Published in final edited form as:

Neuroscience. 2004 ; 126(3): 677–688. doi:10.1016/j.neuroscience.2004.04.014.

Sprouting and Synaptic Reorganization in the Subiculum and CA1 Region of the Hippocampus in Acute and Chronic Models of Partial-Onset Epilepsy

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Abstract

Repeated seizures induce permanent alterations in the hippocampal circuitry in experimental models and patients with intractable temporal lobe epilepsy (TLE). Most studies have concentrated their attention on seizure-induced reorganization of the mossy fiber pathway. The present study examined the projection pathway of the CA1 pyramidal neurons to the Subiculum, which is the output of the hippocampal formation in five models of TLE. We examined the laminar pattern of Timm's histochemistry in the stratum lacunosum-moleculare of CA1 in three acute and two chronic models of TLE: intraventricular kainic acid (KA), systemic KA, systemic pilocarpine, chronic electric kindling and chronic intraperitoneal pentylenetetrazol. The laminar pattern of Timm histochemistry in the stratum moleculare of CA1 was permanently remodeled in epileptic models suggesting sprouting of Timm containing terminals from the adjacent stratum lacunosum. Ultrastructural examination confirmed that Timm granules were localized in synaptic terminals. As the source of Timm labeled terminals in this region was not known, sodium selenite, a selective retrograde tracer for Zinc-containing terminals, was iontophoretically injected in-vivo in rats exposed to systemic pilocarpine, systemic KA or chronic PTZ. The normal projection of CA1 pyramidal neurons to the Subiculum is topographically organized in a lamellar fashion. In normal rats, the extent of the injection site (terminals) and the retrogradely labeled pyramidal neurons (cell soma) corresponded to the same number of lamellas. In epileptic rats, the retrograde labeling extended 81–130% farther than the normal dorso-ventral extent including lamellas above and below the expected. This is direct evidence for sprouting of CA1 pyramidal axons into the Subiculum and stratum lacunosum-moleculare of the CA1 region confirming the alterations of the laminar pattern of Timm's histochemistry. Sprouting of the CA1 projection to Subiculum across hippocampal lamellas might lead to translamellar hyperexcitability, and to amplification and synchronization of epileptic discharges in the output gate of the hippocampal formation.

Additional Key Words

hippocampus; plasticity; seizures; tracing; Timm staining

INTRODUCTION

Recurrent or prolonged partial-onset seizures induce neuronal loss in the limbic system of humans and in animal models of intractable partial-onset epilepsy, leading to sprouting and

reorganization of limbic pathways and persistent cellular hyperexcitability (Cavazos et al., 1991, 1994; Represa et al., 1989a, 1989b; Sutula et al., 1988, 1989; De Lanerolle et al., 1990). Reorganization of the epileptic circuitry with newly sprouted recurrent excitatory synapses is a putative mechanism explaining, at least in part, the cellular hyperexcitability observed in intractable partial-onset epilepsy (Buckmaster and Dudek, 1997; Meier et al., 1992; Meier and Dudek, 1996; Sayin et al., 1999, 2003; Smith and Dudek, 2001, 2002). This hypothesis is supported by the demonstration that even a small increase of 4% in the number of recurrent excitatory collaterals in the circuitry **is sufficient** to cause persistent cellular hyperexcitability in the abnormally connected dentate gyrus (Lytton et al., 1998). The computer modeling demonstration is just one of several lines of evidence that strongly support the hypothesis that sprouted mossy fibers plays a key role underlying the intractability of human temporal lobe epilepsy, the most common type of intractable partial-onset human epilepsy. However, mossy fiber sprouting does not appear to be a necessary pre-condition to establish intractability in animal models of partial epilepsy as there are rare examples where this form of morphological plasticity is not present or does not appear critical for epileptogenesis (Longo and Mello, 1998; Mohapel et al., 2000). This apparent discrepancy does not invalidate the hypothesis that circuit reorganization is necessary for epileptic intractability as the only evidence thoroughly investigated is limited to mossy fiber sprouting. Reorganization of synaptic circuitry might be occurring elsewhere in the limbic system initiating pharmacologically intractable temporal lobe epilepsy.

Sprouting and synaptic reorganization induced by seizures has been well studied in the mossy fiber pathway due to the ease of detecting changes in the laminar pattern of this pathway using Timm histochemistry or Dynorphin-A immunocytochemistry (Houser et al., 1990; Cavazos et al., 1991, 2003). Studies investigating sprouting and synaptic reorganization beyond the mossy fiber pathway are limited. In experimental models of epilepsy and in humans with intractable partial-onset epilepsy, the CA1 pyramidal neurons show persistent cellular hyperexcitability and a pattern of neuronal loss similar to those observed in the epileptic dentate gyrus (Babb et al., 1984; Babb and Brown, 1987; Meier et al., 1992; Meier and Dudek, 1996). The CA1 region is the main projection to the Subiculum (Amaral et al., 1991), the final output of the hippocampal formation, and thus, plasticity of CA1 projection to the Subiculum might have a major role underlying persistent cellular hyperexcitability in the hippocampal formation (Greene and Totterdell, 1997; Menendez de la Prida et al., 2003). There are three prior reports in two experimental models that have examined the branching pattern of individual axons of CA1 pyramidal neurons labeled during neurophysiological investigations in conventional transverse hippocampal slices (Esclapez et al., 1999; Lehmann et al., 2000; Smith and Dudek, 2002). Although these reports are important in establishing that seizure-induced plasticity occurs in another hippocampal pathway, they also have significant limitations because: 1) they only used acute chemoconvulsant models of severe prolonged seizures (i.e.: status epilepticus), 2) provide information about a limited number of individual neurons, 3) examine only the proximal axonal branches of the CA1 pyramidal neurons, and 4) omit analysis of axonal processes that project outside of the 300–400 μ m thickness of the hippocampal slice. These reports have shown increased branching in the proximal axons of CA1 pyramidal neurons in the stratum oriens and radiatum of CA1 region within the limited plane of the transverse hippocampal slice of epileptic rats in the Pilocarpine and Kainic acid models of adult status epilepticus. There is no information regarding: 1) the extent of sprouting in the “population” (as compared to individual neurons) of CA1 pyramidal neurons, 2) the extent of remodeling of the projection field of CA1 pyramidal neurons across hippocampal lamellas (longitudinal axis) within the CA1 region, 3) the extent of seizure-induced remodeling of the distal projection of the CA1 pyramidal neurons to the Subiculum, which is the normal efferent projection of the CA1 pyramidal neurons and the final output of neuronal computation of the hippocampal formation, and 4) whether similar alterations develop in chronic models of

recurrent brief seizures or in non-chemoconvulsant models (as compared to acute status epilepticus).

The present study examines the possibility that the distal axonal projection of the epileptic CA1 pyramidal neurons reorganize outside of its normal laminar boundaries within the subiculum and CA1 hippocampal region. If present, sprouting of the CA1 projection to its final output, the subiculum, might provide a mechanism for the enhanced and persistent cellular hyperexcitability in this region that has been observed using a variety of neurophysiological techniques (Meier et al., 1992; Meier and Dudek, 1996). The major finding of this study was that there is substantial plasticity of the terminal field (distal axonal branches) of the CA1 axonal projection to subiculum in five experimental models (three models of acute prolonged seizures and two models of chronic recurrent brief seizures) of intractable partial-onset epilepsy using two histological methods. Furthermore, there is also substantial plasticity along the septotemporal axis of the hippocampus allowing for increased transverse connectivity among hippocampal lamellas above and below the normal circuitry. This might allow that epileptic activity in a hippocampal lamella to recruit additional lamellas, amplifying the epileptic response, and may play a role in the persistent hyperexcitability observed in intractable partial-onset epilepsy.

EXPERIMENTAL PROCEDURES

In this study, we examined the morphological alterations of the projection field of the CA1 pyramidal axons induced by seizures using five experimental animal models of epilepsy. Three experimental models, intraventricular administration of Kainic Acid, systemic administration of Kainic Acid, and systemic administration of Pilocarpine, were used to examine the consequences of *acute prolonged seizures* mimicking human convulsive status epilepticus, which often leads to intractable partial-onset epilepsy. Two additional experimental models, recurrent seizures induced electrically with kindling stimulation and repeated administration of Pentylenetetrazol (Metrazol or PTZ), were used to examine the consequences of *chronic, recurrent, and brief seizures of partial-onset*, which more accurately represent intractable partial-onset epilepsy. Male Sprague-Dawley rats (Harlan) were used in this study to avoid the great variability of seizure threshold associated with cyclic hormonal rhythms. The procedures and protocols discussed in this paper were approved by the institutional animal care and use committees in our institutions, and follow international guidelines for vertebrate research.

Systemic Pilocarpine model

Male Sprague Dawley rats (250 – 325 g) were pretreated with 1mg/kg (i.p.) of methylscopolamine nitrate (Sigma) 30 minutes prior to receive 350mg/kg (i.p.) of Pilocarpine (Sigma). The rats were observed for signs, latency and duration of clinical status epilepticus according to standard procedures for at least two hours after the injection (Cavalheiro et al., 1982; Cavalheiro, 1995). Rats that did not exhibit convulsive status epilepticus within two hours of the injection were excluded from the study. For Timm histochemistry, rats were perfused two to three weeks after the pilocarpine injection according to procedures described below. A control group consisted of normal littermate rats. For retrograde labeling experiments, rats underwent stereotactic surgery for iontophoretic injection of Sodium selenite aiming at the Subiculum, three weeks after the pilocarpine injection according to procedures described below. The control group consisted of age-matched rats.

Systemic Kainic Acid model

Male Sprague-Dawley rats (150 – 250 g) were injected subcutaneously with three hourly injections of 5 mg/kg of Kainic acid (OPIKA-1, Ocean Produce, Inc.). Rats were monitored for signs of convulsive seizures for 6 hours. The seizure severity was scored every 15 minutes using a previously validated scale (Hu et al. 1998; Koh et al., 1999). Animals were included only if they showed convulsive seizures. The rats were then perfused two to three weeks later for Timm histochemistry. An additional group was perfused for Timm histochemistry at least six months after receiving Kainic acid (KA) to assess the permanence of histological alterations induced in this model. Control rats included littermate matched controls. For retrograde labeling experiments, rats underwent stereotactic surgery for iontophoretic injection of Sodium selenite aiming at the Subiculum three weeks after the KA injection according to procedures described below. The control group consisted of age-matched rats that were given three hourly injections of isotonic saline in the same manner than the KA treated rats, and perfused three weeks after saline injection.

Intraventricular Kainic Acid model

Male Sprague Dawley rats (275 – 325 g) were pretreated with atropine and anesthetized with pentobarbital (50–60 mg/kg, i.p.). They received an intraventricular injection of 0.75 µl of a Kainic acid (Sigma) solution (0.5 µg in 1.0 µl of deionized H₂O) using a Hamilton syringe positioned stereotactically over the left lateral ventricle according to a previously published protocol (Sutula et al., 1992). The rats were allowed to recover and were observed for signs of clinical status epilepticus for at least three hours after the injection according to standard procedures. Rats that did not exhibit clinical status epilepticus were excluded from the study. The rats were then perfused two weeks after the Kainic acid injection for Timm histochemistry as it is described below. The control group consisted of normal age matched rats.

Chronic PTZ model (chemical kindling)

Male Sprague-Dawley rats (300 – 325 g) were injected with Pentylentetrazol (PTZ i.p. - Sigma) in isotonic solution at a subconvulsive dose (24 mg/kg) three times per week according to previously published method (Golarai et al, 1992). The rats were observed for at least three hours after the injection and seizure manifestations were classified according to standard scale of seizure behavior. This seizure scale has been validated and correlates quite closely to the spread of ictal epileptiform activity to limbic structures (Golarai et al., 1992). The control group consisted of age-matched normal rats that received repeated injections of isotonic saline solution (i.p.) in an identical manner as the PTZ injected rats, and were similarly handled and observed for the same length of time. The PTZ rats were injected until 10–15 class V (generalized tonic-clonic seizures with loss of postural tone) seizures were evoked, typically about 3 months after receiving the first injection. At that point, rats were either perfused for Timm histochemistry or underwent stereotactic surgery for Sodium Selenite iontophoretic injections as described below.

Electrical Kindling model

Sprague-Dawley male rats (250 – 350 g) were anesthetized with pentobarbital (60 mg/kg, i.p.) and stereotactically implanted with an insulated stainless steel bipolar electrode for stimulation and recording. The electrode was implanted in the perforant path near the region of the angular bundle (8.1 mm posterior, 4.4 mm lateral, 3.5 mm ventral from bregma), in the amygdala (1.5 mm posterior, 4.2 mm lateral, 8.8 mm ventral from bregma), or in the olfactory bulb (9 mm anterior, 1.2 mm lateral, 1.8 mm ventral from bregma). After a recovery period of two weeks, the unrestrained awake animals received twice daily kindling stimulation (5 days per week) with a 1 second train of 62 Hz biphasic constant current 1.0

ms square wave pulses. The stimulation was delivered at the lowest intensity that evoked an afterdischarge (AD) according to the standard protocol until at least 50 generalized tonic-clonic seizures with loss of postural tone (class V seizures) were observed (Goddard et al., 1969; Cavazos et al., 1991). At least 24 hours after their last class V seizure, the rats were perfused for Timm histochemistry according to the protocol described below. Another group of rats was perfused 3 months after the last class V seizure to determine the permanence of the morphological plasticity induced by kindling. The electroencephalogram and AD were recorded from the bipolar electrode, which could be switched to the stimulator by a digital circuit for the delivery of the kindling stimulation. The evoked behavioral seizures were classified according to standard criteria (Cavazos et al., 1991). The control groups consisted of normal age matched rats, and unstimulated rats implanted with chronic angular bundle electrodes for three months.

Timm Histochemistry – Light and Ultrastructural examination

At the appropriate time, rats from experimental and control groups were deeply anesthetized with lethal dose of a pentobarbital (100 mg/kg, i.p.) or Beuthanasia-D (Schering, 1 ml i.p.) and perfused transcardially with an aqueous solution of 500 ml of 0.4% (w/v) sodium sulphide, followed by 500 ml of 1.0% (w/v) paraformaldehyde/1.25% (w/v) glutaraldehyde solution according to previously published procedures (Cavazos et al., 1991). In brief, the brains were removed and left overnight in a 30% (w/v) solution of sucrose in fixative. Horizontal 40µm frozen sections were developed in the dark for 30–60 minutes in a 12:6:2 mixture of gum arabic (20% w/v), hydroquinone (5.6% w/v), citric acid-sodium citrate buffer with 1.5 ml of a silver nitrate solution (17% w/v). Alternate sections were stained with cresyl violet. Two naïve rats, two rats that received electrical kindling from the angular bundle, and two rats that received repeated PTZ injections were also examined for electron microscopy of the apical region of the CA1 region. 400 µm coronal blocks were cut with a vibratome, developed in the dark for 15–20 minutes in the Timm developer solution, and exposed to light for one minute after 10 minutes of developing time. After a thorough rinse in 0.1M NaPO₄ buffer, the blocks were postfixated in 1% OsO₄ for one hour, dehydrated in graded alcohols, and embedded in Durcupan. Serial 0.1 µm sections were cut and mounted on Formvar coated single-slot grids that were stained with uranyl acetate and lead citrate for ultrastructural examination in a JEOL-100S electron microscope. Alternate 40 µm sections were stained for light microscopy examination. Labeled synaptic profiles were identified by the presence of at least 5 silver grains in a synaptic terminal profile that contained synaptic vesicles (Cavazos et al., 2003). The stratum moleculare of CA1 was defined conservatively as the CA1 region within the 40 µm adjacent to the hippocampal fissure.

The laminar pattern of Timm histochemistry of the ventral CA1 region was examined with light microscopy. Several attempts of quantifying using light densitometry were made. There is considerable variance in the background of the sections that masks the obvious pattern of punctate Timm granules, which labels the pattern of synaptic terminals. A scoring scale has been used previously to overcome this problem, and it has been validated to describe the intensity of sprouting observed with Timm histochemistry (Cavazos et al., 1991). This scale was simplified to indicate either the presence or absence of punctate Timm granules in the two adjacent layers (str. moleculare and str. radiatum) to the stratum lacunosum of the ventral CA1 region. Three observers blinded to the identity of the slides from the experimental or control animals reviewed selected sections from the ventral CA1 region. The observers scored slides independently using the following scoring scale:

- 1 – there was a dense extension of the laminar pattern of Timm punctate staining in the stratum moleculare near the hippocampal fissure.
- 0 – the laminar pattern of Timm histochemistry in this region was almost devoid of Timm punctate granules.

There were only 8 out of 128 discordant scores (6%) between the three scorers, which were resolved by consensus while maintaining the blinding to the slide identity. In those eight cases, the consensus was conservative, and a score of no alteration (0) was assigned. The discordant scores were later found to be equally distributed among experimental and control rats. The scores were statistically analyzed using Chi-square test for each of the experimental models.

Sodium Selenite retrograde tracing

Zinc containing buttons are present in excitatory glutaminergic neurons and also serve an important modulatory role in GABA-A receptor blockade (Cohen et al., 2000, Frederickson and Moncrieff, 1990). A retrograde neuroanatomical tracing method using iontophoretic injection of Sodium Selenite (Na_2SO_3) was selected to assess the location of the cell bodies of origin of the Zinc-containing terminals of the ventral CA1 region because of the high specificity of this technique to selectively label this subset of synaptic terminals (Howell and Frederickson, 1990). Selenite anions are taken up by a heavy metal transporter that is present in Zinc-containing pre-synaptic terminals to re-uptake released Zinc (Danscher, 1984). The Selenite anions get into the presynaptic terminal and precipitate with ionic Zinc to form a ZnSe molecule, which is retrogradely transported to the cell soma to lysosome-like organelles and later visualized using an autometallographic Silver enhancement stain (Howell et al., 1989; Mandava et al., 1993). The histochemical reaction is quite similar to the developing process of Timm histochemistry. Sodium Selenite will also bind to zinc that is present in inhibitory neurons of the cerebellum and spinal cord (Wang et al., 2002, Wang et al., 2001). Survival of only one day after iontophoretic injection is sufficient to transport to local synaptic circuits. Although this histochemical method is highly specific and clearly identifies the cells of origin of the Zinc-containing terminals (Slomianka, 1992), it only faintly stains the axonal arbor not showing their trajectory as exquisitely as other neuronal tracers such as neurobiotin, biocytin or PHA-L. In a lamellar structure, examining the ratio between the dorso-ventral extent of labeled cell bodies and the extent of the injection site can be used to assess the dorso-ventral extent of the projection field.

Rats were anesthetized with sodium pentobarbital (50–60 mg/kg, i.p.), and placed in a Kofis stereotactic apparatus for iontophoretic infusion with micropipettes filled with sodium selenite (Sigma; 0.2% in deionized water) aimed to the stratum lacunosum of the ventral CA1/subiculum transitional region (5.90 mm posterior, 5.65 mm lateral and 4.65mm ventral from bregma). The micropipettes tips had a 2–5 μm outside diameter and the tip was fire polished. Iontophoresis was performed using a Midgard constant current source (Stoelting) of 2 μA negative current for 0.2–1 min with a 7 sec duty cycle, and according to previously published protocols (Howell and Frederickson, 1990; Mandava et al, 1993). The rats were allowed to survive for 24–48 hours, and then were deeply anesthetized pentobarbital (100mg/kg i.p.), and perfused with 2% glutaraldehyde in buffered Soreson's solution. The brains were removed and left overnight in a 30% (w/v) solution of sucrose in fixative. Horizontal 40 micron frozen sections were obtained, mounted in gelatin-coated slides, and developed in the dark using the Timm histochemistry protocol described above. Every fifth section was stained with developer. The injection site and the area of retrograde labeled cell bodies were mapped using an image analysis system (Neurolucida). As the hippocampal formation is organized in a lamellar distribution, the statistical analysis was performed using the ratio of dorsal-ventral extent of the retrograde labeling / injection site. The ratios obtained from the pilocarpine and naïve control groups were statistically compared using standard Student's *t* test.

Photography

Micrographs were photographed using a Zeiss Axioskop microscope and printed using 8×10 photographic paper. The micrographs were scanned at 300 dpi resolution. No alterations to these micrographs were made, other than the autoadjust contrast setting in Adobe Photoshop. Reconstructions of the contour of the injection site, hippocampal formation, and the location of retrograde labeled CA1 soma were made by serial tracings of slices every 200 μm using a Zeiss Axioskop microscope with a motorized stage and Neurolucida software. Portions of preliminary experiments were published in abstract form (Cavazos et al., 1989, 1999).

RESULTS

Timm Histochemistry

The normal pattern of Timm histochemistry in the CA1 region is characterized by punctate Timm granules in the stratum lacunosum and oriens, where the CA1 pyramidal neurons extend their apical and basal dendrites, respectively. This laminar pattern is more prominent in the ventral region but it is present throughout the dorsal-ventral axis. In the present study, we examined the laminar pattern of Timm histochemistry in the stratum lacunosum-moleculare of the CA1 region in five experimental models of partial-onset epilepsy: electrical kindling, repeated injections of pentylenetetrazol (PTZ), intraventricular kainic acid (KA), systemic KA, and systemic pilocarpine. In these five experimental models, there was an expansion of these punctate Timm granules into the two adjacent layers to the stratum lacunosum of the ventral CA1 region, namely the stratum moleculare and the stratum radiatum, that was not present in any of the control rats. The presence or absence of Timm granules in the stratum moleculare was evaluated by three observers blinded to identity of each rat in each of these five experimental models of partial-onset epilepsy. Timm granules were observed in those experimental models in the stratum moleculare near the hippocampal fissure, and were not observed in several types of control rats.

Prominent Timm granules were found in the stratum moleculare of the ventral CA1 in eleven of the twelve rats kindled to at least 50 class V (generalized tonic-clonic-atonic) seizures by stimulation from the olfactory bulb ($n=4$), the amygdala ($n=4$), or the angular bundle ($n=4$) (Figs. 1B and 1E). These changes were not present in the naïve animals ($n=8$) (Figs. 1A and 1D), or in the unstimulated rats that were implanted with electrodes in the perforant path ($n=6$). Eight rats that received intraventricular Kainic acid and experience convulsive status epilepticus also demonstrated a similar increase of Timm punctate staining in the stratum lacunosum-moleculare of the ventral CA1 region of the hippocampus (Figs. 1C and 1F). A similar expansion of band of Timm punctate staining was observed in ten rats that received chronic intraperitoneal PTZ injections ($n=10$) (Figs. 2B and 2E) but it was not seen in the ten paired rats that received saline injections (Figs. 2A and 2D). Ten out of twelve rats that experienced clinical status epilepticus after an i.p. injection of Pilocarpine demonstrated a similar extension of the band of punctate Timm granules in the ventral CA1 hippocampal region. Eleven out of twelve rats that received systemic Kainic acid (s.c.) and experienced status epilepticus with convulsive seizures demonstrated punctate Timm granules in the stratum moleculare of the CA1 region, as compared to none of ten rats that received a similar volume of isotonic saline. The newly formed Timm granules in the stratum moleculare were still present in ten out of ten rats that received systemic KA and were perfused 6 months after experiencing convulsive status epilepticus. None of the ten rats who received isotonic saline showed this alteration. The alteration in the pattern of Timm histochemistry was also unchanged in a group of five rats kindled through the angular bundle to at least 50 class V seizures but perfused 3 months after the last convulsion (as compared to 24 hours after the last of 50 convulsions). Thus, the expansion of the pattern of

Timm histochemistry in the ventral CA1 region appears a permanent alteration of the hippocampal circuitry in a variety of experimental models of partial-onset epilepsy. All of the groups of epileptic rats were statistically significant different than their controls at $p < 0.01$ or less using Chi-square tests.

Ultrastructural analysis of the stratum lacunosum and stratum moleculare of the CA1 region was performed in normal ($n=2$) rats, rats treated with repeated injections of PTZ ($n=3$), and rats that received electrical kindling through the perforant path ($n=3$). Timm punctate granules in the stratum lacunosum of the CA1 region corresponded to clusters of small labeled presynaptic profiles that made synaptic contacts with small profiles, presumably spines of CA1 pyramidal neurons. Both pre-synaptic and post-synaptic profiles were typically small, typically less than one micron in diameter. In the stratum moleculare of the CA1 region, abundant profiles were observed in the two experimental models of epilepsy, whereas no labeled pre-synaptic terminals were observed in the control rats within $40\text{ }\mu\text{m}$ of the stratum moleculare adjacent to the hippocampal fissure. The limited sampling ultrastructurally supported the findings obtained in light microscopy. The ultrastructural localization of the punctate Timm granules in the ventral CA1 corresponded to pre-synaptic terminals that contained spheroid vesicles with asymmetric synapses (Gray type I synapse) suggestive of excitatory nature (Fig.3). The post-synaptic structures were profiles with no microtubular structures and of small rounded size suggesting that they correspond to dendritic spines. The ultrastructural appearance is similar to other Timm labeled excitatory synaptic terminals such as the mossy fiber synaptic terminals but of smaller size.

Tracing Studies of Zinc-containing Terminals

The source of the Timm granules in the Subiculum and CA1 region was investigated using the retrograde tracer sodium selenite, which is selectively taken up by Zinc containing synaptic terminals and retrogradely transported to their cell bodies. Representative examples of the anatomical distribution of the sodium selenite injection site and retrogradely labeled cell bodies in the hippocampal formation of normal and epileptic rats are shown in Figs. 4 and 5, and the data is shown in table 1. Iontophoretic injection of sodium selenite into the stratum lacunosum of the ventral CA1 and subiculum of normal and epileptic (chronic PTZ, pilocarpine, systemic KA) rats demonstrated retrograde transport of into the cell bodies of CA1 pyramidal neurons ipsilateral to the injection site. Injection sites were aimed to the subiculum along the dorsal-ventral axis of the hippocampal formation. Injection sites that were larger than $1600\text{ }\mu\text{m}$ along the dorso-ventral axis were excluded from the analysis. In few of the larger injection sites, cell bodies of pyramidal neurons from the ipsilateral entorhinal cortex were also labeled. The smaller injection sites had no labeled cell bodies in the entorhinal cortex but just in the CA1 pyramidal region. A representative injection site is shown in figure 4. The epicenter of this iontophoretic injection of sodium selenite is at the Subiculum, while still demonstrating specific labeling of the cell bodies of CA1c pyramidal neurons away $4000\text{ }\mu\text{m}$ from the epicenter in the transverse plane. A representative Neurolucida reconstruction of the site of injection and the extent of retrogradely labeled CA1 pyramidal neurons is shown in figure 5A. The figure demonstrates that the dorsoventral extent of retrograde labeling of CA1 pyramidal neurons is similar to the dorsoventral extent of the injection site in the Subiculum, supporting the concept of a lamellar organization. In normal rats, the CA1 projection to the Subiculum tended to shift about $400\text{--}600\text{ }\mu\text{m}$ ventral from their corresponding lamella. Thus, this projection would be cut in most cases of standard transverse hippocampal slices, but periodically, slices cut at a slight angle would contain this circuitry.

The injection site of sodium selenite typically was about $400\text{ }\mu\text{m}$ ventral from the center of the dorsoventral extent of the retrogradely labeled CA1 pyramidal neurons. In the control adult rats ($n=9$), the dorsoventral extent of the injection site was $867 \pm 145\text{ }\mu\text{m}$ (mean $\pm$ SD).

SEM) while the dorsoventral extent of the retrogradely labeled pyramidal neurons is $911 \pm 164 \mu\text{m}$. The ratio of dorsoventral extent of the retrogradely labeled neurons to the injection site was 1.03 ± 0.02 (retrograde transport extent/injection site). In the group of adult rats that had experienced at least 10–15 generalized seizures induced by repeated administration of PTZ ($n=7$), the dorsoventral extent of the injection site was $886 \pm 86 \mu\text{m}$ while the dorsoventral extent of the retrogradely labeled pyramidal neurons was $1514 \pm 190 \mu\text{m}$. The ratio of dorsoventral extent of the retrogradely labeled neurons to the injection site was 1.81 ± 0.30 . A standard two-tailed Student's *t* test revealed a significant difference between the two ratios with unequal variance (1.03 vs 1.81 , $p = 0.04$), between the dorsoventral extent of the retrogradely labeled neurons ($911 \mu\text{m}$ vs $1514 \mu\text{m}$, $p = 0.03$), and no significant difference between the size of the injection sites ($p = 0.91$). We also tested the same hypothesis in acute experimental models of severe prolonged seizures using the systemic injection of pilocarpine and systemic KA. Only rats that experienced convulsive status epilepticus with motor manifestations were included in the analysis. In the group of adult rats that had experienced convulsive status epilepticus induced by pilocarpine ($n=10$), the dorsoventral extent of the injection site was $960 \pm 98 \mu\text{m}$ while the dorsoventral extent of the retrogradely labeled pyramidal neurons was $1920 \pm 108 \mu\text{m}$. The ratio of dorsoventral extent of the retrogradely labeled neurons to the injection site was 2.17 ± 0.24 . Significant difference between the two ratios (1.03 vs 2.17 , $p = 0.0009$), between the dorsoventral extent of the retrogradely labeled neurons ($911 \mu\text{m}$ vs $1920 \mu\text{m}$, $p = 0.0001$), and no significant difference between the size of the injection sites ($p = 0.60$). In the group of adult rats that had experienced convulsive status epilepticus induced by Kainic acid ($n = 8$), the dorsoventral extent of the injection site was $975 \pm 45 \mu\text{m}$ (mean $\pm$ SEM) while the dorsoventral extent of the retrogradely labeled pyramidal neurons was 2200 ± 53 (mean $\pm$ SEM) μm . The ratio of dorsoventral extent of the retrogradely labeled neurons to the injection site was 2.30 ± 0.15 (mean $\pm$ SEM). A standard two-tailed Student's *t* test revealed a significant difference between the two ratios (1.03 vs 2.30 , $p = 0.00005$), between the dorsoventral extent of the retrogradely labeled neurons ($911 \mu\text{m}$ vs $2200 \mu\text{m}$, $p = 0.00003$), and no significant difference between the size of the injection sites ($p = 0.49$).

The results show that in normal rats the axons of CA1 pyramidal neurons grossly project in a lamellar fashion with the extent of retrograde labeling from an injection site being essentially limited to same number of lamellas where sodium selenite was injected. However, in epileptic rats, the retrograde labeling extends beyond those lamellas to include lamellas above and below the injection site. This is direct evidence for axonal terminals from CA1 pyramidal neurons of acute or chronic epileptic rats to sprout and extend their axonal terminals into the area of injection site where they are not normally present.

DISCUSSION

The main finding of this study is the demonstration that the output of the hippocampal formation, the CA1 pyramidal axons, showed exuberant plasticity making synaptic contacts across several lamellas in acute and chronic experimental models of epilepsy. CA1 synaptic reorganization has a potential of permanently enhance synaptic interactions between CA1 pyramidal neurons via recurrent excitatory collaterals in the stratum lacunosum-moleculare as well as increase the activation of subicular neurons that would otherwise not have been activated. In this study, we described that reorganization of synaptic projections develops and becomes a permanent feature in the Subiculum and the CA1 region of the hippocampus in three acute and two chronic experimental models of partial-onset epilepsy. The experiments confirmed and extend the previous observations in the pilocarpine and Kainic acid models of status epilepticus that used hippocampal slices (Esclapez et al., 1999; Lehmann et al., 2000; Smith and Dudek, 2002). Prior studies showed reorganization of the

proximal aspect of the CA1 axons including the formation of recurrent excitatory collaterals. The present study extends these observations to the distal projection of the CA1 pyramidal neurons including to the stratum lacunosum of CA1 as well as the Subiculum. In addition, we describe CA1 sprouting for the first time in two chronic models to repeated brief seizures, the electrical kindling model and the repeated pentylenetetrazol model. The presence of another form of morphological plasticity in the epileptic limbic brain should not be surprising as the Subiculum and the CA1 region of the hippocampus exhibit cellular hyperexcitability and neuronal injury as a consequence of acute or prolonged seizures in striking similarity to the development of mossy fiber sprouting in the dentate gyrus in animal and humans with partial-onset epilepsy (Babb and Brown 1984, 1987; Meier and Dudek, 1992, 1996; Cavazos et al., 1994; Esclapez et al., 1999; Lehmann et al., 2000, 2001; Smith and Dudek, 2001, 2002; Harris and Stewart, 2001).

The first finding in this study was the recognition that there was an alteration in the laminar pattern of punctate Timm granules in the ventral CA1/subicular region in five experimental models of partial-onset epilepsy. This alteration consisted in the appearance of punctate Timm granules in the stratum moleculare of CA1, which is adjacent to the stratum lacunosum of ventral CA1 region of the hippocampus. The source(s) that extend this afferent projection of Timm labeled synaptic terminal in this region is not precisely known (Amaral et al., 1991; Long et al., 1995). Thus, we performed additional experiments using a retrograde tracer that selectively labels the afferent source of Zinc-containing synaptic terminals. The source of this projection to the stratum lacunosum of CA1 was a recurrent projection from the CA1 pyramidal neurons, which provides for a source of collateral excitation that is present in normal rats but demonstrate considerable sprouting along the dorsal-ventral axis in the epileptic models. Our experiments are in agreement with the only two anterograde tracing rats that were injected in this region by Amaral et al. (see in fig. 10 of Amaral et al. 1991). However, in the epileptic rats, the CA1 projection expanded into the adjacent stratum moleculare, which is not normally innervated by Zinc-containing synaptic terminals. The ultrastructural examination of the newly formed synaptic terminals in this area suggests that most of the contacts were excitatory synapses with small profiles such as dendritic spines. Because the CA1 pyramidal neurons are covered with dendritic spines, while most of the sparse interneurons in the stratum moleculare are aspiny (Bannister and Larkman, 1995; Megias et al., 2001), our findings suggest that the CA1 sprouted synaptic terminals primarily make recurrent synaptic contacts with distal dendrites of CA1 pyramidal neurons. This interpretation is supported by the physiological studies in hippocampal slices (Smith and Dudek, 2001, 2002).

Investigators have suggested that seizure-induced CA1 hyperexcitability is due to the formation of recurrent excitatory collaterals in the CA1 region (Meier and Dudek, 1992, 1996; Esclapez et al., 1999; Lehmann et al., 2000, 2001; Smith and Dudek, 2001, 2002). These investigators have used the KA and Pilocarpine models and examined the emergence of hyperexcitability in transverse hippocampal slices. Prolonged excitatory post-synaptic potentials (EPSPs) bursts observed in bicuculline treated Kainate slices showed an all-or-none behavior (Meier and Dudek, 1992, 1996). This all-or-none behavior can not be explained by changes of the intrinsic properties of CA1 pyramidal neurons because the EPSP bursts would then be graded. Thus, the prolonged EPSP bursts are mediated by synaptic transmission reflecting a network-driven hyperexcitability. However, the prolonged EPSP bursts were only seen in 28% of the isolated CA1 transverse slices spontaneously (Meier and Dudek, 1992, 1996). Although this may be due to a sampling error, one possibility is that the critical recurrent CA1 collaterals are cut-away of the hippocampal slice because of the slight variability in the plane of cutting slices. Subsequently, Smith and Dudek (2001, 2002) using isolated CA1 transverse slices demonstrated that glutamate microapplication to the CA1 pyramidal cell layer increased excitatory postsynaptic current

(EPSC) frequency only in slices of kainate-treated rats but not in control slices. These results, along with the work of Esclapez et al. (1999) and Lehmann et al. (2000, 2001), support the hypothesis that the increased recurrent excitatory connections between CA1 pyramidal cells after kainate-induced status epilepticus are functional connections that increase the excitatory drive of the hippocampal circuitry.

The second set of experiments described were the retrograde tracing studies, which indicated that in normal rats the axonal projection of CA1 pyramidal neurons to the Subiculum and stratum lacunosum of CA1 project in a lamellar fashion with the extent of CA1 lamellas labeled by retrograde transport was similar than the extent of lamellas injected in the Subiculum. This is in agreement with the anterograde tracing experiments of Tamamaki et al., (1988) and Tamamaki and Nojyo (1990). In three experimental models of epilepsy, our experiments showed that the retrograde labeling extended 81–130% beyond the normal lamellar organization to include lamellas above and below the injection site. This is direct evidence for sprouting of CA1 axonal projection to the Subiculum and stratum lacunosum-moleculare of CA1 in a manner that is position to activate the output of lamellas that would not be expected to receive a projection from CA1. The hypothesis of the lamellar organization of the hippocampus was derived from mapping of the hippocampal formation using extracellular field evoked recordings (Andersen et al., 1971, 1973). The lamellar hypothesis proposes that the hippocampal formation consists of a stack of hippocampal transverse slices that are functionally connected uni-directionally along the transverse axis of the structure. In this manner, the entorhinal cortex projects to the dentate gyrus via the perforant pathway. The dentate gyrus then projects to the CA3 pyramidal region via the mossy fiber pathway. The CA3 region projects to the CA1 pyramidal region through the Schaffer collaterals. The CA1 region projects to the Subiculum, which in turn, projects back to the entorhinal cortex completing the loop within the hippocampal formation. In essence, the function of the structure is primarily organized topographically in the transverse hippocampal slices or lamellas, and interconnections with lamellas above or below have no major significant importance for its primary function. As better neuroanatomical tracers became available, more careful mapping of the extent of the principal unidirectional projections revealed more extensive projections from principal hippocampal neurons along the dorso-ventral axis that was predicted by the lamellar hypothesis. Experiments demonstrated entorhinal projection to the dentate gyrus and the CA3 projection to the CA1 region with considerably divergence from the proposed lamella. For example, an anterograde tracer injection that span 10% of the ventro-dorsal axis of the entorhinal cortex projected to about 25% of the dentate gyrus (Amaral and Witter, 1989). Furthermore, it was also shown that some types of hilar polymorphic neurons give rise to highly dense projections to the dentate gyrus for almost two thirds of the entire ventro-dorsal axis (Laurberg, 1979; Laurberg and Sorensen, 1981). The presence, density and extent of these associational interconnections seem to invalidate the lamellar hypothesis, but functional studies are lacking. It can be surmised that some of the hilar polymorphic neurons could be activating local inhibitory circuits tuning out lamellas above or below the activated lamella (Zappone and Sloviter, 2003). Nevertheless, the projections in the hippocampal formation clearly follow a topographical organization that, as shown in the present article, is altered in experimental models of epilepsy providing pathways for further activation of hippocampal circuitries that do not normally receive these projections. The functional consequences of these alterations are not completely understood, but might contribute to the state of hyperexcitability seen in epilepsy. experiments demonstrated prominent excitatory projections along the ventro-dorsal axis of the hippocampal formation suggesting a greater amount of interconnection between hippocampal lamellas that was predicted by the theory (Witter and Amaral, 1989). However, the functional importance of these ventro-dorsal interconnections, also known as associational projections, is not well understood. A revision of the lamellar hypothesis using extracellular field potentials in-vivo (Andersen et al., 2000)

showed that it still “remains a useful concept for understanding of hippocampal connectivity.” Andersen showed that the amplitude of the compound action potential was largest in a slightly oblique transverse band across the CA1 towards the subicular region with decreasing activation in the lamellas above and below the activated lamella despite the larger dorso-ventral extent of the neuroanatomical projection. Recent experiments (Buckmaster and Dudek, 1997; Zappone and Sloviter, 2003) have also shown that the larger extent of dorso-ventral projections actually serves to inhibit the surrounding lamellas to limit the spread of activation. In the Dentate Gyrus, the excitatory associational projections play a major role for activating inhibitory hilar circuits in the lamellas away from the source of the associational projection. This pattern of organization strengthens the lamellar hypothesis by fine-tuning the activation to the “on lamella” while there is increase inhibition in the “off lamella”. Furthermore, Zappone and Sloviter suggest that hilar neuronal loss in epileptic models leads to **translamellar disinhibition** in the dentate gyrus. Although the functional consequences of remodeling of the CA1 projection to the Subiculum in acute and chronic epilepsy models is not known, we suggest that the enhanced excitatory spread of CA1 axonal collaterals that make contacts with spines profiles would enhance **translamellar hyperexcitability** in the Subiculum, the output gate of the hippocampal formation.

ABBREVIATIONS

EPSPs	Excitatory Post-Synaptic Potentials
KA	Kainic Acid (a.k.a.: Kainate)
PHA-L	Phaseolus vulgaris Leucoagglutinin
PTZ	Pentylenetetrazol (a.k.a.: Metrazol)
SEM	Standard Error of the Mean
TLE	Temporal Lobe Epilepsy

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Fig. 1.

A and D. The normal pattern of Timm's stain sections obtained in the horizontal plane of the ventral CA1 is demonstrated at low and high power magnification. (Abbr.: DG: dentate gyrus, SUB: subiculum, str lac-mol – stratum lacunosum-moleculare of CA1, str mol – stratum moleculare of the DG, str gr – stratum granulare of the DG, HF – hippocampal fissure). **B and E.** Photomicrographs from horizontal sections obtained from a rat 2 weeks after being injected with Kainic acid. Prominent Timm granules in the inner molecular layer (MFSR) and in the stratum lacunosum-moleculare of the ventral CA1 region. More diffuse changes in the pattern of Timm histochemistry at the stratum radiatum of ventral CA1 were also observed. **C and F.** Photomicrographs from horizontal sections obtained from a rat that had experienced 50 kindling seizures and perfused 24 hour after the last seizure. Timm granules are seen in the stratum lacunosum-moleculare extending to the hippocampal fissure. A similar alteration in the pattern of Timm histochemistry in the ventral CA1 region was observed in kindled rats perfused up to 6 months after the last of 30 class V seizures.

Fig. 2.

A and D demonstrate the normal pattern of Timm's stain sections obtained in the coronal plane of the ventral CA1 at low and high power magnification. (Abbreviations: DG: dentate gyrus, SUB: subiculum, str rad: stratum radiatum of CA1, str lac-mol: stratum lacunosum-moleculare of CA1, str mol: stratum moleculare of the DG, str gr : stratum granulare of the DG, HF – hippocampal fissure). **B and E** were obtained from a rat 2 weeks after the last of 10 generalized tonic-clonic- atonic seizures induced by repeated injections of PTZ. Prominent Timm granules in the inner molecular layer (MFSR) and in the stratum moleculare of the ventral CA1 region. More diffuse changes in the pattern of Timm histochemistry at the stratum radiatum of ventral CA1 were also observed. **C and F** were obtained from a rat 2 weeks after Kainate-induced seizures. Prominent Timm granules in the inner molecular layer (MFSR) and in the stratum lacunosum-moleculare of the ventral CA1 region.

Fig. 3.

A is taken from a Timm's stain section of a rat that received 10 seizures induced by PTZ, and shows the punctate staining extending through the stratum moleculare of CA1 up to the hippocampal fissure. **B** is a thin section contiguous to the fig. 3A that was stained with toluidine blue. The asterisk depicts the location where fig. 3C was taken. **C**. The ultrastructural characteristics of the Timm granules from the stratum lacunosum and moleculare of CA1 region are shown. Labeled Timm granules clustered in presynaptic terminals that make two asymmetric synapses with dendritic spines and contain spheroid vesicles, both features suggestive of a Gray type I synapse (excitatory).

Fig. 4. Photomicrographs of the hippocampus demonstrating the maximum extent of the injection site after a iontophoretic injection with sodium selenite. The micrograph on top shows a low magnification view of the injection site. The micrograph in the bottom demonstrates retrograde transport to the CA1 pyramidal neurons at the level indicated with a star in the low magnification view.

Fig. 5. Anatomical distribution of the sodium selenite injection site and retrogradely labeled cell bodies in the hippocampal formation of normal and epileptic rats. **A.** Superimposed contours of horizontal sections obtained every 200 μm from the dorsal through ventral hippocampus of normal rats. The large solid black blot represents the injection site of sodium selenite, which its center is about 400 μm ventral from the center of the dorsoventral extent of the retrogradely labeled CA1 pyramidal neurons. The CA1 pyramidal layer was represented with a solid line and the groups of labeled pyramidal neurons were indicated as small dots along the pyramidal layer. In this case of a control rat, the dorsoventral extent of the injection site is also 1400 μm while the dorsoventral extent of the retrogradely labeled

pyramidal neurons is 1600 μm . The ratio of dorsoventral extent of the retrogradely labeled neurons to the injection site was 1.14, which was representative among other controls. **B.** Superimposed contours of horizontal sections obtained every 200 μm from the dorsal through ventral hippocampus of rats that had experienced at least 10–15 generalized seizures induced by repeated administration of PTZ. The large solid black blot represents the injection site of sodium selenite and the CA1 pyramidal layer was represented with a solid line and the groups of labeled pyramidal neurons were indicated as small dots along the pyramidal layer. In this case of an epileptic rat, the dorsoventral extent of the injection site is 1200 μm while the dorsoventral extent of the retrogradely labeled pyramidal neurons is 2200 μm . The ratio of dorsoventral extent of the retrogradely labeled neurons to the injection site was 1.83, which was representative among other epileptic rats. **C.** The drawing indicates the results of the experiment described above. The axons of CA1 pyramidal neurons project in a lamellar fashion. In control rats, the extent of retrograde labeling from an injection site is essentially limited to same lamella where sodium selenite was injected. In epileptic rats, the retrograde labeling extends beyond the lamella where sodium selenite was injected to include lamellas above and below the injection site. This is direct evidence that axonal terminals from neurons in those layers extend their axons into the area of injection. This could not be a nonspecific enhanced diffusion of sodium selenite in the extracellular space because in few animals that were incorrectly injected in neighboring regions extending into the lateral ventricle had a larger than expected injection site.

TABLE 1

Iontophoretic injections in the Subicular region of Sodium Selenite in the chronic pentylenetetrazol (PTZ), acute systemic Kainic acid (KA) and acute systemic Pilocarpine (PILO) models of epilepsy.

	Injection Site - Subiculum Control (uM)	PTZ (uM)	KA (uM)	PILO (uM)	Extent of labelled soma in CA1 Control (uM)	PTZ (uM)	KA (uM)	PILO (uM)	RATIO (soma/inj) Control (uM)	PTZ (uM)	KA (uM)	PILO (uM)
	600	600	800	600	600	1000	2400	1200	1.00	1.67	3.00	2.00
	800	1000	1000	600	800	1000	2000	2200	1.00	1.00	2.00	3.67
	600	1000	1000	1000	600	1000	2200	2200	1.00	1.00	2.20	2.20
	800	600	1000	1200	800	2000	2200	2200	1.00	3.33	2.20	1.83
	1400	1200	800	1400	1600	2000	2200	2200	1.14	1.67	2.75	1.57
	1600	800	1200	1000	1600	1600	2000	1800	1.00	2.00	1.67	1.80
	400	1000	1000	800	400	2000	2200	2200	1.00	2.00	2.20	2.75
	1200		1000	600	1400		2400	1800	1.17		2.40	3.00
	400			1000	400			1600	1.00			1.60
				1400				1800				1.29
average	866.667	885.714	975.000	960.000	911.111	1514.286	2200.000	1920.000	1.034	1.810	2.302	2.171
std	435.890	226.779	128.174	309.839	491.031	501.427	151.186	342.540	0.069	0.790	0.418	0.746
n	9	7	8	10	9	7	8	10	9	7	8	10
sem	145.297	85.714	45.316	97.980	163.677	189.521	53.452	108.321	0.023	0.299	0.148	0.236
p value	ctl vs PTZ	0.911892			ctl vs PTZ	0.031694			ctl vs PTZ	0.040895		
	ctl vs KA		0.493654		ctl vs KA		0.00003		ctl vs KA		0.00005	
p value	ctl vs PILO			0.6024983	ctl vs PILO			0.000146	ctl vs PILO			0.0009326