



# Early Aberrant Growth of Mossy Fibers after Status Epilepticus in the Immature Rat Brain

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## Abstract

Axonal sprouting is recognized to be an important mean of repair after neurologic injury. Some characteristic aftermaths of pilocarpine-induced status epilepticus (SE) in the immature rat are nerve cell loss and rearrangement of neuronal fibers. SE induced cell degeneration exclusively in the hippocampal CA1 subfield. Development of neuronal death becomes evident within hours after SE, following a delayed time course ranging from 6 to 48 h post-SE. An incidental finding is that pilocarpine induces within 48 h an aberrant growth of hippocampal mossy fibers in the hippocampus, especially in the infrapyramidal region of the CA3-subfield. We found a strong infrapyramidal band of mossy fibers along the entire stratum oriens of the CA3-region. No mossy fibers sprouting into the inner molecular layer of the dentate gyrus, or CA1 sprouting into the stratum moleculare of CA1 were noted. Signs of aberrant connectivity were found in six of the 10 pilocarpine-treated animals. This study provides the demonstration that pilocarpine within 48 h consistently results in the formation of ectopic hippocampal mossy fibers in a 2-week-old pup. This indicates a high degree of axonal reorganization in the hippocampus. It remains controversial whether such reorganization is the cause or consequence of chronic seizures. We assume that these additional infrapyramidal mossy fibers may influence the way in which granule cells drive pyramidal cells in CA3.

**Keywords** Status epilepticus · Pilocarpine · Hippocampus · Mossy fibers · Immature rat

## Introduction

The hippocampal formation in animal models of epilepsy exhibits several anatomical malformations, including neuronal loss, aberrant growth of neurites [1], dispersion of the granule cell layer, enhanced neurogenesis [2], and sclerosis [3, 4]. These events may alter physiological properties of the hippocampal network. It is likely, that newborn and dispersed granule cells may lead to aberrant neuronal integration, including abnormal axonal and dendritic

reorganizations. These changes seem to be closely related with frequent seizures and status epilepticus [4, 5].

In adult animals, status epilepticus induces cell death in all hippocampal subfields as well as in the dentate gyrus [6] and leads to mossy fibers sprouting exclusively in the fascia dentata [5, 7]. In contrast, SE in the immature animal results in less cell loss and sprouting [8]. However, by analyzing the relevant scientific literature, we observe that the mossy fiber sprouting in adult and immature animals has been extensively evaluated in the dentate gyrus, and less in the regio inferior (CA3) of the hippocampal formation.

Therefore, we evaluated the effects of pilocarpine on changes in mossy fibers distribution in the regio inferior (CA3) of the hippocampal formation in the immature rat. There were clear differences in the amount of mossy fibers seen in the pilocarpine-treated animals compared to controls. We found signs of aberrant connectivity in six of the 10 pilocarpine-treated animals. This study provides the demonstration that pilocarpine within 48 h consistently results in the formation of ectopic hippocampal mossy fibers in a 2-week-old pup.

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## Materials and Methods

**Animals** P14 Wistar rat pups of either sex (Simonsen Lab, Gilroy, CA) were used. The day of birth was considered as day 0. Pups were housed with their dams in a temperature- and humidity-controlled room with 12 h light–dark cycles (7 a.m.—7 p.m.) and had free access to food. All experiments were conducted with the approval and in accordance with the regulations of the Institutional Animal Care and Use Committee of West Los Angeles VA Medical Center (USA).

**Induction of SE** Thirteen-day-old (P13) rat pups were administered with lithium chloride (3 mEq/kg; L-0505 Sigma, St. Louis, MO, USA) intraperitoneally (i.p.), and 20 h later, SE was induced with subcutaneous injection of pilocarpine hydrochloride (60 mg/kg; P6503 Sigma) as described previously [9]. Control rats were given an equal volume of saline subcutaneously. Only animals reaching SE (defined as near-continuous seizure activity lasting over 10 min) were included in the study. After SE, pups received 5% of their body weight of isotonic 5% dextrose in water subcutaneously to avoid dehydration without stressing the cardiovascular system. Temperature and time of separation from the mother were strictly controlled since separation alone can trigger neuronal apoptosis in rat pups.

**Preparation of Tissue for Immunohistochemistry** Rats were anesthetized with an overdose of pentobarbital (100 mg/kg i.p.) 6 ( $n = 6$ ), 24 ( $n = 6$ ), and 48 h ( $n = 6$ ) after induction of SE. Then, pups underwent transcardiac perfusion with 4% phosphate-buffered paraformaldehyde. Control animals ( $n = 6$ ) were subjected to the same procedure. Brains were kept in situ at 4 °C overnight, after which they were removed and postfixed in the same perfusate for 2–3 h. The brains were removed from the skull, postfixed in the same fixative, and placed overnight in PBS containing 30% sucrose. The brains were kept at 4 °C in PBS until coronal sections (12- $\mu$ m thick) were cut on a cryostat. Due to the curvature of the hippocampus, with the septal end extending along the anterior-posterior axis and the temporal end extending along the dorsal-ventral axis, all analyses were limited at the mid-septo-temporal levels of the hippocampus.

For immunofluorescence, slides were preincubated for 1 h at room temperature in PBS, 5% NGS (Sigma), and primary antibodies (calbindin-D28 (Swant), synaptophysin and PSD95- (cell signaling)) were applied at 4 °C for 24 h in PBS plus 5% normal goat serum. Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit secondary antibodies were used at a 1:200 dilution (Molecular Probes, Göttingen, Germany). After several washes in 0.1 M PBS, sections were incubated in Alexa 488- or 568-conjugated anti-mouse IgG (1:200, 2 h, room temperature, in 0.1 M PBS; Molecular Probes, Eugene). After rinsing the sections with 0.1 M PBS, they were mounted in Dako fluorescent mounting medium (Dako). To ensure comparable immunostaining,

sections were processed together at the same time under the same conditions. For the assessment of non-specific immunostaining, alternating sections from each experimental group were incubated without the primary antibody. All tissue sections examined in this study were processed simultaneously so that they would be exposed to exactly the same concentrations for exactly the same periods of time.

Quantification of the infrapyramidal mossy fibers bundle (IMF) length was performed using the ratio of the IMF length to the length of the CA3 (“normalized IMF length”) as described [10]. The IMF length was measured from the tip of the inferior blade of the granule cell layer (“x”). The length of the CA3 was measured from the tip of the inferior blade to the apex of the curvature of the CA3 pyramidal cell layer (“y”). Normalized IMF length was taken as  $x/y$ . The statistical significance of differences in normalized IMF length between the groups was analyzed by the two-tailed  $t$  test.

### Detection of Neuronal Degeneration, Fluoro-Jade Staining

For Fluoro-Jade (FJ) staining, sections were initially incubated in 1% alcohol-NaOH for 5 min and then transferred to 70% ethanol for 2 min. They were then washed in aqua bidest. For 2 min and transferred to a 0.06% potassium permanganate (Sigma-Aldrich, St. Louis, USA) solution for 10 min in order to reduce background staining. The actual FJ staining was performed by opaquely incubating the sections in a solution of 0.0004% Fluoro-Jade B (Millipore, Billerica, USA) for 20 min with gentle shaking. After washing the sections ( $3 \times 1$  min), they were dried for 1 h and finally mounted by using Entellan (Merck, Darmstadt, Germany) after they were rinsed in xylene for 1 min. Fluoro-Jade-positive cells in the CA1 pyramidal cell layer were then visualized by using fluorescence microscopy.

### Determination of Neuronal Apoptotic Death, TUNEL Staining

In Situ Cell Death Detection Kit (Roche, Germany) was employed for the detection of DNA fragmentation and apoptotic bodies in hippocampal cells. Normal nuclei did not stain with the TUNEL method and cells exhibiting a necrotic morphology in some instances, displayed a faint, diffuse-staining pattern.

**Digital Illustrations** Fluorescent images were acquired using an Axio-Cam digital camera mounted on a Zeiss microscope (Carl Zeiss, Jena, Germany). Single-fluorescent images of the same section were digitally superimposed.

## Results

### Cell Damage Induced by SE in the Hippocampus

The cholinergic agent pilocarpine produces limbic and generalized SE in rodents accompanied by a widespread

brain damage. Our results show the enhanced vulnerability of neurons in the hippocampal CA1-subfield. SE induced cell degeneration exclusively in the hippocampal CA1 subfield. Development of neuronal death becomes evident within hours after SE, following a delayed time course ranging from 6 to 48 h post-SE (Fig. 1A–B). Necrotic as well as apoptotic cell death was observed upon induction of SE. Fluorescent labeling was never observed in controls (data not shown).

### Identification of the Aberrant Infrapyramidal Mossy Fibers Bundle

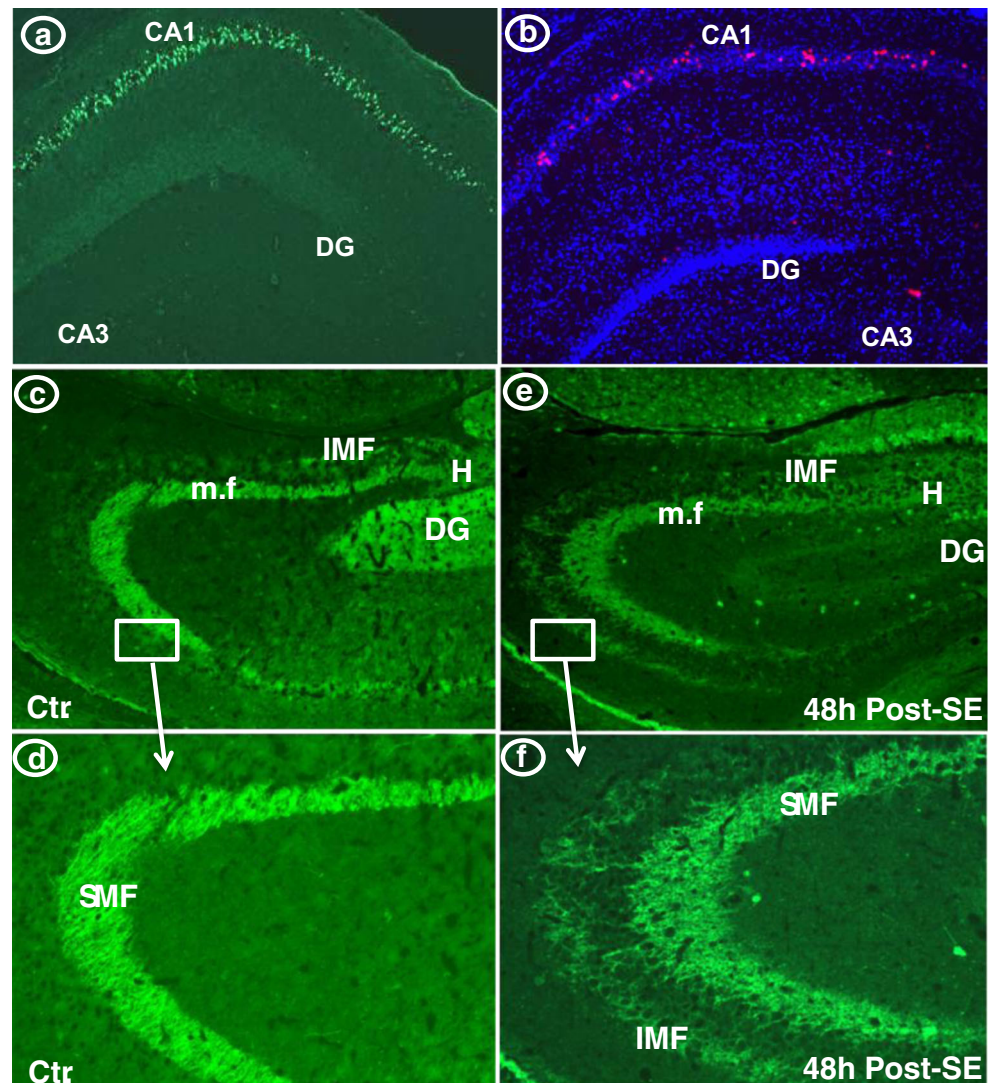
We visualized the mossy fibers by calbindin staining. Calbindin is a  $Ca^{2+}$ -binding protein which shows a characteristic spatial pattern of expression in the hippocampus and is a marker of the mossy fibers [11]. In control rats, stained mossy fibers originated from the granule cells and entered the hilus, where they build a dense plexus (Fig. 1C–D). In the hilus, a band of fibers was

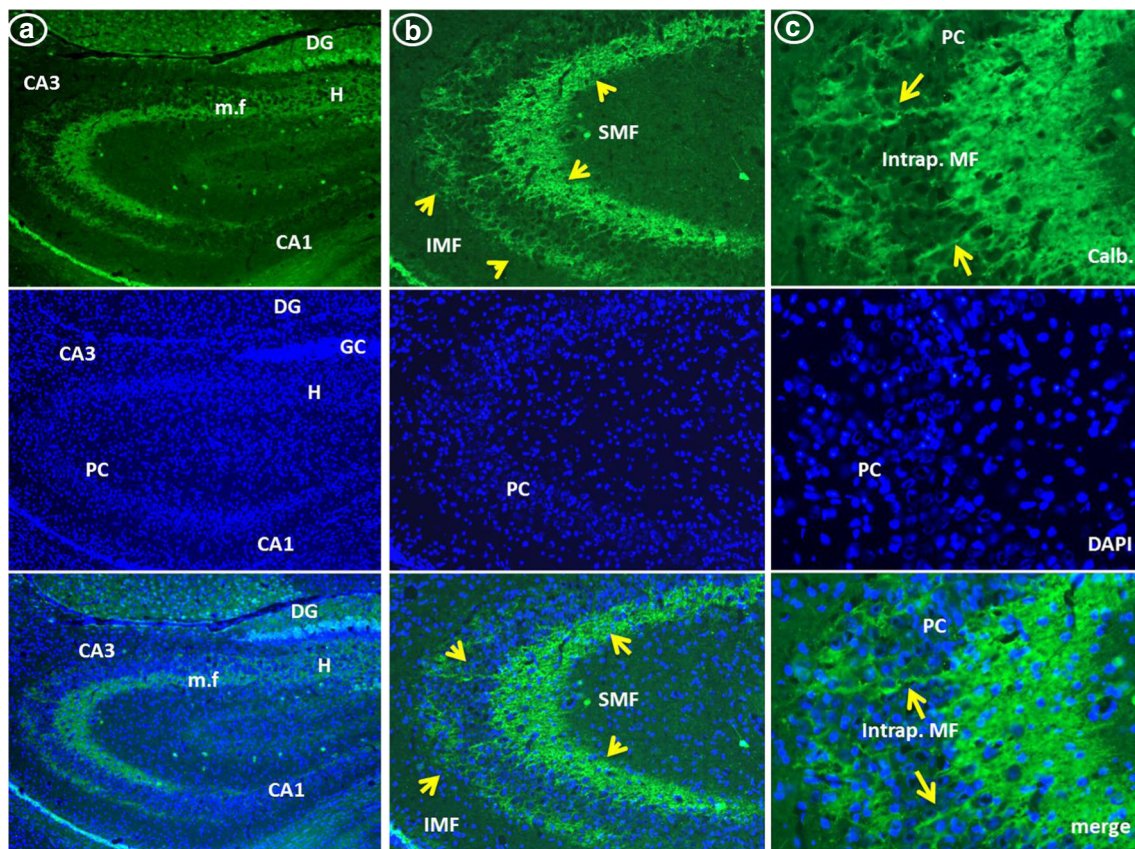
formed transversing the long axis of the hippocampus, pursuing a close course to CA3, and ending in a dense band in the stratum lucidum. The stratum lucidum corresponds to the suprapyramidal band where mossy fibers innervate the apical dendrites of the pyramidal cells. The infrapyramidal bundle of the mossy fibers crossed the stratum pyramidale to join the suprapyramidal bundle before extending to the apex of the curvature of the CA3-region (Fig. 1C, D).

In 60% of animals treated with pilocarpine, a dense layer of immunostaining is consistently located in the infrapyramidal section of the CA3-region (Fig. 1E, F & Fig. 2). Some of these axons cross the pyramidal cell layer, suggesting that some infrapyramidal mossy fibers originate from the suprapyramidal bundle. The abnormally long IMF bundle is likely to form ectopic synapses. Quantitative analysis showed that the IMF in the hippocampus of pilocarpine treated was much longer than that in the control rats (Fig. 2D).

In addition, we have used immunostaining of synaptophysin and PSD95 to identify mossy fibers synaptic structures in the

**Fig. 1** Fluoro-jade B histochemistry indicating neuronal injury in the rat hippocampus 24 h following SE. Note the selective neurodegeneration (in green) in the CA1-region (A) and TUNEL-positive cells (in red) in the CA1-region (B), indicating occurrence of apoptotic cell death. Abbreviations: CA1 and CA3 are hippocampal subfields; DG, dentate gyrus. The mossy fiber trajectory in the hippocampus of both control rats (C, D) and rats surviving 2 days after SE (E–F). The mossy fibers were stained with the anti-calbindin. (DG, dentate gyrus; H, Hilus; mf, mossy fibers; PC, pyramidal cells; GC, granule cells; SMF, suprapyramidal mossy fibers; IMF, infrapyramidal mossy fibers; CA1 and CA3 are hippocampal subfields)





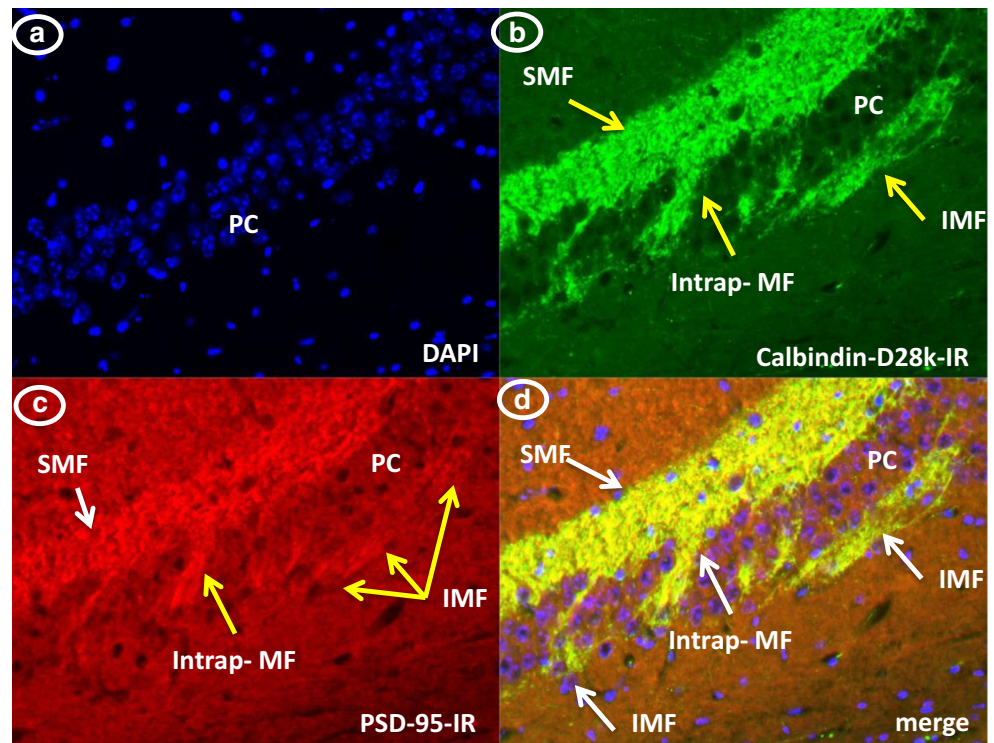
**Fig. 2** Calbindin-staining in pilocarpine-treated animals. Digital photomicrographs of transverse section through the mid-septotemporal part of the hippocampal formation indicate calbindin-staining in the dentate gyrus and mossy fibers (A). Higher power images (B, C) of the dentate gyrus from the section shown in A, indicating a clear calbindin-staining in the infrapyramidal mossy fibers bundle (DG, dentate gyrus; H, Hilus; mf, mossy fibers; PC, pyramidal cells; GC, granule cells; SMF,

suprapyramidal mossy fibers; IMF, infrapyramidal mossy fibers, Intrap. MF, intrapyramidal mossy fibers; Calb, calbindin; CA1 and CA3 are hippocampal subfields). D Quantitative analysis of the normalized length of the IMF in controls and in post-SE animals (\*\* $P < 0.01$  vs. controls). The results shown in panels A and B and C and D are the representatives of three independent experiments. The normalized lengths of the IMF shown in panel E are the mean of  $\pm$  SEM

hippocampal CA3-subfield. Synaptophysin antibodies were used previously to identify giant mossy fiber terminals in the hippocampal tissues, and PSD95 serves as a useful marker of excitatory synapses on neurons. Because of densely packed

neuropil in brain sections, it was difficult to isolate clear immunostaining for synaptophysin (data not shown). Nevertheless, we succeeded in detecting the postsynaptic marker PSD-95 on aberrant mossy fibers (Fig. 3). The immunostaining pattern we

**Fig. 3** Digital photomicrographs indicate calbindin-D28k (green in B) and PSD-95 (red in C) immunostaining in the hippocampal CA3 subfield indicating a clear colocalization of calbindin-D28k and PSD-95 (yellow in D) in the aberrant infrapyramidal mossy fibers bundle. A shows DAPI (blue) positive nuclei in the CA3 pyramidal cells (PC, pyramidal cells; SMF, suprapyramidal mossy fibers; IMF, infrapyramidal mossy fibers, Intrap. MF, intrapyramidal mossy fibers)



observed was consistent with a distribution on mossy fibers. Calbindin-D28- and PSD-95-immunolabeling patterns were relatively identical, and there was a clear spatial colocalization of calbindin-D28 with PSD95 puncta.

## Discussion

Granule cells of the dentate gyrus have the typical morphology of bipolar cells. Their apical dendrites arise from one pole and form the molecular layer, while the axon originates from the base of the cell body and extends into the hilus subjacent to the granule cell layer. The mossy fibers pass into the hilus of the dentate gyrus, then along the pyramidal cell layer largely transverse to the septotemporal axis of the hippocampal formation [12]. Most of these axons project on the apical dendrites of pyramidal cells, forming a suprapyramidal mossy fibers bundle, which extends until the border between the regio inferior (CA3) and regio superior (CA1). In the hilus, some of these axons enter through and under the pyramidal cell layer, making synapses with both basal and apical dendrites of pyramidal cells and joining finally the suprapyramidal bundle of mossy fibers [12]. Normally, mossy fibers terminate exclusively on dendrites in stratum lucidum of the CA3 pyramidal cells and hilus respectively. However, here, they terminate in addition in the stratum oriens along the whole CA3 region. These aberrant infrapyramidal mossy fibers are most likely mossy

fiber synapses according to colocalization of calbindin, which is mainly expressed in granule cells.

Synaptic reorganization induced by excitotoxic injury in the immature brain has been shown in the distal projection of the mossy fibers to the stratum oriens of the CA3 pyramidal region when the animals were tested as adults [13, 14]. In contrast, our study describes similar reorganization of the mossy fiber projection to the CA3 region when animals are tested in an early life (2 days after SE). This novel and robust sprouting response from granule neurons into the CA3-subfield adds another excitatory circuit in the hippocampus. The temporal course of development of this novel projection into the hippocampus is unknown. However, unlike the short mossy fibers sprouting in the fascia dentate seen in adults, the sprouting into the CA3 seen here is relatively long because of the distance between the granule cells and pyramidal cells.

The morphogenesis of the hippocampal formation during development depends on chemical and temporal factors that control the spatiotemporal relationships between pyramidal and granule cells. The formation of apical dendrites which takes place earlier during development than basal dendrites [12], may explain the high density of mossy fibers on the suprapyramidal part comparing to the infrapyramidal one. It seems that pilocarpine alters consistently these processes and it is not excluded that the postnatal pilocarpine-treatment led to the ectopic

formation of synapses infrapyramidally. In this context, Wasterlain et al. [15] have shown that seizure-induced slightly granule cell neurogenesis at a time of intense granule cell neurogenesis. This corroborates the assumption that the abnormally distributed mossy fibers in the pilocarpine group may be a result of the exacerbated growth of postnatally generated granule cell axons and of an acceleration of granule cell differentiation.

Another mechanism by which pilocarpine results in the formation of ectopic hippocampal mossy fibers in our animals may be that seizures in rat pups trigger a cascade of physiological changes that alters the balance between neurogenesis and synaptogenesis.

Many studies have reported an increase in dentate granule cell neurogenesis after seizures in adult animals [16, 17]. Increased neurogenesis after seizure-induced cell loss may result in new and aberrant neuronal circuits. The effects of seizures on granule cell proliferation in the newborn rats, at a time of intense granule cell neurogenesis, remain primarily unknown and are very complex. However, it is intriguing to see an early rapid growth of aberrant mossy in the hippocampus within 2 days after SE in the immature rat brain. Similarly, Ribak et al. [18] have shown that the formation of hilar basal dendrites on granule cells of the rodent dentate gyrus takes place within 1 day after seizures were induced. Recently, Häussler et al. [19] have shown a sprouting of mossy fibers into the CA2 pyramidal cell layer. Römer et al. [20] found that environmental enrichment and seizure activity influenced mossy fiber distribution and size in the adult hippocampus. Animals living in an enriched environment showed a significant increase in the volume of the infrapyramidal mossy fibers bundle compared to control animals. Alterations in mossy fibers in the CA3 stratum pyramidale have been reported in a variety of experimental conditions [21, 22] including malnutrition [23], CA3 lesions [24], and prenatal exposure to methylazoxymethanol [25]. Mossy fiber sprouting has also been found in mature animals after treatment with kainic acid [7] and amygdala kindling [26]. In this context, we and others [27, 28] have reported on the induction of a similar infrapyramidal bundle of mossy fibers during postnatal hyperthyroidism. The fact that aberrant mossy fibers contain PSD-95 clusters suggests that they are potentially functional and might be able to create an abnormal excitatory input. It is not excluded that the infrapyramidal mossy fiber bundle is a reactive event that happens in the hippocampus after SE and this may correlate with an elevated predisposition for enhanced excitability. Thus, under these conditions, the CA3-pyramidal cells should receive abnormally high levels of synaptic input from the granule cells.

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