Characterization of Target Cells for Aberrant Mossy Fiber Collaterals in the Dentate Gyrus of Epileptic Rat

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Previous studies have demonstrated formation of recurrent excitatory circuits between sprouted mossy fibers and granule cell dendrites in the inner molecular layer of the dentate gyrus (9, 28, 30). In addition, there is evidence that inhibitory nonprincipal cells also receive an input from sprouted mossy fibers (39). This study was undertaken to further characterize possible target cells for sprouted mossy fibers, using immunofluorescent staining for different calcium-binding proteins in combination with Timm histochemical staining for mossy fibers. Rats were injected intraperitoneally with kainic acid in order to induce epileptic convulsions and mossy fiber sprouting. After 2 months survival, hippocampal sections were immunostained for parvalbumin, calbindin D28k, or calretinin followed by Timm-staining. Under a fluorescent microscope, zinc-positive mossy fibers in epileptic rats were found to surround parvalbumin-containing neurons in the granule cell layer and to follow their dendrites, which extended toward the molecular layer. In addition, dendrites of calbindin D28k-containing cells were covered by multiple mossy fiber terminals in the inner molecular layer. However, the calretinin-containing cell bodies in the granule cell layer did not receive any contacts from the sprouted fibers. Electron microscopic analysis revealed that typical Timm-positive mossy fiber terminals established several asymmetrical synapses with the soma and dendrites of nonpyramidal cells within the granule cell layer. These results provide direct evidence that, in addition to recurrent excitatory connections, inhibitory circuitries, especially those responsible for the perisomatic feedback inhibition, are formed as a result of mossy fiber sprouting in experimental epilepsy.

INTRODUCTION

Mossy fiber sprouting is a well-known phenomenon both in human temporal lobe epilepsy and in experimental models of epilepsy (3, 16, 17, 29, 42, 43, 44). The axons of the granule cells, the mossy fibers, which normally terminate in the hilus and stratum lucidum of the CA3 subfield, sprout and project through the granule cell layer toward the molecular layer, forming an abnormal termination field in the inner molecular layer (24).

It has been proposed that sprouting of the mossy fibers results in formation of recurrent excitatory granule cell–granule cell circuits. This, in turn, has been hypothetized to be the mechanism which leads to increased seizure susceptibility in the dentate gyrus (3, 29, 42, 43). Indeed, anatomical findings have provided strong support for this assumption. First Frotscher and Zimmer (9) demonstrated at the electron microscopic level synaptic contacts between the mossy fibers and the apical dendrites of the granule cells in lesion-induced sprouting in the rat. Later Represa et al. (30) and Okazaki et al. (28) found that similar connections are also formed in kindling and in the kainic acid model of epilepsy. However, recent findings have indicated that inhibitory nonprincipal cells are also involved in newly formed circuitries (7). Sloviter (39) found that in animals treated with kainic acid, granule cell hyperactivity was present before reorganization of mossy fibers had taken place, but after sprouting had occurred, recurrent inhibition increased. Furthermore, in the same study it was found that two months after treatment with kainic acid, some nonprincipal cells in the inner molecular layer and the granule cell layer were surrounded by Timm-stained mossy fibers. It was suggested that these new contacts between aberrant granule cell axon collaterals and inhibitory nonprincipal neurons may be responsible for the observed restoration of recurrent inhibition.

The dentate gyrus contains several types of nonpyramidal cells which are in a position to receive an input from the mossy fiber collaterals that sprout toward the molecular layer in epilepsy; i.e., either their soma is located within the granule cell or the molecular layer or their dendrites extend there (13, 14). Typically these include parvalbumin- (PV), calbindin D28k- (CaBP), calretinin- (CR), neuropeptide Y-, cholecystokinin-, and vasoactive intestinal polypeptide-containing neurons (12, 23, 27, 37, 40). However, although their location and pattern of dendritic arborization may resemble each other, their input–output relationships vary considerably. Findings obtained in anatomical and electrophysiological studies indicate that different neurope-
tides and calcium binding proteins are useful markers for distinct subgroups of interneurons that may have different functional role in the neuronal circuits of the dentate gyrus (1, 11, 19, 26, 34, 40). Therefore the present study was undertaken to characterize the target cells for the sprouted mossy fibers using a combination of immunofluorescent staining for PV, CaBP, and CR and histochemical Timm-staining for mossy fibers on the same hippocampal sections. Synaptic contacts between sprouted mossy fibers and target cells were studied at the electron microscopic level.

MATERIALS AND METHODS

Animal Treatment

Sixteen male Han:Wistar rats weighing 250–350 g were used at the age of 10 to 12 weeks. The rats were housed individually and were maintained at constant temperature (20 ± 1°C), humidity (50–60%), and light-dark cycle (lighting on 0700–1900). Standard food and water were available ad libitum. The animal studies were approved by the Ethics Committee of the University of Kuopio and by the Provincial Government of Kuopio.

Kainic acid (2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine, Sigma K-0250) was dissolved in 0.9% NaCl and the concentration adjusted to 5 mg/ml. It was injected intraperitoneally in a volume of 1.8 ml/kg (9 mg/kg) to 11 rats. Five control rats received only 0.9% NaCl. The rats were observed for at least 3 h after kainic acid injection. Only those animals which developed generalized seizures were included in the study.

Histological Procedures

Two months after kainic acid treatment, the animals were deeply anesthetized with chlorpromazine and perfused transcardially first with 0.37% sodium sulphide solution in 0.1 M phosphate buffer, pH 7.4 (PB), followed by 4% paraformaldehyde in PB (36). After 4 h postfixation with 4% paraformaldehyde in PB, coronal sections of 40-µm thickness were cut with a freezing microtome.

The sections were Nissl-stained to demonstrate loss of neurons. Adjacent sections were immunostained either for PV, CaBP, or CR using fluorescent-labeled antibodies. The following procedure was carried out at room temperature with constant agitation, unless stated otherwise. The sections were first washed in PB and incubated in 10% normal goat serum for 40 min. This step was followed by incubation in rabbit anti-PV (37) (R301, 1:300, a gift from Dr. K. G. Baimbridge, University of British Columbia, Vancouver, Canada), rabbit anti-CaBP (2) (R202, 1:1000, also a gift from Dr. K. G. Baimbridge) or rabbit anti-calretinin (1:3000, Swant) for 1 day. The second layer was biotinylated goat anti-rabbit IgG (1:50, Vector, overnight at 4°C). The third layer was fluorescein (FITC)-labelled avidin (1:100, Vector, 3 h). All washing and antibody dilutions were done in 0.05 M Tris-buffered saline, pH 7.4, containing 1% normal goat serum and 0.5% Triton X-100. Triton X-100 was omitted from the FITC-avidin solution. After immunostaining, the sections were mounted on the gelatin-coated slides and, to demonstrate the mossy fibers, stained using the Timm sulphide silver histochemical procedure of Danscher (6) modified by Sloviter (36).

Fluorescent Microscopy

Sections were analyzed under a Nikon Optiphot-2 fluorescence microscope equipped with a mercury lamp and epifluorescence optics. FITC was excited at 450–490 nm and emission was detected using a 520-nm barrier filter. Photomicrographs were taken from areas of interest during the analysis.

Electron Microscopy

For electron microscopy, adjacent sections (without immunostaining) were Timm-stained, osmicated, dehydrated, and embedded in Durcupan ACM. Areas of interest were cut out of the sections and reembedded. Ultrathin serial sections were cut with an ultramicrotome on copper grids and stained with uranyl acetate for 60 min and with lead citrate for 4 min.

RESULTS

Timm-Staining

Timm-staining was used to demonstrate mossy fibers in the dentate gyrus, because it selectively labels mossy fiber terminals due to their high content of zinc (15, 35). In the dentate gyrus of a control animal, the darkest Timm-staining was found in the hilus. Only a few Timm-labeled mossy fibers extended into the granule cell layer (Fig. 1). In the dentate gyrus of epileptic rats, an aberrant distribution of mossy fibers was observed. Numerous collaterals of Timm-labeled mossy fibers ran through the granule cell layer toward the inner molecular layer where they formed a dark band of terminals (Fig. 1). Nissl-staining demonstrated loss of hilar neurons and CA3c pyramidal cells together with accumulation of gial cells in the epileptic rats compared to the controls (Fig. 1).

Fluorescent Microscopy

In the present study the combination of Timm-staining for mossy fibers together with immunofluorescent staining for PV-, CaBP-, and CR-containing neurons on the same sections was found to be useful technique for characterizing possible target cells. The FITC-label produced a bright green fluorescent color.
for the immunopositive neurons, and the Timm-staining labeled terminals of the mossy fibers as dark brown or black. The combination of these two stainings produced a fine contrast between black fibers and green neurons, such that possible contacts were easy to observe.

The distribution and morphology of PV-, CaBP-, and CR-immunoreactive neurons in the rat dentate gyrus were similar to that described previously (2, 12, 27, 33, 37). The majority of PV-immunopositive cell bodies were located within or near the granule cell layer and less frequently in the hilus and molecular layer of the dentate gyrus. In the granule cell layer, some PV-positive cell bodies showed typical features of the pyramidal basket cells. The cell bodies of the granule cells were surrounded by PV-immunoreactive terminals. CaBP-immunoreactivity was present in the granule cells and their axons, the mossy fibers. In addition, a subpopulation of hilar nonprincipal cells were also immunopositive for CaBP. Soma of CR-positive nonprincipal cells were found in all layers of the dentate gyrus. In the molecular layer and the hilus, CR-containing cells were multipolar, bipolar, or irregularly shaped, but in the granule cell layer, some CR-positive cells were pyramidal shaped. The CR-immunoreactive dendrites were weakly stained which did not permit the analysis of possible mossy fiber contacts on CR-containing dendrites.

The proportion of PV-immunopositive cells contacted by aberrant mossy fibers in the granule cell layer of the control and epileptic rats was evaluated semiquantitatively. This approximation was made by investigating 230 PV-containing cells in the granule cell layer of six epileptic rats and 42 PV-containing cells of four control rats. It was found that in epileptic rats approximately 80% of PV-positive neurons in the granule cell layer

FIG. 1. Photomicrographs of the Nissl-stained (A, B) and Timm-stained (C, D) dentate gyrus of a control (A, C) and a kainate-treated rat (B, D). The Nissl-staining of the control animal (A) demonstrates the normal distribution of the CA3c pyramidal cells (arrows) and hilar nonpyramidal cells (small arrows). Two months after kainate-treatment (B) only a few CA3c pyramidal cells (arrows) and hilar nonpyramidal cells (small arrows) are present. Note the accumulation of glial cells (arrowheads) in the hilus of the kainate-treated animal. In the dentate gyrus of the control animal, the mossy fibers are restricted to the hilus (C). Only a few mossy fiber collaterals (open arrows) can be observed in the granule cell layer. In contrast, in the kainate-treated rat (D), several mossy fibers extend through the granule cell layer and form an extensive band of mossy fiber terminals to the inner molecular layer (large arrowheads). Scale bars: A, 50 μm; B, 100 μm.
received contacts from the mossy fibers (Fig. 2), whereas in control rats, intranuclear mossy fibers established contacts with about 40% of PV-immunoreactive cells. Furthermore, in all of the epileptic rats the PV-containing cell bodies, as well as their dendrites were almost entirely covered by the Timm-labeled fibers, whereas in controls, only a few boutons were found per PV-positive cells in the granule cell layer.

Analysis of double staining for Timm and CaBP or CR, revealed that also a few CaBP- or CR-positive neurons received contacts from sparse Timm-stained granules in the granule cell layer of control animals. In kainate-treated animals, the CaBP-containing dendrites in the inner molecular received multiple contacts from the mossy fiber collaterals (Fig. 2). In addition, in those epileptic rats that had extensive mossy fiber sprouting, the CaBP-containing cell bodies and their dendrites running through the granule cell layer were covered with numerous mossy fiber terminals (Fig. 2). In contrast, none of the kainate-treated rats were found to have aberrant mossy fiber terminals which had established multiple contacts with the CR-immunoreactive cell bodies in the granule cell layer (not shown).

Electron Microscopy

In the hilus of the control animals, Timm-staining was restricted to the synaptic terminals. These termi-
nals had the ultrastructure characteristic of the mossy fiber terminals: they were notably large, they made multiple asymmetrical synapses with postsynaptic structures, and they were full of tightly packed round vesicles (4, 10). Similar terminals were never found in the molecular layer of the control animals. In contrast, in animals treated with kainic acid, a large number of Timm-positive terminals, which had characteristic features of mossy fiber terminals, were found in the granule cell layer and the inner molecular layer (Fig. 3).

In the granule cell layer, the aberrant mossy fiber terminals established asymmetrical synapses with the soma of large neurons. The nucleus of these neurons contained euchromatin and a centrally located nucleolus and nuclear inclusions such as intranuclear rods and sheets. In addition, the nuclear membrane of these neurons was usually ruffled or infolded (Fig. 3). In serial sections of these neurons, it was possible to follow the apical dendrite that passed through the granule cell layer toward the inner molecular layer. The basal dendrites extended to the hilus. The apical dendrites of these cells received multiple asymmetrical synaptic contacts from Timm-labeled axon terminals (Fig. 3). In addition, similar terminals were found to establish asymmetrical synapses with some large and round cell bodies in the inner molecular layer. The nucleus of these neurons was also invaginated and contained nuclear inclusions.

**DISCUSSION**

Our present findings at both the light and electron microscopic level show that the basket cells are among the target neurons for the sprouted mossy fiber collaterals in epileptic rats. By combining fluorescent immunostaining with Timm-staining, we were able to demonstrate that the target cells contained PV, a marker for nonpyramidal basket cells (18, 22, 27, 37). Furthermore, the electron microscopic analysis revealed that the aberrant mossy fiber terminals in the granule cell layer and the inner molecular layer made multiple contacts with the neurons that had typical ultrastructural features of nonpyramidal basket cells (31). These cells had abundant cytoplasm with rough endoplasmic reticulum. The nucleus often had inclusions such as intranuclear rods and sheets and the nuclear envelope was infolded. In addition, the nucleolus of these cells was usually centrally located.

The CaBP-containing dendrites were also found to receive multiple contacts from the mossy fibers in the inner molecular layer. These contacts may represent the recurrent inputs on the CaBP-containing granule cells (9, 28, 30). However, we cannot rule out the possibility that some of the CaBP-positive dendrites that received innervation from the aberrant mossy fibers in the inner molecular layer were the dendrites of the CaBP-immunoreactive nonpyramidal neurons. Nevertheless, in rats that had robust mossy fiber sprouting, the CaBP-containing neurons, which were the size and shape of granule cells, were intensively innervated by the recurrent collaterals of the mossy fibers. This suggests that at least in the most severe cases, granule cells are included in the targets for the sprouted mossy fibers. Furthermore, the above observations show—in agreement with previous reports (see review Dudek et al., 1994)—that the innervation pattern of the sprouted mossy fibers can vary between the cases.

The analysis of double staining for CR and Timm revealed that although the granule cell layer and the molecular layer contain a considerable number of CR-positive cell bodies that could also have been in a position to receive input from the sprouted mossy fibers, mossy fibers were found not to establish more contacts with them in epileptic rats than in controls. However, the weak dendritic staining of CR-immunoreactive cells did not allow us to investigate in detail the possible contacts on CR-positive dendrites that arborized in the inner molecular layer. However, the soma of the CR-containing neurons did not receive multiple contacts from aberrant mossy fibers even in those epileptic rats that had massive sprouting.

The above findings agree with the earlier observations that the mossy fibers may form recurrent collaterals with the granule cells (9, 28, 30). However, our study shows that inhibitory neurons belong to the target cells. Intragranular mossy fibers have been found to establish synaptic contacts with the basket cells in the granule cell layer also in normal rats (32). Analyses of epileptic rats in the present study revealed that the number of PV-neurons which received innervation from the mossy fibers in the granule cell layer was increased in epileptic rats when compared with the controls. In addition, in epileptic rats, Timm-labeled terminals established abundant contacts on PV-positive cells covering almost entirely their cell bodies and dendrites extending through the granule cell layer. In the controls, there were only a few contacts on the average PV cell.

The increased innervation of the PV-containing cells after epileptic seizures may be of crucial functional importance. PV is present in the basket (33) and chandelier cells (25, 41), which are known to terminate in the perisomatic region of the granule cells. They can cause powerful feedback inhibition on the granule cells. This is probably mediated via the GABA<sub>A</sub> receptors (19, 20, 21). Therefore, since we found that the mossy fibers increased innervation, especially of PV-containing inhibitory cells during sprouting, our observations provide an anatomical basis for the restoration of GABA<sub>A</sub>-receptor mediated feedback inhibition that has been observed to occur in the epileptic dentate gyrus (39).
FIG. 3. Electron micrograph of the nonpyramidal cell in the granule cell layer of the kainate-treated rat. (A) The apical dendrite of the basket cell is running through the granule cell layer toward the molecular layer. Several mossy fiber terminals are in synaptic contact with the aspinous proximal dendrite. Some of those terminals are indicated by black boxes and shown at higher magnification in the corresponding
This mechanism could be the reason why mossy fiber sprouting could have a strong inhibitory effect on the hyperactive dentate gyrus and restore the fine balance between excitation and inhibition. Electrophysiological studies have indeed shown that granule cells exhibit normal responses to the hilar- and perforant pathway-stimulation at the time points when mossy fiber sprouting has taken place (5, 39).

Sloviter suggested in his “dormant basket cell hypothesis” that loss of inhibition in epileptic hippocampus may be a consequence of decreased innervation of inhibitory basket cells by the excitatory hilar mossy cells that become degenerated soon after the excitotoxic insult (38). He also observed that the lost inhibition can possibly be restored by activation of basket cells via intact connections from contralateral hippocampus. With respect to the “dormant basket cell hypothesis” one could speculate that initially inputs originating from the hilar mossy cells are lost and the synaptic sites are vacated on the surface of the PV-containing nonpyramidal neurons. Subsequently, these synaptic sites are occupied by the mossy fiber collaterals that already existed on the PV-neurons normally, though to a lesser extent. This agrees with the finding that during the first week after kainate injection, inhibition of the granule cells is decreased, but after 2 months the inhibition is restored (39). Since these changes coincide with the occurrence of the mossy fiber sprouting, normalization of the granule cell responses at longer time delays indicate that inhibitory cells may have been recruited somehow into the circuits of the granule cells. This proposal is in line with our present findings of enhanced innervation of PV-containing neurons by the mossy fibers in epileptic rats. Furthermore, since excitability of the granule cells was found to be sensitive to a GABA_A receptor antagonist, bicuculline (granule cells showing hyperexcitability only after blockade of GABA_A receptors (5)), involvement of PV-containing cells may be physiologically relevant. It is interesting to note that similar electrophysiological data has also been obtained in slices of human epileptic hippocampus that have undergone mossy fiber sprouting (8). Therefore, anatomical studies similar to our current experiment could be very useful for investigating the complex network interactions that underlie synaptic reorganization also in human temporal lobe epilepsy.

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REFERENCES


figures, C–G. Note the relative thickness of the dendrite when compared to the size of the surrounding granule cells. (B) In serial ultrathin sections, the soma of this cell is found. The nucleus of the cell contains intranuclear sheets (open arrow) and a large centrally located nucleolus. Mild ruffling of the nuclear envelope can also be seen (arrows). All these ultrastructural features suggest that the nonpyramidal cell is a basket cell. At higher magnifications (C–G) the asymmetrical synaptic contacts (arrowheads) are shown between the Timm-stained terminals of the sprouted mossy fibers and the apical dendrite. The synaptic cleft and postsynaptic density can be seen clearly in most of these terminals. Note that black silver granules of the Timm-staining are completely restricted to the mossy fiber terminals, where they often are concentrated near the synaptic site. Scale bars: A, 2 μm; B, 1 μm; C–G, 200 nm.


