiber connections are composed of large dynorphin A-positive varicosities contacting complex spines (but without a restricted localization). The elementary synaptic connections are glutamatergic, inhibited by dynorphin A, and exhibit N-methyl-D-aspartate-independent long-term potentiation. Thus, the simplicity and experimental accessibility of this enriched in vitro mossy fiber pathway provides a new perspective for studying nonassociative plasticity in the mammalian central nervous system.

Several features of the hippocampal mossy fiber (MF) pathway are interesting for studying synaptic plasticity (1). First, repeated activation of MFs results in long-term potentiation (LTP) in the CA3 subfield (2) that does not require activation of the N-methyl-D-aspartate (NMDA)-type glutamate receptor. Second, MFs are capable of extensive sprouting following epilepsy (3) and contribute to the susceptibility of CA3 neurons to epileptic seizures (4, 5). Finally, the en passant connections established by MF with denticulate spines of CA3 pyramidal cells have unique morphological characteristics. The presynaptic endings of the MF axons are among the largest in the brain, and the postsynaptic target of the MFs often take the form of giant, multi-invagination spines termed thorny exocytoses, making these connections readily identifiable (1, 6, 7).

Study of the MF pathway in hippocampal slices has been limited by the complex circuitry of the CA3 region and the extremely low incidence of MF synapses (1, 2). Here we describe a dissociated cell culture system where mossy fiber plasticity can be characterized at the level of individual neurons and elementary synaptic connections. In culture, the MF connections retain the major morphological and immunocytochemical characteristics of the MF pathway in vivo. Thus, this reduced preparation provides an experimentally accessible, in vitro model of the MF pathway that allows the study of its cellular and molecular properties and plastic capabilities at the level of individual, identified synapses. In a companion study, López-García et al. (8) have further demonstrated that the in vitro granule-to-pyramidal connection exhibits NMDA-independent LTP similar to that evident in vivo.

MATERIALS AND METHODS

Cell Culture. Hippocampi of P1-P5 rat pups were split longitudinally along the midportion of the CA3 region (see Fig. 1 A). Tissue was treated for 30 min at 37°C with 0.25% trypsin (type XI; Sigma), and then gently triturated and the dissociated cells either plated at a concentration of 2 × 10^5/ml onto poly-D-lysine (20 μg/ml; Sigma) and laminin (10 μg/ml; Collaborative Research)-coated glass coverslips or sedimented through a Percoll gradient before plating as described (9).

Cells were plated in Eagle’s minimal essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (HyClone), 2 mM glutamine, and 0.76% glucose. On the following day, the medium was replaced with fresh SF1 medium (10), including B-27 supplements (GIBCO). The cells were grown for up to 12 weeks in a humid incubator containing 5% CO_2/95% air at 37°C.

Cell Type-Specific Labeling. Cells were intracellularly injected with 6% carboxyfluorescein by using sharp electrodes (70 MΩ resistance). Impaled neurons were allowed to fill by diffusion for ~2–3 min.

The dentate gyrus area was labeled following its selective dissection. Cells were concentrated to 1 dentate gyrus/0.5 ml and incubated for 1 h with 40 μM Dil (Sigma) in plating medium, washed, and plated at 500 cells/cm^2.

To label CA3 cells, microspheres (0.2 μl; Lumafluor, New York) were injected into the CA3 subfield of P0-P1 pups, 0.5 mm posterior to lambda and 2.5–3 mm lateral to the sagittal suture at a depth of 2–3 mm as described (11). After 48 h, the injected hippocampus was removed, fixed for 4 h with 4% paraformaldehyde, and incubated overnight at 4°C with 30% sucrose. Frozen sections were taken at 30 μm intervals in the coronal plane and examined with the fluorescence microscope. The contralateral hippocampus was coronally sliced, and cultures were prepared from those cases demonstrating retrograde transport to the CA3 subfield.

Immunocytochemistry. Cells were immunolabeled as described by Craig et al. (12). The mouse antibodies used in this study were monoclonal anti-MAP2 (5 μg/ml; Sigma) and monoclonal anti-synaptophysin (5 μg/ml; Boehringer Mannheim). The rat antibodies were α-MAPs (Sigma), α-dynorphin A (10 μg/ml; Chemicon), and α-glutamate receptor R1 (Glur1; provided by R. L. Huganir, Johns Hopkins University School of Medicine). For dynorphin A and GluR1 double labeling, cells were first incubated for 48 h with α-GluR1, labeled with secondary Cy3-conjugated antibody, fixed with 4% paraformaldehyde, and then labeled with α-dynorphin A.

Abbreviations: MF, mossy fiber; LTP, long-term potentiation; DG, dentate gyrus; NMDA, N-methyl-D-aspartate; mEPSC, miniature excitatory postsynaptic current.
Cl =

Table 1. Morphological and electrophysiological characteristics of granule and pyramidal neurons in dentate gyrus-CA3 culture

<table>
<thead>
<tr>
<th></th>
<th>Pyramidal, µm ± SEM</th>
<th>Granule, µm ± SEM</th>
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<tbody>
<tr>
<td>Width of cell body</td>
<td>28.5 ± 5.7</td>
<td>11.4 ± 0.9</td>
</tr>
<tr>
<td>Length of cell body</td>
<td>29.2 ± 2.5</td>
<td>13.4 ± 1.3</td>
</tr>
<tr>
<td>No. of apical dendrites</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No. of basal dendrites</td>
<td>2–6</td>
<td>0–1</td>
</tr>
<tr>
<td>Length, apical</td>
<td>194.0 ± 14.5</td>
<td>113 ± 8.0</td>
</tr>
<tr>
<td>Length, basal</td>
<td>107.6 ± 17.9</td>
<td>96.1 ± 8.9</td>
</tr>
<tr>
<td>Branching points Apical</td>
<td>3–5</td>
<td>0–3</td>
</tr>
<tr>
<td>Basal</td>
<td>0–3</td>
<td>0–2</td>
</tr>
<tr>
<td>Oblique distance from apical origin</td>
<td>37.3 ± 15.9</td>
<td>62.6 ± 13.7</td>
</tr>
<tr>
<td>Oblique distance from basal origin</td>
<td>38.5 ± 10.2</td>
<td>106 ± 22.7</td>
</tr>
<tr>
<td>Width of apical dendrite, origin</td>
<td>5.7 ± 1.3</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>Width of basal dendrite, origin</td>
<td>3.9 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Resting potential, mV</td>
<td>-49.77 ± 2.63</td>
<td>-61.69 ± 0.43</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>491.7 ± 58.3</td>
<td>658.3 ± 50.31</td>
</tr>
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Cells were photographed with an MRC-1000 laser confocal microscope after staining with α-MAP2. Measurements of the width were taken from phase contrast pictures of identified neurons. Shown are the mean ± SEM of 50 (granule) and 15 (pyramidal) cells. Apical dendrite for both cell types was defined as the thickest and longest. In the case of pyramidal cells, this always occurred at the apex of the perikarya. Measurements of resting membrane potential and input resistance were done using the perforated patch technique (n = 13).

followed by IgG-fluorescein isothiocyanate-conjugated labeling. Cells were examined with an Axiosvert-100 Zeiss fluorescent microscope. Pictures were taken using a MC-80 Zeiss camera, a Hamamatsu (Middlesex, NJ) C-2400 SIT camera, or a MRC-1000 Laser confocal microscope (Bio-Rad).

Electron Microscopy. Cells were allowed to grow for 18–28 days on poly-d-lysine and laminin-coated Aclar 33c coverslips. Cultures were fixed in place by slow perfusion with a trialedehyde solution containing 1% paraformaldehyde, 1% acrolein, 2.5% glutaraldehyde, and 2.5% dimethylsulfoxide plus CaCl2 (0.05%) in 0.1 M cacodylate buffer (pH 7.4). After 1 h at room temperature, this fixative was replaced with 2% paraformaldehyde and 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 16–20 h at 4°C. The cultures were treated with 2% O3O4 in 0.1 M cacodylate buffer (pH 7.4) for 1 h at room temperature. After the cultures were embedded in Epon 812, serial thin sections (50–100 sections per block) were cut parallel to the substrate surface, stained with lead and uranyl acetate, and photographed with a Phillips model 301 electron microscope (Phillips Electronic Instruments, Mahwah NJ).

Electrophysiology. Recording of spontaneous activity was done by whole-cell patch clamp in the presence of 0.5 µM tetrodotoxin (8). Five minutes after gaining stable access into the cell, the occurrence of spontaneous miniature excitatory postsynaptic currents (mEPSCs) was recorded for 1 min using pCLAMPx 6 (Axon Instruments, Foster City, CA), and the frequency and amplitude of the events were analyzed by visual inspection of the traces.

FIG. 2. In vitro dentate gyrus neurons have MF-like axons. (a) Dynorphin A (green) is expressed in axon-like processes but not in dendrites (red). Arrows point to filopodia-like structures. (b) Varicosities of a dynorphin A-positive axon in putative contact with pyramidal cells on their apical dendrite (arrowheads), the basal dendrite (arrow), and cell body (open arrow). (c) MF varicosities in close association with a nonpyramidal cell. Arrows indicate varicosities with a diameter of 4–4.5 µm. (Bar = 20 µm.)

FIG. 1. (On previous page) Hippocampal culture enriched for dentate gyrus granule versus CA3 pyramidal cells. (A1-A3) Hippocampi of P1-P5 rat pups were split through the longitudinal axis of the CA3 region close to the dentate gyrus region. (A4) In several experiments, enrichment was increased by separation through a Percoll gradient. MAP2 staining of 2-week-old CA3-CA1 culture (B1) and dentate gyrus-CA3 culture (B2). (B3) Higher magnification of the area boxed in B2. The arrow indicates a pyramidal cell. A pyramidal (B4) and a granule (B5) neuron from dentate gyrus-CA3 culture intracellularly filled with carboxyfluorescein. Arrow points to the apical dendrite. (B6) A granule cell of the dentate gyrus-CA3 culture identified by prelabeling the dentate gyrus with Dil. (C1) Microspheres taken up by cells positioned in the CA3 subfield of a hippocampus contralateral to the one injected in the CA3 region. (C2) MAP2 staining of dentate gyrus-CA3 culture prepared from the section shown in C1. (C3) Only the pyramidal cell, indicated by an arrow in C2, contains microspheres (arrow). (Bars: B1 and B2 = 90 µm; B3-B6, C2, C3 = 35 µm; C1 = 900 µm.)
RESULTS

Enrichment of Granule Cells Versus Pyramidal Cells in Culture. To enrich primary neuronal cultures for MF connections to eliminate as much as possible the collateral connections between the pyramidal cells, we increased the ratio between granule cells and CA3 pyramidal cells by coculturing the entire dentate gyrus with only a portion of the CA3 subfield (dentate gyrus-CA3 culture) (Fig. 1A). In dentate gyrus-CA3 cultures, only 6.3% (±2% SEM) of the total neurons displayed a pyramidal-like shape, whereas 50.6% (±2.8% SEM) of the cells were granule-like (Fig. 1B2; Table 1). The remainder of the cells were classified as nonpyramidal, nongranule cells. Ten to 15% of the nonpyramidal, nongranule cells were glutamic acid decarboxylase-positive (not shown) and presumably represent inhibitory interneurons. The average ratio of putative granule to pyramidal cells under these conditions was 13:1 (measured by shape and dimension of cell body and dendritic ramification; see below). By contrast, in CA3-CA1 cultures, pyramidal-like neurons occupied ~60% of the total neuronal population (Fig. 1B1; Table 1) with granule-like cells making up <6%, probably reflecting contamination from the dentate gyrus.

To verify that pyramidal cells in dentate gyrus-CA3 cultures originate from the CA3 region, cells from the CA3 subfield were retrogradely labeled with fluorescent microspheres through the commissural pathway and were found to exhibit CA3 pyramidal-like structure when placed in culture (Fig. 1C). The pyramidal cells in culture acquired the appearance of their mature counterparts, having large triangular cell bodies, one major apical dendrite, and 2–6 basal dendrites (Fig. 1B3 and B4; Table 1). As first shown by Boss et al. (13), dentate gyrus granule cells labeled with Dil (Fig. 1B6) appeared immature, exhibiting smaller, round, or oval cell bodies as in vivo (14), and 90% bore a basal dendrite (Fig. 1B2, B3, and B6; Table 1), similar to the immature granule cells described in rats (15), monkeys, and humans (16). Ten percent of the granule cells lacked basal dendrites (Fig. 1B5). A second approach to verify that granule cells survived in dentate gyrus-CA3 culture was the use of dynorphin A as a specific marker for MF axons (17).

We found that during the second week in vitro, dynorphin A staining was present in the axons of dentate gyrus-CA3 cells (Fig. 2). By contrast, examination of CA3-CA1 cultures revealed no significant dynorphin A staining, suggesting that the positive labeling in the dentate gyrus-CA3 culture is due to granule cell axons. In support of this view, dynorphin A was found to inhibit synaptic transmission of the MF in the dentate gyrus-CA3 culture (8) as reported for these synapses in the intact hippocampus (18).

Similar to their in vivo counterparts, the MF axons in culture displayed varicosities of various shapes and sizes (1–4.5 μm) (Fig. 2a) and filopodia-like structures (1). However, unlike the situation in vivo, MF contacts were not restricted to the proximal portion of the CA3 cell apical dendrite (Fig. 2b). Perhaps, as suggested by Boss et al. (13), the correct localization of MF axon connections in vivo is extrinsically regulated and this control is absent from isolated cells in dissociated culture.

One of the features that distinguishes the MF pathway from other hippocampal excitatory pathways is a late onset of activity due to the late generation of granule cells (19). We found that at 7 days in vitro, synaptophysin-positive presynaptic structures and mEPSC frequency (Fig. 3B and C) were approximately four times lower in the dentate gyrus-CA3 compared to the CA3-CA1 culture (Fig. 3C). By contrast, no differences were seen in the amplitude distribution of the spontaneous miniature synaptic potential between the two cultures, with both showing a peak at 4 pA in the amplitude histograms (Fig. 3C). At 14 days in vivo, the concentration of synaptophysin-positive terminals was equal between the two cultures but the differences in the frequency of mEPSC persisted (not shown). Thus, in culture as in vivo, the granule to pyramidal cell connections are formed later than the pyramidal to pyramidal cell connections.

Characterization of the Postsynaptic Site. Similar to their in vivo counterparts, the spine repertoire of the dentate gyrus-CA3 culture included several morphologically identifiable types, including thin, stubby, and mushroom (Figs. 4 and 5). Spines in close contact with MFs tended to be more complex (Fig. 4A and B) but were not restricted to the proximal portion...
of apical dendrites of pyramidal cells. They often consisted of a widened neck and an enlarged head composed of several protrusions, resembling immature “thorny” spines (1). Electron microscopy confirmed these different classes of spines (Fig. 4 C and D) and also revealed evidence of possible structural remodeling in the form of “spinules” and perforated synapses (Fig. 4E), both of which have been suggested to play a role in some types of synaptic plasticity (20–22).

The absence of a restricted localization of complex spines to the proximal portion of pyramidal cell apical dendrites might be due to an abnormal spine distribution in culture. We have found this is not the case. In vivo, hippocampal pyramidal cells have the highest spine frequency on distal parts of the dendrite (23).

Similarly, in culture, we observed a 53% higher concentration of spines on the distal versus proximal portions of pyramidal cells (Fig. 5 b and c; Table 2), suggesting that CA3 neurons have an intrinsic capability to enhance spine formation at the distal part of their apical dendrite but are incapable of concentrating thorny spines of the mossy fiber pathway on their proximal segment.

**DISCUSSION**

We have been able to develop the procedures necessary for reconstituting the MF pathway in dissociated cell culture. In this culture the presynaptic granule cells and their connections with CA3 pyramidal cells are enriched. Moreover, both the pyramidal and granule cells, identified by independent criteria, are readily
produce both the MF connections to mature neurons. However, the ratio of spine concentration between the distal versus the proximal parts along the apical dendrite is 3.9-fold higher on pyramidal than on granule cells (calculated as: \( \frac{\text{distal/proximal (pyramidal)}}{\text{distal/proximal (granule)}} = 3.9 \)). The concentration of spines on the basal dendrite of pyramidal cells is higher than that of the apical dendrite. For granule cells, this distribution is reversed. The ratio for apical/basal was calculated as (average between proximal and distal along the apical portion of the dendrite)/(spine concentration on the basal dendrite).

The relative simplicity and experimental accessibility of the dentate gyrus-CAl culture now allows study, on an elementary level, of the development and plastic properties of both the MF connections between dentate granule cells and hippocampal pyramidal cells from the CA1 region as well as the axon collateral connections between the pyramidal cells from the CA1 region (associational-commissural). Indeed, the ability to produce MF LTP under controlled conditions while simultaneously monitoring identified connections combined with the large size of the MF terminals and their ability to sprout following seizure provides a novel and potentially useful in vitro model for examining the functional and structural components of learning-related plasticity in a mammalian nonassociative synapse.

We thank Drs. Mark Mayford, Robert D. Hawkins, Ottavio Arancio, and Steven A. Siegelbaum for critical comments on earlier versions of this manuscript and Harriet Ayers for typing the manuscript. We also thank Drs. Steven Rayport and Ottavio Arancio for technical advice in cell culturing and Mr. Len Zahlow and the Presbyterian Hospital Photo Department for processing the pictures and images. This research was supported by the Howard Hughes Medical Institute (E.R.K.) and National Institutes of Health Grants MH57134 and GM32099 to C.H.B.