Synaptic and Axonal Remodeling of Mossy Fibers in the Hilus and Supragranular Region of the Dentate Gyrus in Kainate-Treated Rats

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ABSTRACT

Seizures evoked by kainic acid and a variety of experimental methods induce sprouting of the mossy fiber pathway in the dentate gyrus. In this study, the morphological features and spatial distribution of sprouted mossy fiber axons in the dorsal dentate gyrus of kainate-treated rats were directly shown in granule cells filled in vitro with biocytin and in vivo with the anterograde lectin tracer Phaseolus vulgaris leucoagglutinin (PHAL). Sprouted axon collaterals of biocytin-filled granule cells projected from the hilus of the dentate gyrus into the supragranular layer in both transverse and longitudinal directions in kainate-treated rats but were not observed in normal rats. The sprouted axon collaterals projected into the supragranular region for 600–700 µm along the septotemporal axis. Collaterals from granule cells in the infrapyramidal blade crossed the hilus and sprouted into the supragranular layer of the suprapyramidal blade. Sprouted axon segments in the supragranular layer had more terminal boutons per unit length than the axon segments in the hilus of both normal and kainate-treated rats but did not form giant boutons, which are characteristic of mossy fiber axons in the hilus and CA3. Mossy fiber axons in the hilus of kainate-treated rats had more small terminal boutons, fewer giant boutons, and there was a trend toward greater axon length compared with mossy fibers in the hilus of normal rats. With the additional length of supragranular sprouted collaterals, there was an overall increase in the length of mossy fiber axons in kainate-treated rats. The synaptic and axonal remodeling of the mossy fiber pathway could alter the functional properties of hippocampal circuitry by altering synaptic connectivity in local circuits within the hilus of the dentate gyrus and by increasing the divergence of the mossy fiber terminal field along the septotemporal axis. J. Comp. Neurol. 390:578–594, 1998.

Indexing terms: hippocampus; granule cells; plasticity; seizures; epilepsy

The hippocampus is a distinctive structure of the limbic forebrain that has been implicated in memory formation and epilepsy by experimental, clinical, and pathological observations (for reviews, see Squire and Zola-Morgan, 1991; Zola-Morgan and Squire, 1993; McNamara, 1994). Pathways of the hippocampus have been attractive for anatomical and physiological analysis because of the relative simplicity of their laminated organization compared with other brain regions, such as neocortex. Improved understanding of hippocampal organization may provide insights into computational processing in the hippocampus and also could lead to better understanding of memory disorders and epilepsy.

Pathways of the hippocampal formation undergo reorganization of their synaptic connections during the development of epilepsy. There is considerable histochemical evidence that mossy fiber axons in the dentate gyrus undergo sprouting and reorganization of their terminal field in a variety of experimental models of epilepsy and in the human epileptic temporal lobe. Mossy fiber synapses, which can be identified by Timm histochemistry (Tauck and Nadler, 1985; Sutula et al., 1988, 1989; Houser et al.,

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1990), immunochemistry with antibodies to dynorphin (de Lanerolle et al., 1989; Houser et al., 1990), and high-affinity kainate binding (Represa et al., 1989), are observed after a single prolonged seizure or repeated brief seizures in the supragranular region of the dentate gyrus, where they are not normally found (Claiborne et al., 1986). Although the presence of mossy fiber synapses in this location is consistent with axonal sprouting and synaptic reorganization of the mossy fiber pathway, these histochemical methods reveal alterations in the location of the terminal field of the pathway but do not provide specific anatomical details about the arborization of sprouted axons or the spatial distribution of the reorganized connections of individual neurons.

Many potentially important anatomical details about the sprouted mossy fibers that might influence functional effects of the reorganized circuitry are uncertain. For example, better characterization of the spatial distribution of the sprouted mossy fiber pathway in hippocampal slices oriented to optimally preserve recurrent circuits for physiological investigation might facilitate analysis of the functional effects of sprouting.

The aim of this study was to characterize the morphology of sprouted mossy fibers in additional detail in rats treated with kainic acid. The anatomical features and spatial distribution of the sprouted mossy fiber collaterals in the dorsal dentate gyrus were directly shown by analysis of the axonal arbors of granule cells that were individually filled in vitro with biocytin and by study of the terminal projections of the mossy fiber pathway in vivo with the anterograde lectin tracer Phaseolus vulgaris leucoagglutinin (PHAL). A preliminary report has appeared in abstract form (Zhang et al., 1993).

**MATERIALS AND METHODS**

**Administration of kainic acid**

Adult male Sprague-Dawley rats (250–300 g) were injected with kainic acid (9–12 mg/kg IP or SC) and were observed for signs of behavioral seizure activity, which typically consisted of altered responsiveness to environmental stimuli, irregular tonic-clonic movements of the extremities, and loss of postural tone. The injected rats were observed and returned to their cages after 2–3 hours, when the most severe behavioral seizures usually diminished. Previous studies have documented that kainic acid initially produces intense electrographic seizures that gradually diminish and usually cease by 4–5 days (Sutula et al., 1992) and that are eventually followed by spontaneous recurrent seizures (Cronin et al., 1992). Methods of animal handling and all experimental procedures were approved by the Research Animal Care Committee of the University of Wisconsin.

**Analysis of granule cell morphology and mossy fiber axon arborization with intracellular biocytin**

**Procedures.** Granule cell morphology and arborization of mossy fiber axons were studied in vitro by intracellular filling of individual granule cells with biocytin in hippocampal tissue slices. Rats treated with kainic acid were studied at least 1 month after the administration of kainic acid, which is an interval sufficient to permit development of mossy fiber sprouting (Nadler et al., 1980).

Normal rats and the rats treated with kainic acid were decapitated after induction of anesthesia by ether. The brains were rapidly removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) with the following composition (in mM): 124 NaCl, 4.4 KCl, 1.2 KH$_2$PO$_4$, 2.4 CaCl$_2$, 1.3 MgSO$_4$, 26 NaHCO$_3$, 10 glucose, which was saturated with 95% O$_2$-5% CO$_2$ at pH 7.4. Transverse hippocampal slices were cut from the dorsal hippocampus with a Vibratome at a thickness of 400 µm perpendicular to the septotemporal axis of the hippocampus. The slices were specifically selected from the dorsal hippocampus to exclude the ventrolateral (temporal) regions of the hippocampus, where there is a supragranular projection of the mossy fiber pathway in normal rats (Cavazos et al., 1992). The slices were then transferred to a submersion recording chamber and bathed in ACSF at 31–32°C. Longitudinal slices were cut parallel to the
Fig. 2. Camera lucida drawing of a biocytin-filled granule cell in the suprapyramidal blade of the dentate gyrus from a normal rat. The axon has an extensive collateral plexus in the subgranular region of the hilus and projects into CA3. The border of the granule cell layer is indicated by the solid line. Broken lines indicate the border of the CA3 pyramidal cell layer. The inset (upper right) in this and subsequent figures shows the location of the biocytin-filled granule cell in the transverse plane of the slice. GL, granule cell layer; H, hilus. a: Photomicrograph at higher magnification of the section from the region of the axon labeled “a” in CA3 shows multiple small, irregularly spaced synaptic varicosities of 0.5–1 µm and two larger varicosities that measure 2–5 µm in the longest dimension, which correspond to giant mossy fiber synaptic boutons. b: The photomicrograph from the region labeled “b” within the pyramidal layer CA3, shows another giant mossy fiber synaptic bouton. Scale bars = 50 µm in top, 10 µm in A, B.
Fig. 3. Photomicrograph of a cresyl violet-stained section of a transverse hippocampal slice from a kainate-treated rat showing a biocytin-filled granule cell. A: The main trunk of the axon from the biocytin-filled granule cell in the suprapyramidal blade of the dentate gyrus forms a subgranular plexus and then extends a sprouted axon into the supragranular region where it forms an extensive collateral plexus (dark arrows) approximately 150 µm from the granule cell body and dendrites. A sprouted axon segment is also apparent in the supragranular region near the dendrites (open arrow). B: Higher magnification of the supragranular sprouted plexus indicated by the dark arrows area in (A) shows numerous irregularly spaced small synaptic varicosities. There was an increase in the number of small synaptic boutons in supragranular axon segments compared with axons in the hilus (see text for details). Giant mossy fiber terminal boutons were not observed in the sprouted axon collaterals. C: Higher magnification of the supragranular sprouted plexus indicated by the open arrow in (A), photographed in a different focal plane for clarity, also shows numerous irregularly spaced small synaptic varicosities. ML, molecular layer; GL, granule cell layer; H, hilus. Scale bars = 50 µm in A, 20 µm in B, 25 µm in C.
septotemporal axis of the hippocampus and were used to assess the projection of the mossy fiber pathway along this axis.

Granule cells were impaled with glass micropipettes (100–150 MΩ) filled with biocytin (2% wt/vol in 4 M potassium acetate, adjusted to pH 7.4). The impaled granule cells were filled with biocytin by passage of depolarizing current pulses of 2.0 nA for 8–10 minutes. The tissue slices were maintained in the recording chamber for 1–2 hours for additional physiological studies and were then placed into freshly prepared 4% paraformaldehyde (wt/vol) and stored at 4°C for at least 24 hours. After freezing on dry ice, tissue sections of 60 µm thickness were cut by a freezing microtome in the same plane as the transverse hippocampal slice. Biocytin-filled neurons, dendrites, and axons were visualized by the biotin-avidin reaction by using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) following intensification with 1% (wt/vol) CoCl2 and 1% (wt/vol) Ni(NH4)2(SO4)2. The slices were counterstained with cresyl violet.

**Technical considerations.** Each filled neuron included in the analysis was identified as a granule cell based on physiological criteria (Lambert and J ones, 1991) and morphological features (Amaral, 1978; Claiborne et al., 1986). Neurons were included in the study only when resting membrane potentials were at least −65 mV and stable impalements were obtained for at least 1 hour. Because preparation of hippocampal slices inevitably cuts portions of the axons along the edge of the cut section, an extensive axonal plexus was not always available for study in a given slice. In each camera lucida drawing, the cut ends of axons on the surface of the 400-µm-thick slices were identified. Detailed examination of axon collateral patterns by using camera lucida drawing and analysis with the NeuroLucida image analysis system was performed only on axons that arborized extensively in the hilus and extended into CA3. Because it is difficult to exclude the possibility that the labeling of analyzed axon collaterals was not complete, the observations of this study should not be regarded as a definitive or complete description, but as a description of the more prominent features of an inevitably limited sample of granule cells.

**Analysis of mossy fiber axons with PHAL**

**Procedures.** The spatial distribution of sprouted mossy fiber axons was studied in vivo by using the anterogradely transported lectin PHAL according to previously described methods (Gerfen and Sawchenko, 1984; Ishizuka et al., 1990). Rats were studied at least 1 month after injection of the kainic acid and were compared with normal rats without sprouting of the mossy fiber pathway. After induction of anesthesia with ketamine (80–90 mg/kg IP) and xylazine (7–13 mg/kg IM), the rats were placed in a stereotaxic frame, and a glass micropipette (5–7 µm in diameter) containing PHAL (2.5% wt/vol in 0.1 M phosphate-buffered saline) was stereotaxically placed into the molecular layer of the dorsal dentate gyrus (3.5 mm posterior, 1.75 mm lateral, 3.5 mm deep to bregma) through a small craniotomy. PHAL was iontophoretically injected by passage of 5 µA of positive DC current pulses (7-second duration delivered every 7 seconds) for 3–5 minutes. The rats were allowed to recover for 10–14 days, an interval sufficient for PHAL transport into axons, and were then deeply anesthetized with pentobarbital (50 mg/kg IP) and sequentially perfused with 4% solutions of paraformaldehyde at pH = 6 and pH = 9.5. Coronal sections were cut with a freezing microtome at a thickness of 30 µm and were reacted with goat anti-PHAL antibodies, followed by visualization with the Vectastain ABC kit and intensification with OsO4. Sequential coronal sections were examined by both darkfield and lightfield microscopy and were then sketched by camera lucida drawing and photographed.

**Technical considerations.** The intracellular transport of PHAL is exclusively anterograde. There is no significant uptake by axons of passage and only very limited uptake by injured or cut axons (Gerfen and Sawchenko, 1984). Interpretation of the patterns of anterogradely transported PHAL for the purpose of identifying the terminal field of an injected neuronal population is, therefore, critically dependent on accurate localization of the injection site and identification of the cells that take up the PHAL. Although PHAL is in many respects ideal for anterograde tracing of the terminal fields of pathways that project for relatively long distances beyond the site of PHAL injection, it is more difficult to distinguish and accurately identify the terminal fields of local circuits or other pathways that may terminate in close proximity to the injection site. Although the hippocampus and dentate gyrus have laminar organization and relatively well-defined populations of neurons that can facilitate tract tracing and analysis of terminal fields, correct interpretation of patterns of PHAL transport from injection sites in the dentate gyrus may be particularly difficult due to the

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**TABLE 1. Morphological Characteristics of Normal and Sprouted Mossy Fibers**

<table>
<thead>
<tr>
<th></th>
<th>Normal (n = 3)</th>
<th>Kainate-treated (n = 3)</th>
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<tbody>
<tr>
<td></td>
<td>Hilus and CA3</td>
<td>Supragranular</td>
</tr>
<tr>
<td>Giant boutons (&gt;2.5 µm per 1,000 µm)</td>
<td>F = 0.08, ANOVA</td>
<td></td>
</tr>
<tr>
<td>Synaptic varicosities (&lt;2.5 µm per 100 µm)</td>
<td>F = 18.95, P = 0.003, ANOVA</td>
<td></td>
</tr>
<tr>
<td>Branch points per 1,000 µm</td>
<td>F = 0.03, ANOVA</td>
<td></td>
</tr>
<tr>
<td>Axon length (µm)</td>
<td>F = 0.01, ANOVA</td>
<td></td>
</tr>
</tbody>
</table>

1None observed.
2Versus normal hilus, P = 0.03, t-test.
3Versus normal hilus, P = 0.04, t-test.
4Versus normal hilus, P = 0.002, t-test.
5Versus kainate-treated hilus, P = 0.007, t-test.

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**TABLE 2. Spatial Distribution of Mossy Fiber Axons in the Dentate Gyrus of Normal and Kainate-Treated Rats With Sprouting**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Kainate-treated</th>
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<tbody>
<tr>
<td>Supragranular granule cells (no.)</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Subgranular axon plexus same blade</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Subgranular axon plexus opposite blade</td>
<td>10/16</td>
<td>16/16</td>
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<tr>
<td>Supragranular axon plexus same blade</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Supragranular axon plexus opposite blade</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Infragranular granule cells (no.)</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Subgranular axon plexus same blade</td>
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<td>4/4</td>
</tr>
<tr>
<td>Subgranular axon plexus opposite blade</td>
<td>7/10</td>
<td>4/4</td>
</tr>
<tr>
<td>Supragranular axon plexus same blade</td>
<td>3/4</td>
<td>3/4</td>
</tr>
<tr>
<td>Supragranular axon plexus opposite blade</td>
<td>3/4</td>
<td>3/4</td>
</tr>
</tbody>
</table>

1Proportions differ between blades, P = 0.002, Fisher exact test.
2Proportions differ between normal and kainate-treated groups, P = 0.017, Fisher exact test.
3Not observed.
4Proportions differ between blades, P = 0.003, Fisher exact test.
heterogeneity of the neuronal populations in the hilus and reciprocal local circuit connections between cells in the hilus and the principal neurons in the granule cell layer. For example, uptake of PHAL by basket cells or mossy cells in the hilus of the dentate gyrus, which project into the inner molecular layer, could result in fiber labeling by PHAL in the inner molecular layer that would overlap with the terminal field of sprouted mossy fibers.

To avoid these potential interpretive problems, serial sectioning through the injection sites was performed in all cases to distinguish injection sites that were confined to the granule cell and molecular layers from injections that involved the hilus of the dentate gyrus. Cases with PHAL injection that involved the hilus were excluded.

RESULTS

Anatomical features of biocytin-filled granule cells were studied in a total of 26 granule cells from 16 normal rats. The relative locations of 24 of these cells in transverse slices from dorsal hippocampus are indicated in Figure 1A. Two biocytin-filled granule cells from 2 normal rats were examined in longitudinal hippocampal slices cut parallel to the septotemporal axis of the hippocampus. The relative locations of 21 granule cells in transverse hippocampal slices from 9 kainate-treated rats are illustrated in the schematic of Figure 1B. Seven additional granule cells from 6 kainate-treated rats were examined in longitudinal hippocampal slices cut parallel to the septotemporal axis of the hippocampus.

Biocytin-filled axons were observed in the supragranular region of the dorsal dentate gyrus in 18 of the 28 granule cells from kainate-treated rats but were not
observed in the supragranular region in any of the 26 neurons from normal rats (compare Figs. 2 and 3, $P < 0.0001$, $\chi^2$). Because mossy fibers are rarely found in the supragranular region of the dorsal dentate gyrus in normal rats (Claiborne et al., 1986), the observation of mossy fiber axons projecting into this region is evidence of axon sprouting. The morphological features of mossy fiber terminal boutons and the spatial distribution of mossy fiber axons were compared in biocytin-filled granule cells from normal and kainate-treated rats.

**Morphological features of mossy fiber synapses and axons in normal and kainate-treated rats**

The mossy fiber axons of biocytin-filled granule cells from normal rats typically extended as a single trunk from the granule cell body, entered the hilus, and branched into an extensive plexus of fine caliber axons of 0.1–0.2 µm in diameter. There were numerous irregularly spaced varicosities of 0.5–1 µm in diameter along the length of the axons (see Fig. 2). After reaching the CA3 pyramidal layer, occasional larger irregularly shaped varicosities that were 2–6 µm in diameter were observed in the mossy fiber axons (Fig. 2). The smaller irregularly spaced varicosities, which were observed only after the axon had penetrated into the hilus, resembled terminal boutons described previously in mossy fiber axons studied in hippocampal slices by horse-radish peroxidase and ultrastructural techniques (Claiborne et al., 1986) and were presumed to be small terminal boutons. The less frequently observed 2–6 µm...
Figure 7
varicosities, which were also irregularly spaced along the length of the filled axons, corresponded in size, appearance, and location to giant mossy fiber terminal boutons, which are most often observed after the axon enters the pyramidal cell layer of CA3 and occasionally in the hilus of normal rats (Claiborne et al., 1986). The features of the mossy fiber axons and putative terminal boutons observed in the individually labeled granule cells of this study thus conformed to descriptions of mossy fiber axons and synapses available in previous Golgi and ultrastructural studies (Amaral, 1978; Seress and Pokorny, 1981; Claiborne et al., 1986).

Detailed analysis of terminal bouton size, number, and axon length was performed in granule cells from three normal and three kainate-treated rats by using camera lucida reconstruction and the NeuroLucida image analysis system. Sprouted axon segments, defined as mossy fiber axons that projected into the supragranular layer, showed more small varicosities (less than 2.5 µm) per 100 µm than axon segments in the hilus of both normal (P < 0.002) and kainate-treated rats (P < 0.007; compare Figs. 2, 3, and 1). Giant mossy fiber terminal boutons were not observed in sprouted axon segments in the supragranular layer. There was a trend toward an overall increase in the mean number of terminal boutons per granule cell in kainate-treated rats (1,199 ± 180) compared with normal controls (610 ± 172, P = 0.07).

Analysis of axon length and numbers of terminal boutons also revealed evidence of axonal and synaptic remodeling of mossy fibers in the hilus and CA3 region of kainate-treated rats. There was an increase in the number of small varicosities per 100 µm (P = 0.04) and a decrease in the giant mossy fiber terminal boutons per 1,000 µm (P = 0.03) in the hilus and CA3 region of kainate-treated rats (Table 1).

Although preparation of hippocampal slices often cuts axons at the edges of slices, there was a trend toward an overall increase in mossy fiber axon length in the hilus and CA3 (Table 1), and with the additional supragranular sprouted collaterals, there was an increase in mean total axon length in granule cells of kainate-treated rats (7,162 ± 761 µm) compared with controls (4,430 ± 667 µm; P = 0.038, n = 4; compare Figs. 2 and 4 with Figs. 5 and 6). There was no change in the frequency of branch points in axon arbors of kainate-treated rats (Table 1).

**Spatial distribution of mossy fiber axons in normal and kainate-treated rats**

The spread of mossy fiber collaterals was examined in both the transverse and longitudinal axes of the hippocampus in both normal and kainate-treated rats.

**Mossy fiber projections in the transverse hippocampal axis of normal rats.** The patterns of mossy fiber axon distribution in the hilus of the dentate gyrus in normal rats are summarized in Table 2. Fourteen of the 24 biocytin-filled granule cells studied in transverse slices from normal rats were in the suprapyramidal (dorsal) blade. In previous studies of horseradish peroxidase-filled mossy fibers in normal rats, collateral plexuses of granule cells in the one-third of the suprapyramidal blade closest to the tip were confined to the region of the hilus directly beneath the suprapyramidal blade (Claiborne et al., 1986). In this series of 14 biocytin-filled granule cells in comparable locations in the suprapyramidal blade of the dorsal dentate gyrus (see Fig. 1A), the mossy fiber axons of cells in the suprapyramidal blade similarly formed a plexus in the subgranular region of the hilus directly beneath the blade but did not cross the hilus or extend beyond the CA3 pyramidal area into the subgranular region of the infrapyramidal blade (Fig. 2). Some axon branches turned toward the granule cell layer from which they originated and typically terminated along the border of the hilus and the granule cell layer, but in no case did the axon project beyond the granule cell layer into the molecular layer of the dentate gyrus (Fig. 2).

Ten of the 24 granule cells studied in transverse slices from normal rats were in the infrapyramidal (ventral) blade (Table 2). All 10 of these granule cells gave rise to an axon plexus in the subgranular region of the infrapyramidal blade, but in addition 7 of these 10 granule cells also projected a collateral that crossed the hilus and extended beyond the CA3 pyramidal area to the subgranular layer of the suprapyramidal blade (Fig. 4). In no case, however, did these axons project through the granule cell into molecular layer.

**Mossy fiber projections in the transverse hippocampal axis of kainate-treated rats.** Anatomical features of 21 biocytin-filled granule cells were studied in transverse slices of dorsal hippocampus from 9 kainate-treated rats. Sprouted collaterals were observed in the supragranular region of 15 of these 21 granule cells (Fig. 1B). Of the 21 biocytin-filled granule cells in transverse slices from the kainate-treated rats, 16 were located in the suprapyramidal blade, 1 in the region of the crest of the dentate gyrus, and 4 were located in infrapyramidal blade. The patterns of sprouted mossy fiber axon distribution in the supragranular region of the dentate gyrus in kainate-treated rats are summarized in Table 2. In 10 of the 16 biocytin-filled granule cells located in the suprapyramidal blade, sprouted axons extended from the axon plexus in the subgranular region of the hilus into the adjacent supragranular region of the suprapyramidal blade (Fig. 5A). The granule cell located in the crest of the dentate gyrus also projected a sprouted axon into the suprapyramidal layer (Fig. 5B). In 3 of 4 biocytin-filled granule cells in the infrapyramidal blade, sprouted axon collaterals extended from the subgranular region of the hilus into the adjacent supragranular region of the infrapyramidal blade (Fig. 6B). In addition, 3 of 4 neurons in the infrapyramidal blade also showed sprouted mossy fiber collaterals from axon branches that crossed the hilus and entered the supragranular region of the opposite suprapyramidal blade (Fig. 6A, B).

**Mossy fiber projections in the septotemporal axis.** In longitudinal slices from normal rats, there was one infrapyramidal and one suprapyramidal biocytin-filled granule cell, and their axons did not project into the supragranular region. Seven biocytin-filled granule cells were examined in longitudinal slices of the hippocampus from six kainate-treated rats.

Sprouted mossy fiber collaterals were observed in three of seven biocytin-filled granule cells examined in longitudinal slices of the hippocampus from six kainate-treated rats. Two of these three granule cells in longitudinal slices of hippocampus from six kainate-treated rats. Two of these three granule cells in longitudinal slices projected sprouted collaterals in both the septal and temporal directions (Fig. 7A). The third granule cell in this group had only a single sprouted collateral that projected in the temporal direction. The terminal field of the granule
cell illustrated in Figure 7A,B was distributed over slightly more than 650 µm along the septotemporal axis (Fig. 7C). The sprouted collaterals of the three granule cells examined in longitudinal slices extended in the septal or temporal direction for distances of 50–328 µm from the cell body. Because the mossy fiber pathway has no supragranular projection in normal rats and its projection in CA3 is lamellar in the transverse axis, the sprouted axon collaterals in the supragranular region increase the divergence of the mossy fiber pathway along the septotemporal axis.

To investigate further the septotemporal divergence of the sprouted supragranular projection in vivo, the spatial distribution of the sprouted terminal field of the mossy fiber pathway was examined in 6 normal rats and 4 kainate-treated rats with injections of PHAL confined to the granule cell layer and molecular layer of the dorsal dentate gyrus. These cases were selected from a larger group of 15 normal and 12 kainate-treated rats. Cases were excluded if examination in serial sections revealed that the injection site included the hilus in addition to the granule and molecular layers of the dentate gyrus. The cases selected for analysis had injection sites that ranged from 100 to 700 µm in diameter. In pilot studies and cases that included an injection in the hilus, the pattern of PHAL labeling in the inner molecular layer was distinct from the patterns encountered in kainate-treated rats with PHAL injections confined to the granule cell and molecular layers. Although it is difficult to completely exclude the possibility that some neurons in the hilus and along the electrode tract were injected, this appeared to be minimal.

Darkfield examination of sections of the dentate gyrus and hippocampus in the six normal rats with PHAL injection confined to the granule cell and molecular layer of the dentate gyrus revealed a dense confluent plexus of PHAL-filled axons in the hilus of the dentate gyrus and in the CA3 subfields of the hippocampus (Fig. 8A). Although there were rare PHAL-filled fibers noted in the molecular layer of the dentate gyrus, the frequency of these fibers was not any more than the background in other extrahippocampal areas (Fig. 8A). There was no observation of PHAL-filled fibers in the supragranular area above background in other regions (Fig. 8A,B).

There was a distinct difference in the pattern of PHAL-filled axons in rats treated with kainic acid. In addition to a dense plexus of PHAL-filled axons in the hilus and CA3, PHAL-filled axons were also observed in the supragranular region of the dentate gyrus in four of four kainate-treated rats with injections confined to the granule cell layer and molecular layer of the dentate gyrus (Fig. 9). In this example from a case with a relatively large injection into the infrapyramidal blade, there was a plexus of PHAL-filled axons in the opposite suprapyramidal blade (Fig. 9A–J) in addition to the dense plexus in the hilus that extended into the CA3 region (Fig. 9F–J). This in vivo observation confirms the infrapyramidal to suprapyramidal axon projection detected in vitro in biocytin-filled granule cells in the infrapyramidal blade (Fig. 6).

In the transverse plane (Fig. 9), the supragranular spatial distribution of PHAL-filled axons and the relatively greater density of PHAL-filled axons in the hilus corresponded to the distribution and relative density of mossy fiber terminals observed in these regions in kainate-treated and kindled rats studied by Timm histochemistry (Sutula et al., 1992; Cavazos et al., 1991). Because transport of PHAL is anterograde and the location of PHAL-filled axons defines the terminal field of the injected neurons, the observation of PHAL-filled axons septal and temporal to the injection sites is consistent with septotemporal spread of sprouted collaterals, which was directly shown in biocytin-filled granule cells (Fig. 7), and enabled additional analysis of the septotemporal distribution of the terminal field that cannot be assessed by Timm histochemistry.

To obtain a rough estimate of the septotemporal distribution of sprouted mossy fiber axons, the four kainate-treated rats with PHAL injections were examined in further detail. An example of this analysis for the case in Figure 9 and comparison with an injection in a normal rat is shown in Figure 10. PHAL-filled axons and the injection site were identified in serial coronal sections and were sketched by camera lucida methods. Sections containing the injection site were identified, and the distribution of the injection site along the septotemporal axis was then determined. Sections containing PHAL-filled axons and terminals in the supragranular layer were similarly identified in each coronal section. The distance of the section with PHAL-filled axons in the supragranular layer that was most distant from the edge of the injection site was determined.

In the analysis for the kainate-treated rat shown in Figure 9, supragranular PHAL-filled collaterals were observed in the septal section located 690 µm from the center of the injection site, which was approximately 300 µm from the septal edge of the injection site. In the temporal direction, supragranular PHAL-filled axons were observed in the temporal section 690 µm from the center of the injection site and were approximately 510 µm from the temporal edge of the injection site. In contrast, there was no septotemporal supragranular projection in the dorsal dentate gyrus of the normal rats (Fig. 10). In the four kainate-treated rats, the sprouted supragranular plexus was observed 100–600 µm in septal and temporal directions beyond the edge of the injection site and was observed only in sections that also included a projection of mossy fibers into the hilus or CA3 (Figs. 9A–E and 10).

**DISCUSSION**

This study of the mossy fiber pathway directly showed sprouted mossy fiber collaterals and provided anatomical details about morphological features of the terminal boutons and spatial organization of the sprouted pathway in the supragranular region and hilus of the dorsal dentate gyrus in kainate-treated rats. In addition to direct demonstration of the sprouted projection in the supragranular region, quantitative analysis of alterations in numbers and distribution of terminal boutons also showed synaptic and axonal remodeling of mossy fibers in the hilus of the dentate gyrus. The study confirmed previous observations about the cellular features and organization of the mossy fiber pathway in normal rats and revealed that the sprouted mossy fiber collaterals increase the septotemporal divergence of the mossy fiber pathway. These observations are potentially of interest for understanding the functional effects of mossy fiber sprouting and the cellular basis of the seizure-induced hippocampal dysfunction, which includes
Fig. 8. Darkfield photomicrographs of a coronal section of hippocampus and dentate gyrus from a normal rat injected with PHAL in the dorsal dentate gyrus. A: The injection site in the infrapyramidal blade is indicated by the asterisk and includes a portion of the molecular and granule cell layer near the tip of the dentate gyrus but does not extend into the hilus. The light staining areas contain axons with PHAL that was anterogradely transported from the injection site and define the terminal field of the injected neurons. The terminal field includes an extensive plexus of axons in the subgranular and central hilus and a projection to the CA3 region of the hippocampus that are easily distinguishable from the occasional isolated PHAL-filled fibers observed in other regions. The granule cell layer in the suprapyramidal blade is indicated by the white arrows. B: Higher magnification view of the dentate gyrus from (A) shows the absence of PHAL staining in the supragranular region, which is indicated by the white arrow. Background area in this figure were retouched with ink in areas of artifacts near schematic arrows. GL, granule cell layer. Scale bars = 110 µm in A; 50 µm in B.
increased susceptibility to epilepsy and memory dysfunction.

**Synaptic and axonal remodeling of mossy fibers in kainate-treated rats**

In this light level analysis, biocytin-filled mossy fiber axons of granule cells formed both small and large varicosities in the hilus of the dentate gyrus and CA3. Although electron microscopic analysis would be required to definitively confirm synaptic ultrastructure of these varicosities, the size, shape, locations, and irregular distribution of the varicosities along biocytin-filled axons corresponded closely to previous descriptions of mossy fiber terminal boutons (Claiborne et al., 1986).

In kainate-treated rats, axons of biocytin-filled granule cells projected from the hilus into the supragranular layer of the dentate gyrus. These results and other studies using retrograde filling of mossy fiber axons with biocytin after extracellular injection into CA3 (Okazaki et al., 1995) directly showed the phenomenon of seizure-induced sprouting, which was detected by histochemical methods in kainate-treated rats (Laurberg and Zimmer, 1981; Nadler et al., 1980), in other experimental models (Sutula et al., 1988; Cavazos et al., 1991; Golarai et al., 1992; Stanfield, 1989; Qiao and Noebels, 1993), and in the human epileptic temporal lobe (de Landerolle et al., 1989; Sutula et al., 1989; Represa et al., 1989; Houser et al., 1990).

The terminal boutons of sprouted collaterals were comparable in size with the small terminal boutons observed in the hilus. Giant mossy fiber terminals, which were observed in the hilus and CA3, were not detected in sprouted axon collaterals in the supragranular region. Ultrastructural studies using Timm histochemistry also have shown that sprouted mossy fibers in the supragranular layer form numerous smaller asymmetric terminal boutons (Sutula et al., 1988). These observations suggested that the terminal boutons formed by mossy fibers appear to be dependent on postsynaptic signals or regional conditions encountered by the axon and are not likely to be determined exclusively by intrinsic axonal signals.

The ability to directly examine the axonal arborization and terminal boutons of mossy fibers in the hilus of the dentate gyrus provided evidence that mossy fiber axonal remodeling induced by kainic acid evoked seizures is not confined to the supragranular area but also involves the circuitry of the hilus. Mossy fibers in the hilus of kainate-treated rats showed an increase in the number of small terminal boutons per unit length of axon and increased total axon length. The methods of this study showed synaptic and axonal remodeling that was not previously detected by Timm histochemical and other methods, which revealed alterations in the location of the terminal field of mossy fibers but provided no information about synaptic remodeling and reorganization within the hilus.

**Effects of sprouting on the spatial distribution of the terminal field of the mossy fiber pathway**

In this study, in vitro filling of granule cells with biocytin and in vivo injections of PHAL confirmed previous observations about the terminal field of the mossy fiber pathway in normal rats (Blackstad, 1958; Blackstad et al., 1970; Amaral, 1979; Gaarskjaer, 1978, 1986; Claiborne et al., 1986; Amaral, 1993). Mossy fiber axons of granule cells that were individually filled with biocytin arborized in the hilus and extended into the subfields of CA3. Of particular importance for purposes of this study, there was no evidence from either the biocytin or PHAL method that the terminal field of the mossy fiber pathway in the dentate gyrus of normal rats extended into the supragranular region. The mossy fiber pathway does project into the supragranular region in the ventral dentate gyrus (Cavazos et al., 1992).

There were differences in the spatial organization of the terminal fields of granule cells located in the suprapyramidal and infrapyramidal blades of normal rats. The mossy fiber axons of granule cells in the infrapyramidal blade arborized most extensively in the subgranular region of the hilus beneath the suprapyramidal blade but did not typically branch across the hilus toward the infrapyramidal blade. In contrast, the mossy fiber axons from granule cells in the infrapyramidal blade not only formed a plexus in the subgranular region of the hilus adjacent to the infrapyramidal blade but also crossed the hilus and formed terminal boutons in the subgranular region of the opposite suprapyramidal blade. These results confirmed previous observations about differences in mossy fiber organization in the blades of the dentate gyrus (Claiborne et al., 1986) and are potentially of interest. There is considerable evidence that the suprapyramidal and infrapyramidal blades develop with a different time course, have different neuronal populations, and show physiological differences in response to activation of afferent input from the entorhinal cortex (Schlessinger et al., 1975; Seress and Pokorny, 1981; Amaral et al., 1990; Golarai and Sutula, 1996).

The ability to directly study the terminal fields of sprouted pathway by using the biocytin and PHAL methods afforded an opportunity for more detailed analysis of the spatial distribution of the sprouted pathway along the transverse and septotemporal axes of the hippocampus. The majority of biocytin-filled granule cells sampled in this study of kainate-treated rats showed sprouted collaterals that projected into the supragranular region within the transverse plane of the hippocampus. Axons projecting from granule cells in the suprapyramidal and infrapyramidal blades sprouted into the adjacent supragranular regions of the blade of origin. In addition, axons arising from granule cells in the infrapyramidal blade also sprouted.
into the supragranular region of the opposite suprapyramidal blade. This finding, which was shown in individual neurons filled with biocytin and by PHAL, is evidence that sprouting may increase neural connectivity both within and between blades in the transverse plane of the dentate gyrus.

The distances spanned by the sprouted axons passing from blade to blade and the overall average increase in axon length due to the sprouted supragranular axon segments (more than 1 mm) may at first seem remarkable, but the actual length of axon growth by specific sprouted segments is most likely considerably less. Because axons of granule cells in the infrapyramidal blade of normal rats commonly project to the subgranular region of the opposite suprapyramidal blade, sprouted axons projecting from the infrapyramidal blade to the suprapyramidal blade in kainate-treated rats may arise from axons in the subgranular region of the suprapyramidal hilus. The axon growth required to extend into the supragranular region of the suprapyramidal blade, therefore, may be considerably less.

FIG. 10. Example of the analysis of the spatial distribution of the terminal field of PHAL-filled axons in a normal rat and the kainate-treated rat in Figure 9. The spatial distribution of the PHAL-filled fibers was determined by evaluating the location of PHAL-filled axons in a series of coronal sections of the dorsal hippocampus oriented as in the schematic inset. The injection sites are indicated by the solid black areas in the infrapyramidal blade, and the stippled black areas indicate the terminal field defined by PHAL-filled fibers. The distance of each section from the section containing the center of the injection site (indicated by "0") is on the right of section. In the normal rat, the PHAL-filled fibers do not extend into the supragranular region. The location of sprouted mossy fiber collaterals are indicated by the black stippled regions (arrows) that extend beyond the granule cell layer in the kainate-treated rat. Sprouted axons in the supragranular layer were observed only in sections that also contained mossy fibers in the hilus or CA3. Asterisks identify sections with sprouted supragranular collaterals that are farthest from the edge of the injection site. In this case, sprouted axons projected 300–500 µm beyond the injection site in the direction of the septotemporal axis.
than 1 mm. Furthermore, the average measured increase in axon length of more than 1 mm does not imply that growth of individual sprouted axons extends a projection for this distance, because the elaborate arborization of sprouted axons may increase the total length of the sprouted axon without extending the terminal field of the pathway by this same distance. In a recent study, it was suggested that aberrant mossy fiber outgrowth in models of temporal lobe epilepsy may arise from newly born granule cells (Parent et al., 1997). If this is indeed the case, growth of sprouted axons could be considerably greater than the above estimates.

In addition to prominent reorganization of the mossy fiber pathway in the transverse plane, analysis of the terminal field of granule cells injected in vitro with biocytin showed that the sprouted pathway also projects along the septotemporal axis of the hippocampal formation. This finding also was suggested by the in vivo observations with PHAL. Although the measured estimates of the septotemporal temporal divergence of sprouted collaterals are at best rough approximations on a limited sample and should be regarded as preliminary, these observations provide evidence that seizures alter neural connectivity not only in the local circuitry of the transverse plane but also in circuitry along the hippocampal septotemporal axis that normally lacks mossy fiber input. Measurement of the sprouted axon projection in this axis showed that individual axons grew for more than 300 μm along this axis. The major hippocampal pathways, which include the entorhinal-perforant path, the associational pathway in...
the dentate gyrus, and the pyramidal neurons of CA3, which are the principal targets of the mossy fibers and project to CA1, have prominently divergent organization along the septotemporal axis (Steward, 1976; Wyss, 1981; Witter and Amaral, 1991; Swanson et al., 1978; Ishizuka et al., 1990; Amaral, 1993). In contrast, the normal mossy fiber pathway in CA3 has a relatively restricted lamellar terminal field that is spatially limited to the transverse plane, except for the region near the transition from CA3 (and CA2) to CA1, where mossy fibers make an abrupt turn and travel caudally in a longitudinal direction (Gaarskjaer, 1986; Swanson et al., 1978; Amaral and Witter, 1989).

Although there is thus some degree of septotemporal divergence of mossy fibers in this location in the normal rat, the mossy fiber pathway is the only hippocampal fiber system that has relatively lamellar organization. From previous autoradiographic and PHAL studies in normal rats, the septotemporal divergence of mossy fibers near the CA3 transition to CA2 has been estimated to be approximately 1,650–2,000 μm (Swanson et al., 1978; Amaral and Witter, 1989). Analysis of biocytin-filled and PHAL-filled axons in this study support the interpretation that sprouted supragranular collaterals also diverge along the septotemporal axis (Fig. 11), and the preliminary estimates of the septotemporal divergence of the sprouted supragranular collaterals are comparable with the divergence of the CA3 projection in earlier studies (Swanson et al., 1978; Amaral and Witter, 1989).

Implications for functional effects of mossy fiber sprouting

The observations of this study may be helpful in efforts to understand the functional effects of the sprouted mossy fiber pathway. Although the methods of this study clearly do not address many critical issues, such as the relative number of reorganized inhibitory versus excitatory recurrent circuits, the extensive spatial distribution of the reorganized circuits found in this study is of interest.

In contrast to the relatively lamellar excitatory mossy fiber projection from the dentate gyrus of the normal rat, the terminal fields of inhibitory interneurons in the dentate gyrus have substantial divergent septotemporal projections (Strube et al., 1978; Buckmaster and Schwartzkroin, 1995). As a consequence of the development of divergent excitatory projections by sprouting of mossy fiber collaterals, it might be anticipated that seizure-induced sprouting could modify the apparent lamellar functional inhibition in the dentate gyrus (Andersen et al., 1971; Bertesaghi et al., 1983; Sloviter and Brisman, 1995).

The reorganization of connections in the supragranular region of the transverse plane, between the infrapyramidal and suprapyramidal blades within the transverse plane, and along the septotemporal axis of the dentate gyrus is consistent with the possibility that there may be substantial topographical and temporal alterations in physiological and computational processing in the reorganized hippocampus. In particular, seizure-induced hippocampal reorganization along the septotemporal axis may result in formation of recurrent circuitry that would be difficult to adequately assess in the typical in vitro transverse hippocampal slice, which could substantially underestimate the functional effects of the synaptic reorganization. The results of this study and continued efforts to elucidate the topography and the relative abundance of sprouted terminal boutons on inhibitory or excitatory neurons may be useful in the design and critical interpretation of physiological experiments that address the effects of activity-induced plasticity in hippocampal circuitry.

The possibility that reorganization of the terminal field of the sprouted pathway contributes to topographical and temporal alterations in physiological and computational processing is supported by recent observations of behavioral dysfunction in kindled rats (Sutula et al., 1995). Although it is difficult to establish the specific contribution of sprouting and other seizure-induced cellular alterations to memory dysfunction and epileptogenesis, continued efforts to characterize how seizure-induced structural plasticity alters hippocampal organization may eventually permit the development of realistic computational models and may help to clarify how alterations in hippocampal circuitry contribute to clinical conditions, such as epilepsy and memory disorders.

LITERATURE CITED


