Dissociation Between Mossy Fiber Sprouting and Rapid Kindling with Low-Frequency Stimulation of the Amygdala

by

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Abstract

To determine whether sprouting of hippocampal mossy fibers is invariably correlated with kindling of seizures, rats were subjected to rapid kindling with long trains of low-frequency stimulation of the amygdala, which resulted in development of generalized seizures within a mean of 5 stimulations. For comparison other rats were subjected to conventional kindling with short trains of high-frequency stimulation of the amygdala, which resulted in development of generalized seizures within a mean of about 13 stimulations. The purpose of using the rapid kindling technique was so that we could attempt to tease apart the time course of mossy fiber sprouting and kindling. There was no evidence of mossy fiber sprouting in the brains of rats killed 1 day after completion of rapid kindling, as compared to yoked controls, although significant sprouting was seen in rats killed 1 day after completion of conventional kindling. Examination of tissue from rats killed 20 days after rapid kindling, however, revealed significant sprouting, suggesting that mossy fiber sprouting can be triggered by rapid kindling if sufficient elapsed time is allowed. The observed disparity between completion of rapid low-frequency kindling and detection of mossy fiber sprouting suggests that mossy fiber sprouting may be associated more with an elapse of time after neuronal activation than with kindling per se. Furthermore, the similar time course of conventional kindling and of mossy fiber sprouting precludes a causal role of mossy fiber sprouting in conventional kindling.

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Dedication

I dedicate this thesis to Grandma "C".

CHAPTER I

INTRODUCTION

In epilepsy seizure susceptibility can depend on the number and type of viable neurons remaining in an injured site as well as the number and type of new circuits formed subsequent to injury. Also, recurrent bouts of seizures likely participate in initiating a cycle of neuronal alterations and synaptic sprouting that leads to increased bouts of seizures or decreased seizure thresholds. However, the specific neuronal alterations and resulting functional changes in association with epilepsy is unclear.

Ectopic mossy fiber sprouting is a specific neuronal alteration that is commonly detected in tissue resected from patients with temporal lobe epilepsy and in tissue taken from brains of animals that have had experimentally-induced seizures. Several reports of mossy fiber sprouting in association with epileptogenesis have led to the suggestion that mossy fiber sprouting may be causally involved in the induction or maintenance of experimentally-induced epilepsy or temporal lobe epilepsy. However, in controlled experiments involving epileptogenic treatments, the time course of mossy fiber sprouting and seizure induction coincide closely and this coincidence precludes any interpretation of a causal role. To further clarify the role of sprouting in epileptogenesis an attempt to tease apart the time course sprouting and seizure induction apart, an epileptogenic treatment that rapidly induces seizures could be applied and the magnitude of sprouting assessed. In theory then, if mossy fiber sprouting is one of the factors that plays a causal role in epileptogenesis, it would likely be detected in the same time course of rapidly induced epileptogenesis.

CHAPTER II

LITERATURE REVIEW

Epilepsy

Epilepsy is a common and debilitating neurological disorder that affects at least 1-2% of the population (Driefuss, Martinez-Lage, Roger, Seino, & Dam, 1985). The epilepsy syndrome manifests several clinical, pathological and electrophysiological symptoms; the most obvious of which are the recurrence of sudden and transient seizures that correspond with synchronous and excessively paroxysmal electrophysiological discharges arising from interconnected populations of neurons. This aberrant seizure activity is often accompanied by motor or sensory phenomena lasting from seconds to minutes and ranging from motor convulsions, sensory dysfunctions and abnormal behaviours to loss of consciousness. Seizure episodes may not initially be associated with neuronal damage but head injuries incurred during a seizure, prolonged episodes of paroxysmal discharges (status epilepticus) or frequent episodes tend to increase the likelihood of permanent morphological, biochemical or physiological alterations[1] (Glass & Dragunow, 1995; McNamara, 1994) and increased susceptibility to seizures.

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[1]Surgical resection of tissue specimens or postmortem autopsy of hippocampal tissue often reveals hippocampal sclerosis (Ammon's horn sclerosis) that is characterized by neuronal death and gliosis. Ammon's horn sclerosis is almost always evident in tissue from patients who have had retractable epilepsy or bouts of status epilepticus. It has been hypothesized that intense or recurring seizures can cause sclerosis and, once developed, the sclerosis can cause epilepsy (McNamara, 1986, see also Gloor, 1991; Sloviter, 1994).

In some cases outright damage at the site of seizure generation (i.e. due to head injury or tumour) or obvious disruptions of central nervous system function (i.e. due to ischemia or infection) can be identified as the cause of the epileptic syndrome, however, in most cases the etiology of epilepsy is unknown. Thus the epileptic syndrome is not classified according to cause but rather according to electrophysiological and concomitant behavioural convulsive profiles. Electrophysiological profiles can differ according to whether the seizure activity originates within a restricted area of the brain (partial or focal epilepsy), then propagates to other regions of the brain (secondary generalized partial epilepsy) or whether the seizure activity is synchronized simultaneously in all regions of the brain (primary generalized epilepsy). Similarly, the behavioural profiles differ in whether there is loss of consciousness, localized body convulsions that spread to other areas of the body or convulsions with clonic, tonic or both clonic-tonic motor components (Fischer, 1989). The different types of epilepsy syndromes and their associated symptoms are outlined in Appendix A.

Kindling

Kindling is a process whereby daily application of an initially subconvulsive electrical stimulation to a discrete brain site leads to the emergence, elongation and propagation of epileptiform discharge (Goddard, 1967; Goddard, McIntyre, & Leech, 1969). Over time, the initially subconvulsive stimulation evokes both electrographic and behavioural convulsions that eventually culminate in generalized convulsive

seizures. The term "kindling" was coined by Goddard (Goddard, 1967; Goddard et al., 1969), who recognized that rats receiving daily stimulation to discrete brain sites had electroencephalogram (EEG) and behavioural convulsive profiles closely resembling seizure profiles seen in humans with epilepsy (see also McNamara, Bonhaus, & Shin, 1985). Goddard et al. (1969) also found that, even after three months devoid of kindling stimulation, application of a single stimulation was sufficient to evoke a generalized seizure suggesting that, similar to epilepsy, kindling results in a permanently increased susceptibility to seizures. Goddard's group did not find gross evidence of tissue damage in kindled brains, but they recognized that neurons remaining at the generator site may have been viable, but were altered making them more susceptible to epileptogenesis. Perhaps the most important finding in Goddard's studies was that kindling could be used to study motor convulsions and the associated morphological, biochemical and electrophysiological alterations in a controlled manner.

In 1972 , Racine published a series of articles that further characterized the kindling phenomenon. He found that electrographic seizure thresholds referred to as afterdischarge thresholds could be lowered in limbic sites such as the amygdala and the hippocampus. Afterdischarge occurs after repeated application of either subthreshold or suprathreshold low-intensity electrical kindling stimulation (Racine, 1972a). He also found that the afterdischarge waveform, frequency, amplitude and duration became increasingly more complex and corresponded with increasingly complex motor convulsions. Racine rated the severity of the convulsive symptoms on

a 6-point scale: Initially, (stage 0) rats display little or no convulsive symptoms but often increase exploratory behaviours for the duration of the afterdischarge. In stage 1, rats become immobile and exhibit eye blinking and rhythmic masticatory movements that, over a few days of stimulation, will progress to rhythmic head nodding (stage 2), unilateral forelimb clonus (stage 3), bilateral forelimb clonus and rearing (stage 4) and culminate in a generalized convulsion (stage 5) that includes all of the above behaviours plus hindlimb clonus, rearing and falling (Racine, 1972b). Once rats reach a stage 5 seizure they are often described as having reached a fully kindled state and it is assumed that, at least by this point, some permanent morphological, biochemical or electrophysiological alterations have occurred within the kindled brain.

Brain sites vary in degree of epileptogenic susceptibility (Mohapel, Dufresne, Kelly, & McIntyre, 1996; Racine, 1972b; Racine, 1978). For instance some cortical areas like the perirhinal (Mohapel & Corcoran, 1995) and piriform (McIntyre & Plant, 1989) cortices can kindle very rapidly, requiring as little as 1 to 3 daily stimulations to reach the first generalized seizure. Other sites such as the hippocampus kindle very slowly, requiring as many as 30-40 daily stimulations (Racine, 1972b) to reach the first generalized seizure.

Amygdala

The amygdala is a phylogenically old structure within the medial temporal lobe that comprises a heterogenous group of cytoarchitectonically distinct nuclear and cortical structures (Amaral, Price, Pitkänen, & Carmichael, 1992; Pitkänen, & Amaral,

1991; Price, Russchen, & Amaral, 1987; Savander, Go, LeDoux, & Pitkänen, 1995) that can be darkly stained and easily visualized with cresyl violet. The major nuclei of the amygdala include the medial, central, accessory basal, basal, basolateral and lateral nuclei. These individual nuclei have been popular sites for kindling because their rate of kindling is reliable and relatively fast (i.e., the basolateral nucleus kindles within 10-15 days; the central nucleus kindles within 7-10 days) (Goddard et al., 1969; Mohapel et al., 1996; Racine, 1972b). The amygdala itself is an attractive site to kindle because it receives massive inputs from several cortical areas including the temporal, frontal, insular and cingulate association cortices (Krettek & Price, 1974) and in many instances it returns projections to these cortical areas, as well as to other cortical areas like the peristriate and primary visual cortex (Amaral et al., 1992). The amygdala also has massive reciprocal connections with subcortical structures. Thus, the amygdala has the properties of a bidirectional conduit capable of relaying sensory information between association cortices and subcortical structures and capable of simultaneously influencing or modulating the excitability of several brain sites at once.

The amygdala is thought to play an important functional role in epileptogenesis because it is almost always involved in seizure activity generated from limbic sites (Goddard et al., 1969; but see McIntyre & Plant, 1989) and there is a strong relationship between the kindling rate of limbic structures and the number of direct synaptic connections that those structures have with the amygdala (Goddard et al., 1969). However, other evidence suggests that an intact amygdala is not necessary for the induction or maintenance of epileptogenesis. McIntyre (1980) found that

destruction of the amygdala did not prevent or slow the rate of dorsal hippocampal kindling suggesting that structures other than the amygdala may play a role in epileptogenesis.

In most mammals, the amygdala lies slightly anterior to the ventral portion of the hippocampus, yet despite this close physical proximity, there are few reciprocal interconnections between the two structures (Amaral et al., 1992). Recent anatomical (Amaral et al., 1992; Krettek & Price, 1974) and electrophysiological (Ikegaya, Saito, & Abe, 1996; Thomas, Assaf, & Iversen, 1984; Racine, Milgram, & Hafner, 1983) evidence of interconnections demonstrates that projections arising from cells in lateral, basolateral and accessory basal nuclei of the amygdala project heavily to layers II and III of the lateral entorhinal cortex, which provides the major input to the hippocampus and dentate gyrus (see below). In contrast, only a few monosynaptic projections (Mello, Cavalheiro, Tan, Kupfer, Pretorius, Babb, & Finch, 1993) arising from cells situated along the border of area CA1 within the hippocampus project to nuclei within the amygdala (Canteras & Swanson, 1992; Otterson, 1982) suggesting that the amygdala potentially has greater influence on the hippocampus than visa versa.

Hippocampus

The hippocampus (Figure 1) is an archicortical structure that partly envelops the thalamus. It is described two ways: the hippocampus proper and the hippocampal region. The hippocampus proper[2], stretches between the dentate gyrus and the subiculum and is divided into four areas CA1-CA4 whereas the hippocampal region includes both the hippocampus proper and the anatomically close dentate gyrus, subiculum and entorhinal cortex (Figure 2).

Most of the extrinsic sensory information received and integrated by the hippocampus is mediated through the entorhinal cortex. A unique trisynaptic excitatory loop (Anderson, Bliss, & Skrede, 1971; Bliss & Lømo, 1973) connects the entorhinal cortex to the hippocampus: layers II and III of the lateral entorhinal cortex give rise to a major projection, the perforant path[3] (Amaral & Witter, 1989; Steward & Scoville, 1976; Swanson & Cowan, 1977; Witter, 1993), that primarily makes excitatory synaptic contact with granule cells located within the granule cell layer (*stratum granulosum*) of the dentate gyrus (see Figure 3). In turn, the granule cells give rise to unmyelinated mossy fiber axons that emerge from the basal pole of the soma, traverse the polymorphic layer of the dentate gyrus (*hilus*) and form excitatory

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[2] The hippocampus proper is also referred to as Ammon's horn from the Latin term *cornu Ammon,* meaning the structure resembles a ram's horn. The divisions of the hippocampus proper (CA1-CA4) were derived from the Latin term *cornu Ammon*.

[3] The perforant path is the most prominent afferent projection to the hippocampus. It arises from cells in the entorhinal cortex, around the region of the angular bundle, and perforates the hippocampal fissure prior to making excitatory synaptic contact primarily, but not exclusively, with granule cells in the dentate gyrus. The perforant path is the first excitatory synapse of the classic "trisynaptic loop" described by Bliss and Lømo (1973).

Figure Caption

Figure 1. 3-D schematic of the rat hippocampus. Note that anterior is closest to the left and posterior is closest to the right side of the figure. All other structures have been omitted so that the shape of the hippocampus can be fully appreciated.

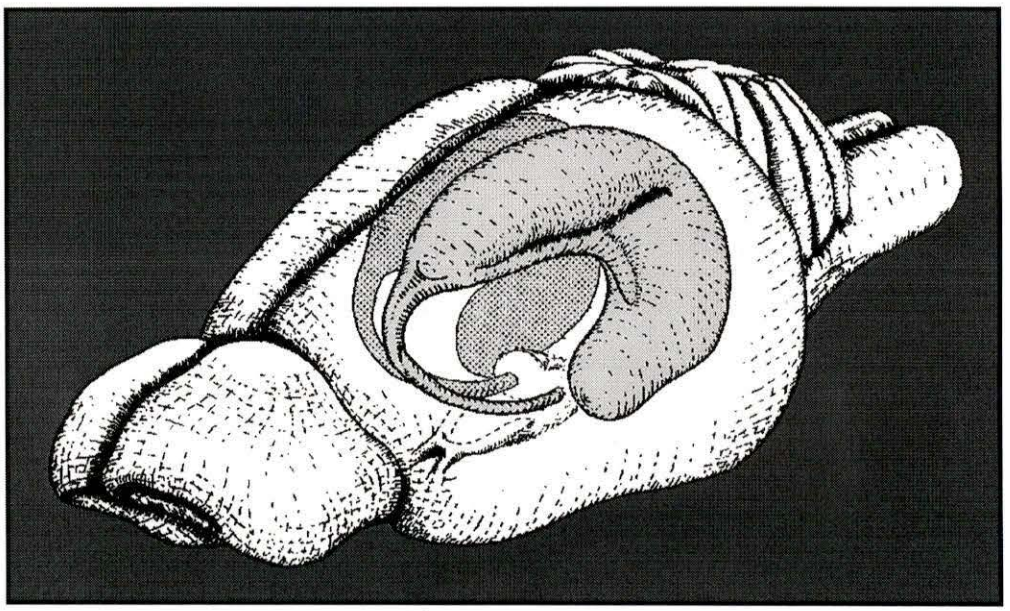


Figure Caption

Figure 2. Schematic of a horizontal section of the hippocampal region (left hippocampus). The hippocampus proper includes areas CA1, CA2 and CA3. The dentate gyrus includes the *hilus*, the granule cell layer (*stratum granulosum*) and the associated molecular layers (represented by the dotted line around the tip of the *hilus*). Note that the anterior hippocampus is closest to the top of the figure.

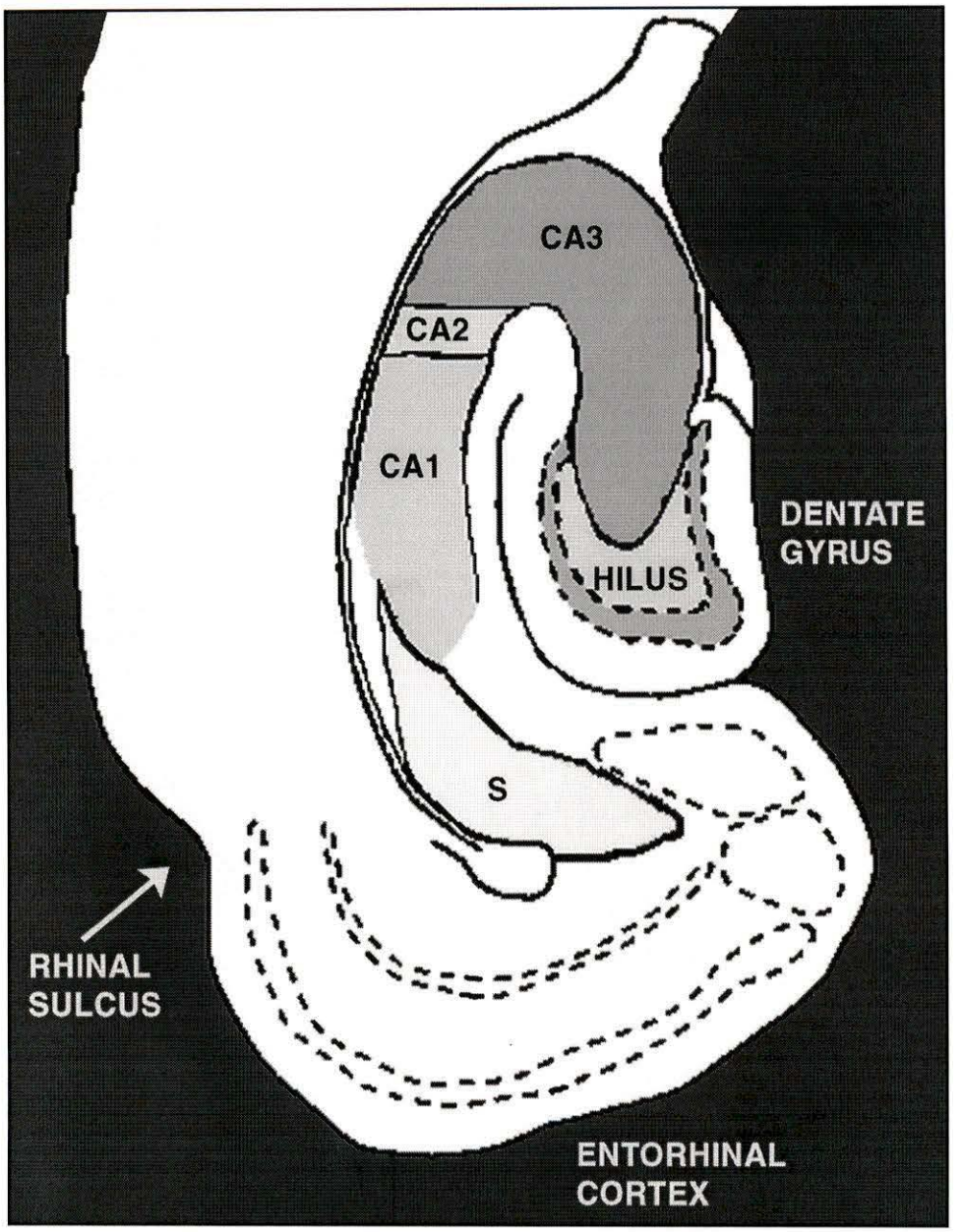

CA3
CA2
CA1
DENTATE
GYRUS
HILUS
S
RHINAL
SULCUS
ENTORHINAL
CORTEX

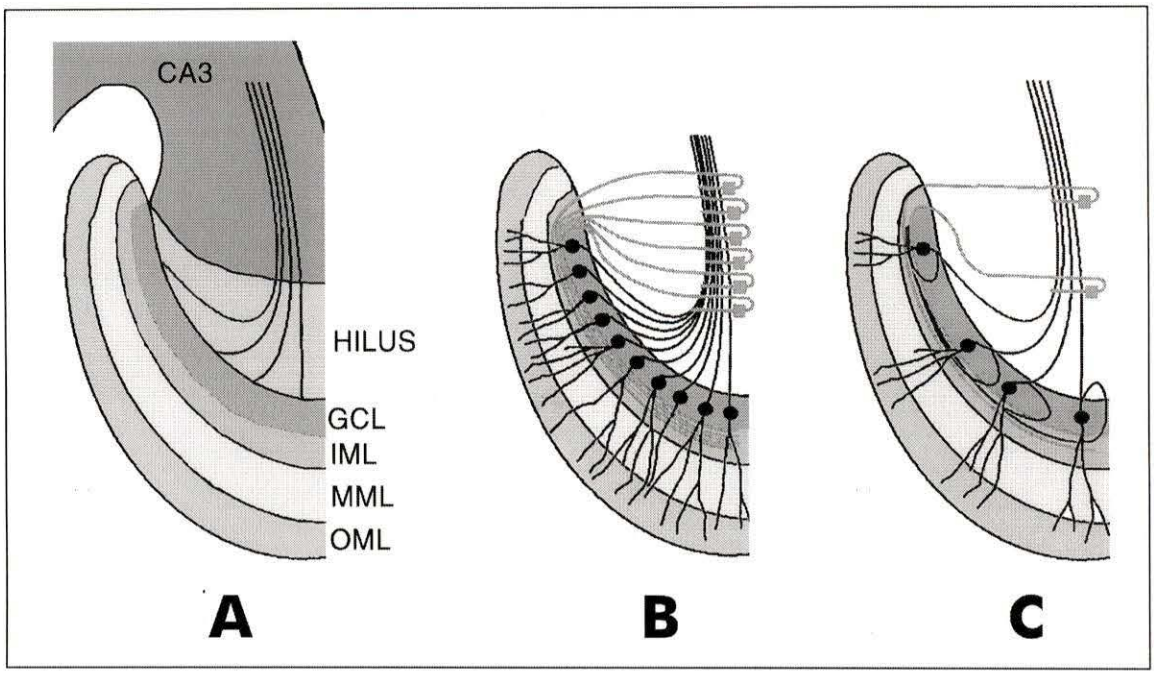
Figure Caption

Figure 3. Schematic of a horizontal section of the left dentate gyrus.

A. Illustration of the posterior blade of the dorsal dentate gyrus: hilus/ *polymorphic layer*, granule cell layer/ *stratum granulosum* (GCL), inner molecular layer (IML), middle molecular layer (MML) and outer molecular layer (OML). Note that the anterior hippocampus is closest to the top of the figure.

B. Schematic of mossy fiber axons in normal brain tissue. Mossy fiber dendrites, located within the MML and OML, are primarily innervated by the perforant path. The mossy fiber axons arise from the granule cells, residing in the GCL, and normally project through the hilus to area CA3 where they primarily make asymmetric synaptic contacts with large pyramidal cells. Mossy fiber collaterals also make some synaptic connections with hilar mossy cells. The mossy cells project axons to the IML where presumably they make excitatory synaptic contact with inhibitory interneurons and nongranule cells.

C. Schematic of mossy fiber sprouting that is found in tissue resected from patients with temporal lobe epilepsy or in tissue taken from rats that have had experimentally-induced seizures. This figure demonstrates that, in epileptogenic conditions, mossy fiber collaterals sprout back into the IML where they make excitatory synaptic contact with at least some of the parent granule cells.


CA3
HILUS
GCL
IML
MML
OML
A
B
C

synapses[4] with large pyramidal cells in area CA3. In turn again, the axons of pyramidal cells in CA3 give rise to Schaffer collaterals that synapse with smaller pyramidal cells in CA1 which complete the trisynaptic loop (Figure 4) by projecting axons to the entorhinal cortex via the subiculum[5].

Projections from several limbic regions including the septal region, the hypothalamus, the thalamus, the claustrum and the amygdala (Fibiger, 1982; Finch, Wong, Derian, Chen, Nowlin-Finch, & Brothers, 1986; Köhler, Swanson, Haglund, & Wu, 1985; Wyss, Swanson, & Cowan, 1979 a,b) in addition to other polysensory association cortices (Amaral, Insausti & Cowan, 1983; Insausti, Amaral, & Cowan, 1987; Kosel, Van Hoesen, & West, 1981; Lopes da Silva, Witter, Boeijinga, & Lohman, 1990; Witter, Room, Groenewegen, & Lohman, 1986), the olfactory cortex (Room, Groenewegen, & Lohman, 1984) and the brainstem (Kohler & Steinbusch, 1982) converge within the entorhinal cortex and disperse throughout the hippocampus. Thus, several structures can potentially regulate excitation in the hippocampus via the trisynaptic loop that connects the hippocampus and the entorhinal cortex.

Within the dentate gyrus, the thin granule cell mossy fibers are easily identified at the ultrastructural level (Amaral & Dent, 1981; Blackstad & Kjaerheim, 1961;

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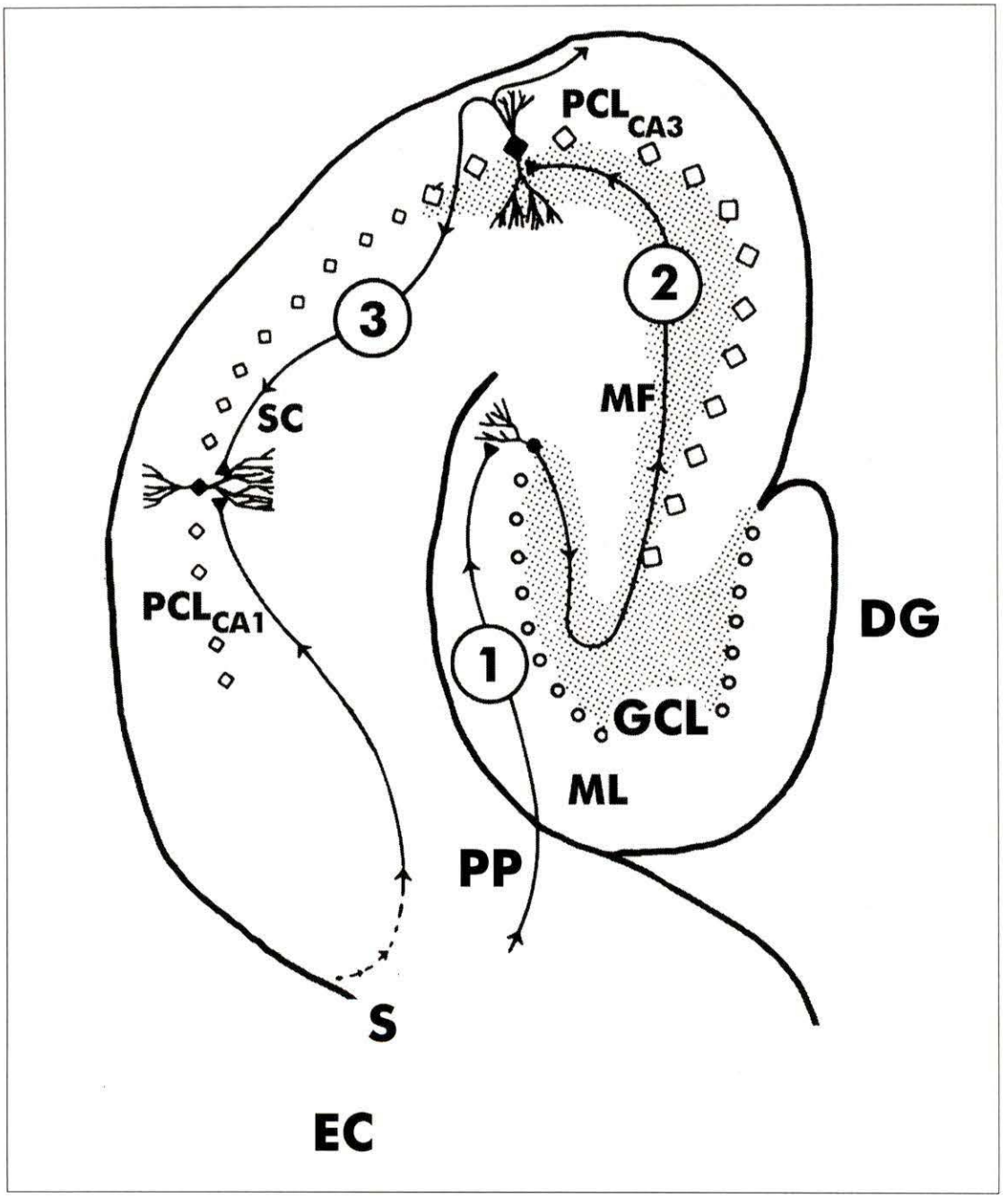
[4] Although mossy fibers do not normally project back into *stratum granulosum* there is some evidence that even in a normal brain mossy fibers can occasionally be observed making synaptic contact with dendrites of granule cells (Gaarskjaer, 1978).

[5] Note that the concept of the "trisynaptic loop" has been simplified to illustrate the point that hippocampal circuitry may be modulated by its connections to the entorhinal cortex. There is evidence of excitatory connections elsewhere in the hippocampus. For instance, neurons in CA3 are capable of exciting dentate granule cells via mossy cells (not to be confused with mossy fibers) (Scarfman, 1994; Scarfman, 1995; Jackson & Scarfman, 1996).

Figure Caption

Figure 4. Schematic of the hippocampal "trisynaptic loop". Abbreviations: PP, perforant path; DG, dentate gyrus; ML; molecular layers; GCL, granule cell layer; MF, mossy fiber axons; PCL_{CA3}, pyramidal cell layer; SC, Schaffer collaterals; PCL_{CA1}, pyramidal cell layer; S, subiculum; EC, entorhinal cortex.


PCL CA3
2
3
SC
MF
PCL CA1
DG
1
GCL
ML
PP
S
EC

Hamlyn, 1962) and after immuno- or Golgi-labelling (Frotscher & Zimmer, 1983) and are distinct because of their mossy-like appearance. The terminal boutons of mossy fibers contain high amounts of metallic zinc so the fibers can also be labelled with a sulfide silver histochemical technique (Danscher, 1981; Danscher & Zimmer, 1978). This silver stain, or Timm stain, labels the mossy fiber terminals a brown-black color that can be visualized at both the light and electron microscopic levels (Claiborne, Amaral, & Cowan, 1986; Danscher & Zimmer, 1978; Haug, 1967; Sloviter, 1982). Dense staining of mossy fibers is typically localized in the *hilus* and area CA3 where the axon terminals are most abundant; however, ectopic staining of mossy fibers has been observed in the inner molecular layer of the dentate gyrus (Figure 3) after lesions of both hippocampal afferents (Frotscher & Zimmer, 1983; Zimmer, 1974) and efferents (Hannesson, Armitage, Mohapel, & Corcoran, 1997) and after various forms of epileptiform activity.

Mossy Fiber Sprouting

Evidence of ectopic mossy fiber sprouting in the inner molecular layer of the dentate gyrus (see Figure 3) has been found in tissue specimens from rats displaying seizures after treatment with kainic acid (Ben-Ari, 1985; Cavalheiro, Riche, & Le Gal La Salle, 1982; Cronin & Dudek, 1988; Davenport, Brown, & Babb, 1990; Laurberg & Zimmer, 1981; Mathern, Cifuentes, Leite, Pretorius, & Babb, 1993; Sundstrom, Mitchell, & Wheal, 1993; Tauck & Nadler, 1985; Wuarin & Dudek, 1996), pentylenetetrazol (Golarai, Cavazos, & Sutula, 1992) or pilocarpine (Mello,

Cavalheiro, Tan, Pretorius, Babb, & Finch, 1992; Mello et al., 1993), from adult *Stargazer* mutant (Qiao & Noebels, 1993) and tottering (tg/tg) (Stanfield, 1989) mice displaying epileptiform activity, and from patients with temporal lobe epilepsy (Babb, Kupfer, Pretorius, Crandall, & Levesque, 1991; de Lanerolle, Kim, Robbins, & Spencer, 1989; Houser, Miyashiro, Swartz, Walsh, Rich, & Delgado-Escueta, 1990; Mathern, Pretorius, & Babb, 1995; Sutula, Cascino, Cavazos, Parada, & Ramirez, 1989). In addition, mossy fiber sprouting has been detected in the supragranular region (Cavazos, Golarai, & Sutula, 1991; Ebert & Löscher, 1995; Sutula, Xiao-Xian, Cavazos, & Scott, 1988), and in the CA3 region of the hippocampus (Ben-Ari & Represa, 1990; Represa & Ben-Ari, 1992; Represa, Le Galle La Salle, & Ben-Ari, 1989) after kindling of sites in the forebrain. There is a strong correlation between mossy fiber sprouting and kindling that has led to the suggestion that mossy fiber sprouting may play a functional role in the development or maintenance of epileptogenesis (Sutula et al., 1988). Specifically, it has been proposed that during kindling the granule cells sprout new excitatory mossy fiber axons back onto the parent cells in the granule cell layer (Tauck & Nadler, 1985) and that the net result is a recurrent excitatory circuit, or epileptic generator, that is responsible for the elaboration and eventual propagation of ictal discharge throughout the brain (Cronin, Obenhaus, Houser, & Dudek, 1992; Sutula, 1990; Tauck & Nadler, 1985).

An Excitatory Role?.

In one of the original electrophysiological studies aimed at understanding the functional significance of mossy fiber sprouting, Tauck and Nadler (1985) demonstrated that 12 to 21 days after rats were treated with kainic acid, antidromic activation of granule cells evoked a response consisting of multiple population spikes. The resulting antidromic response was interpreted as an indirect measure of alterations in granule cell excitability (see also Clusmann, Stabel, Stephens, & Heinemann, 1992; Clusmann, Nitsch, & Heineman, 1994). In addition, Tauck and Nadler found that the response could be potentiated rather than depressed by a "paired-pulse" conditioning stimulus which was subsequently found to correlate to the intensity of mossy fiber sprouting (Sloviter, 1992) thus supporting the previous conclusions that mossy fiber sprouting had an excitatory function. Support for these findings also comes from ultrastructural analysis of mossy fiber sprouting subsequent to partial or complete transection of the hippocampus (Laurberg & Zimmer, 1981), Golgi-electron microscopy of sprouted collaterals subsequent to lesions (Frotscher & Zimmer, 1983) or kindling (Represa, Jorquera, Le Galle La Salle, & Ben-Ari, 1993) of limbic pathways. In addition there is anatomical data that demonstrate sprouting of biocytin labelled mossy fiber boutons subsequent to kainic acid treatment (Okazaki, Evenson, & Nadler, 1995) occurs in the inner molecular layer of the dentate gyrus and synapse on at least some of the parent granule cell dendrites (Laurberg & Zimmer, 1981; Sutula et al., 1988). Furthermore, these anatomical data suggest that mossy fiber sprouting appears and appears to be associated with increased excitability (Babb et al.,

1991; Cavazos et al., 1991; Frotscher & Zimmer, 1983; Laurberg & Zimmer, 1981; Okazaki et al., 1995; Represa et al., 1993; Sutula et al., 1988, 1989; Tauck & Nadler, 1985).

An Inhibitory Role?.

The functional significance of mossy fiber sprouting is however a complex issue whose interpretation is compounded by numerous reports of increased inhibition in the dentate gyrus of kindled brains (deJonge & Racine, 1987; Fricke & Prince, 1984; Milgram, Michael, Cammisuli, Head, Ferbinteanu, Reid, Murphy, & Racine, 1995; Tuff, Racine, & Adamec, 1983) and resected tissue from patients with temporal lobe epilepsy (Uruno, O'Connor, & Masukawa, 1994, 1995). It has been suggested that morphological alterations occurring within the dentate gyrus are associated with increases in inhibition and play a role in resisting or dampening epileptic activity (Ribak & Peterson, 1991; Sloviter, 1991). In support of this hypothesis there is evidence that mossy fiber collaterals make excitatory synaptic contact with non-granule cells and inhibitory interneurons in *stratum granulosum* (Ribak & Peterson, 1991; Ribak, Seress, & Amaral, 1985) that most likely regulate recurrent and feedforward inhibition of granule cells (Frotscher & Zimmer, 1983; Ribak, 1992; Ribak & Seress, 1983). In 1992, Sloviter reported that kainate treated rats display evidence of reduced inhibition (relative to controls) within the dentate gyrus prior to evidence of sprouted mossy fibers. He also found that in kainate treated rats, mossy fiber sprouting appeared to be more closely associated with the recovery rather than

the reduction of inhibition suggesting that sprouting may play a role in *decreasing* rather than increasing hyperexcitability associated with seizures.

A Balancing Role?.

In the same year, an alternative hypothesis was published suggesting that mossy fiber sprouting may increase excitation that is masked by hypo-inhibition at times when there is recovering inhibition in the hippocampi of rats (Cronin et al., 1992; see also Sloviter, 1992) and humans (Masukawa, Uruno, Sperling, O'Connor, & Burdette, 1992). In this hypothesis mossy fiber sprouting may play a role in facilitating epileptogenesis or may play a role in balancing excitation and inhibition (Mody, Otis, Staley, & Kohr, 1992). For instance, in an electrophysiological study performed by Cronin et al. (1992) the association between kainic acid induced seizures, mossy fiber sprouting and intracellular inhibition was assessed. Unexpectedly, Cronin et al. found that 1-4 months after treatment of kainic acid, tissue slices with detectable levels of mossy fiber sprouting expressed normal levels of inhibition. However, after application of bicuculline, a GABA antagonist, they found that excitatory epileptiform activity was unmasked in some (about one-third) but not all of the slices with mossy fiber sprouting. Thus, they concluded that 1) not all sprouted fibers are associated with aberrant excitation and 2) the excitatory function of some sprouted fibers may be suppressed or masked until inhibition is sufficiently reduced for expression of this hyperexcitability.

Mechanism of Kindling?.

Evidence for the hypothesis that mossy fiber sprouting is a mechanism of kindling was provided by Sutula (1991), who described patterns of mossy fiber sprouting in kindled and control rats. Sutula et al., (1988) found that kindling of several limbic sites produced aberrantly located Timm granules in the inner molecular layer of the dentate gyrus in 12 of 13 rats killed 18 hr after one generalized seizure. The sprouting was found in homologous regions in the contralateral hemisphere; however, the most dense sprouting was consistently ipsilateral to the site of stimulation. Ultrastructural analysis of data from 4 rats confirmed that the Timm granules were located in the terminal boutons of the mossy fibers, suggesting that the stained Timm granules represent sprouting and reactive synaptogenesis. Sutula's group (Cavazos et al., 1991) subsequently demonstrated that the magnitude of sprouting correlated with the duration of epileptic activity and that asymptotic levels of sprouting persisted over intervals of 3 to 4 months after kindling of generalized seizures. This therefore was taken to suggest that mossy fiber sprouting could be a mechanism for both the establishment and maintenance of kindling.

Rationale

Cavazos, Golarai and Sutula (1991) have shown that mossy fiber sprouting progresses in parallel to kindling induced by twice daily stimulation of the perforant path and amygdala. These findings suggest that there is a strong correlation between the progression of kindled seizures and mossy fiber sprouting. However, regardless of the functional role of sprouting (excitatory or inhibitory), interpretation of the relation between sprouting and kindling may be obscured by the presence of a strong temporal association. Methodologically, detection of any possible *dissociations* between conventional kindling and mossy fiber sprouting is problematic because the phenomena appear to share parallel time courses.

To determine whether kindling can be dissociated from mossy fiber sprouting, a rapid kindling preparation can be applied, wherein seizures can be kindled very rapidly with long trains of daily low-frequency stimulation at high intensities to the amygdala (Cain & Corcoran, 1981; Corcoran & Cain, 1980; Pelletier & Corcoran, 1992; Pelletier & Corcoran, 1993). Stimulation of the amygdala with 60 sec trains at 3 pps results in development of generalized seizures after a mean of about 3 to 5 stimulations (Corcoran & Cain, 1980; Pelletier & Corcoran, 1992), well below the mean of 13 afterdischarges required with 1 sec trains of conventional high-frequency stimulation at 60 pps. The spike and wave pattern of afterdischarge provoked by both high- and low-frequency stimulations appear to be very similar (Pelletier & Corcoran, 1992). Furthermore, seizure susceptibility established with low-frequency kindling transfers readily to high-frequency stimulation (Cain & Corcoran, 1981) and persists

over an extended period without additional stimulations (personal observations), confirming that low-frequency stimulation induces genuine kindling. Therefore, if mossy fiber sprouting is necessary for kindling, one would expect to be able to detect sprouting 6 days after initiation of low-frequency stimulation, at which point generalized seizures would have been kindled. We therefore attempted to determine whether mossy fiber sprouting occurs subsequent to low-frequency (rapid) kindling.

CHAPTER III

METHOD

Subjects

Eighty-four male Long-Evans hooded rats (Charles River, Quebec) were individually housed in a temperature controlled colony, with unrestricted access to food and water. The rats weighed between 270-370 g at the time of surgery, and all kindling trials occurred during the light portion of the 12 hr light/dark cycle.

Groups

Rats were yoked into pairs that were treated identically with respect to handling, surgery, perfusions, and staining, with the exception that yoked control rats were never connected to the stimulation/recording apparatus or received electrical stimulation. Experimental rats were assigned to one of two kindling conditions, either conventional kindling with high-frequency (60 pps) stimulation or rapid kindling with low-frequency (3 pps) stimulation. Four treatment groups were established as follows: HF1, rats that received once daily high-frequency stimulation and were killed 1 day after the first stage 5 (Racine, 1972b) generalized seizure; HF5, rats that received once daily high-frequency stimulation and were killed 1 day after the fifth of 5 consecutive stage 5 seizures; LF1, rats that received once daily low-frequency stimulation and were killed 1 day after the first stage 5 seizure; LF1-20, rats that received once daily low-frequency stimulation until one stage 5 seizure was evoked and were killed 20 days

after the initiation of kindling. The same afterdischarge threshold procedure was used for both the HF and LF groups.

Surgery

Surgical procedures were conducted according to the guidelines of the Canadian Council on Animal Care. Following 7 days of habituation to the colony, rats were anaesthetized with sodium pentobarbital (Somnotol®; 65 mg/kg, i.p.) and placed in a stereotaxic frame. Unseparated, bare tips of bipolar stimulating/ recording electrodes (127 μm dia., Teflon-coated nichrome wire) were bilaterally implanted into the basolateral nucleus of the amygdala. Stereotaxic coordinates were 0.4 mm posterior to bregma, 4.5 mm lateral to midline and 8.4 mm ventral to the surface of the skull, with the incisor bar angled at +5.0 mm (Paxinos & Watson, 1986). A reference wire and four additional dental screws were anchored to the skull, to which the electrodes were affixed with dental acrylic.

Kindling

Afterdischarge thresholds.

Ten days after surgery, the threshold for amygdaloid afterdischarge and the associated afterdischarge duration were measured at one of the two electrode sites within each experimental rat. Electrical stimulation consisted of a 1-sec train of constant current balanced biphasic square-wave pulses (1-msec duration) at 60 pps. Stimulation was delivered at an initial current of 50 μA (base-to-peak) and increased

in increments of 50 μA until afterdischarge was triggered (Pelletier & Corcoran, 1992; Pelletier & Corcoran, 1993). Afterdischarge threshold was arbitrarily defined as the lowest intensity to evoke an afterdischarge lasting 5 or more sec. Twenty-four hr later the afterdischarge threshold procedure was repeated in the contralateral amygdala.

High-frequency (conventional) kindling.

High-frequency stimulation was delivered 24 hr after afterdischarge threshold determination and was applied once daily at 100 μA above threshold via one of the electrodes. The duration and magnitude of the afterdischarge as well as the behavioural seizure stages were recorded after each stimulation. Daily stimulation continued until 1 generalized stage 5 was evoked (HF1) or 5 consecutive generalized stage 5 seizures were evoked (HF5).

Low-frequency (rapid) kindling.

Electrical stimulation consisted of a 60-sec train of constant current balanced biphasic square-wave pulses (1-msec duration) at 3 pps and was delivered beginning 24 hr after determination of afterdischarge threshold. Low-frequency stimulation was applied once daily via one of the electrodes at 1,000 μA, an intensity generally sufficient to trigger afterdischarge during the train (personal observations; see also Pelletier & Corcoran, 1992), until 1 generalized stage 5 seizure was evoked. Stimulation was terminated if a stage 4 or 5 generalized seizure developed during the train.

Timm Histochemistry

We used Sloviter's (1982) modification of the Timm stain previously employed by Danscher (1981) to stain the mossy fibers. Rats were deeply anaesthetized with an overdose of sodium pentobarbital and perfused transcardially with saline (0.9 g NaCl per 100 ml H_2O) followed by sodium sulfide solution (1.17g $Na_2S.9H_2$, 1.19 g of $NaH_2PO_4 H_2O$ per 100 ml of H_2O), saline (as above), and formalin solution (4.0 g paraformaldehyde, 1.1 g of Na_2HPO_4, 0.34 g of $NaH_2PO_4 H_2O$ per 100 ml of H_2O). Approximately 12 to 16 hr later, brains were removed and fixed in formalin solution (as above) for 2 hours and then were transferred to a 25 % sucrose buffer solution and refrigerated (4 °C) for at least 24 hr. The brains were frozen and horizontal sections at 40 μm were cut on a sledge microtome (Leica, RC2000). Sections were mounted on glass slides coated in chromium potassium sulfate gelatin, and air dried in a dark room. Mounted sections from the four experimental groups were randomly assigned to different staining lots. However, yoked control sections were always stained in the same lot (vial) as the corresponding exerimental sections in order to minimize differences in background staining. Within 2 weeks sections were developed in the Timm stain for 50-60 minutes. The stain was composed of a 120:60:20:1 ml mixture of gum arabic (50 g/100 ml), hydroquinone (5.7 g C_6H_4-1,4-$(OH)_2$/100 ml), citrate solution (23.5 g $C_6H_5Na_3O_7$ $2H_2O$/ 100 ml, 25.5 g $C_6H_8O_7$ H_2O/100 ml), and silver nitrate solution (17 g $AgNO_3$/100 ml). After development, sections were gently rinsed in distilled water, dehydrated in graded ethanol, transferred to xylene and coverslipped

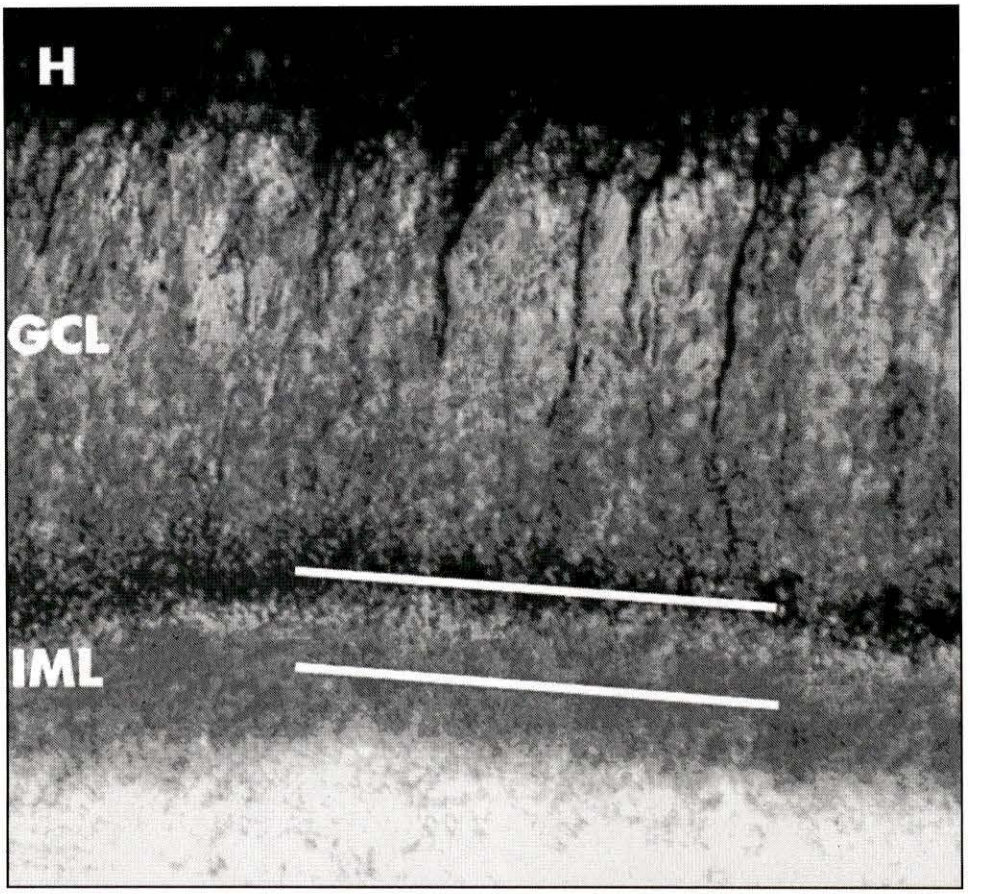
with Permount resin. Electrode placements were verified in 80 μm coronal sections stained with cresyl violet.

Optical Density Analysis

The results of pilot studies indicated that the enhancement of Timm staining associated with moderate levels of kindling is concentrated in the posterior blade of the dorsal (septal) dentate gyrus; therefore we quantified the staining in this region in the present experiment. Stained sections from the dorsal dentate gyrus that corresponded to horizontal sections 3.64 to 4.10 mm ventral to bregma (Paxinos & Watson, 1986) were mounted on a Zeiss microscope, which was equipped with a 6.3x objective lens, a stabilized light source, and an interference filter (546 nm). Digital images were captured by a camera and displayed on a monitor at a resolution of 512 x 512 pixels, wherein the gray scales were analysed with Optimas 3.2 image analysis software. Optical densities of sections were determined by assigning a numerical value between 0 (black) and 255 (white) to each pixel according to its gray scale. The photomicrograph in Figure 5 demonstrates the regions in which samples were taken of the optical density of the Timm stain in the inner edge of the inner molecular layer (IML) and the corresponding optical density of the background stain.

Figure Caption

Figure 5. Photomicrograph representing areas scanned for optical density analysis. Abbreviations: hilus/ *polymorphic layer* (H), granule cell layer/ *stratum granulosum* (GCL) and inner molecular layer/ *stratum moleculare* (IML). One line was drawn across the inner third of the IML, with computer software, to sample the optical density of the area containing Timm granules and a second parallel line was drawn across the outer third of the IML to sample the optical density of the background stain. Each line was the equivalent of 6 pixels wide.


H
GCL
IML

We refer to the difference between these two areas (inner edge of the IML and background) as the percent difference in optical density and calculated it according to the following:

Sprouting Density Formula.

$$\frac{\text{(outer IML - inner IML)}}{\text{(outer IML + inner IML) / 2}} \times 100\ \% = \text{percent difference in optical density}$$

The outer edge of the IML is the background area, where significant Timm staining is not typically detected. The inner edge of the IML is the area where significant Timm staining is typically detected after kindling. Each section received two scores, one for each hemisphere, that were averaged across the six serial sections to give one mean percentage difference score between the background and Timm stain for each rat. The percentage difference score of each kindled rat was then compared to the percentage difference score of their yoked controls.

Statistical Analysis

A one-way analysis of variance (ANOVA) was used to evaluate statistical differences in the kindling parameters and optical density percentage difference scores obtained for all groups. Planned comparisons between individual groups were made with one-tailed *t*-tests. Differences were considered statistically significant when $p < .05$. Values are given as mean ± S.E.M.

CHAPTER IV

RESULTS

Kindling

Kindled rats and their respective yoked controls were included in the data analysis only if the electrode tips were located in the basolateral nucleus of the amygdala, because we have found that low-frequency kindling of the amygdala occurs reliably only with electrode tips located in the basolateral nucleus (Jenkins, 1995). Under this criterion, we excluded 7 yoked pairs of rats because of inaccurate electrode placement, and 6 additional yoked pairs were excluded because of damaged tissue samples that precluded meaningful analysis. Therefore the data of 28 yoked pairs of rats ($N = 56$) were included in the analysis.

The difference in the afterdischarge thresholds of the low-frequency kindled group ($M = 175 \pm 34$ μA; $n = 15$) and high-frequency kindled group ($M = 152 \pm 30$ μA; $n = 13$) was not statistically significant ($p > 0.7$); nor was the difference in initial afterdischarge durations in the low-frequency kindled group ($M = 7.7 \pm 1.0$ sec) and high-frequency kindled group ($M = 8.4 \pm 1.4$ sec) significant ($p > 0.6$). As expected, significantly fewer once daily low-frequency stimulations ($M = 4.8 \pm 0.6$ days; $n = 15$) than once daily high-frequency stimulations ($M = 12.8 \pm 1.3$ days; $n = 13$) were required to kindle the first stage 5 seizure ($F(1,26) = 32.3$, $p < .01$).

To summarize, rats that received low-frequency kindling stimulation required a mean of 5 days to reach a fully kindled state whereas rats that received high-frequency kindling stimulation required a mean of 13 days to reach a fully kindled state.

Optical Density Analysis

Oneway ANOVA on percentage difference scores of kindled and yoked control rats across all kindling trials indicated that kindling produced detectable levels of mossy fiber sprouting (Figure 6) as shown by an overall significant group effect ($F(7,48) = 4.751$, $p < .01$).

Mossy Fiber Sprouting Subsequent to High-Frequency Kindling

Both groups of rats kindled with high-frequency stimulation displayed a patchy but continuous distribution of Timm granules extending from the tips to the crest of the posterior blade of the dorsal (septal) dentate gyrus (Figure 6). In contrast, yoked controls displayed minimal yet visible Timm granules in the inner molecular layer of the dorsal (septal) aspects of the posterior blade (see Figures 6 and 7).

The HF1 ($n=6$) group displayed significantly higher percent differences in optical density than yoked controls ($t(10) = 4.84$, $p < .05$; Figure 7). Similarly, the HF5 ($n = 7$) group displayed significantly higher percent differences in optical density than yoked controls ($t(12) = 2.81$, $p < .05$; Figure 7). Although the magnitude of sprouting appeared to be greater in the HF5 group than in the HF1 group, the difference between them was not statistically significant ($t(11) = -.81$, $p > .05$).

Figure Caption

Figure 6. Photomicrograph representing mossy fiber sprouting. Each photomicrograph is representative of the Timm stained mossy fibers observed in the inner portion of the IML of yoked control rats (left) and experimental rats (right). The arrowheads indicate the region of mossy fiber sprouting and highlights the darkly stained silver-Timm granules. A, HF1; B, HF5; C, LF1; D, LF1-20.

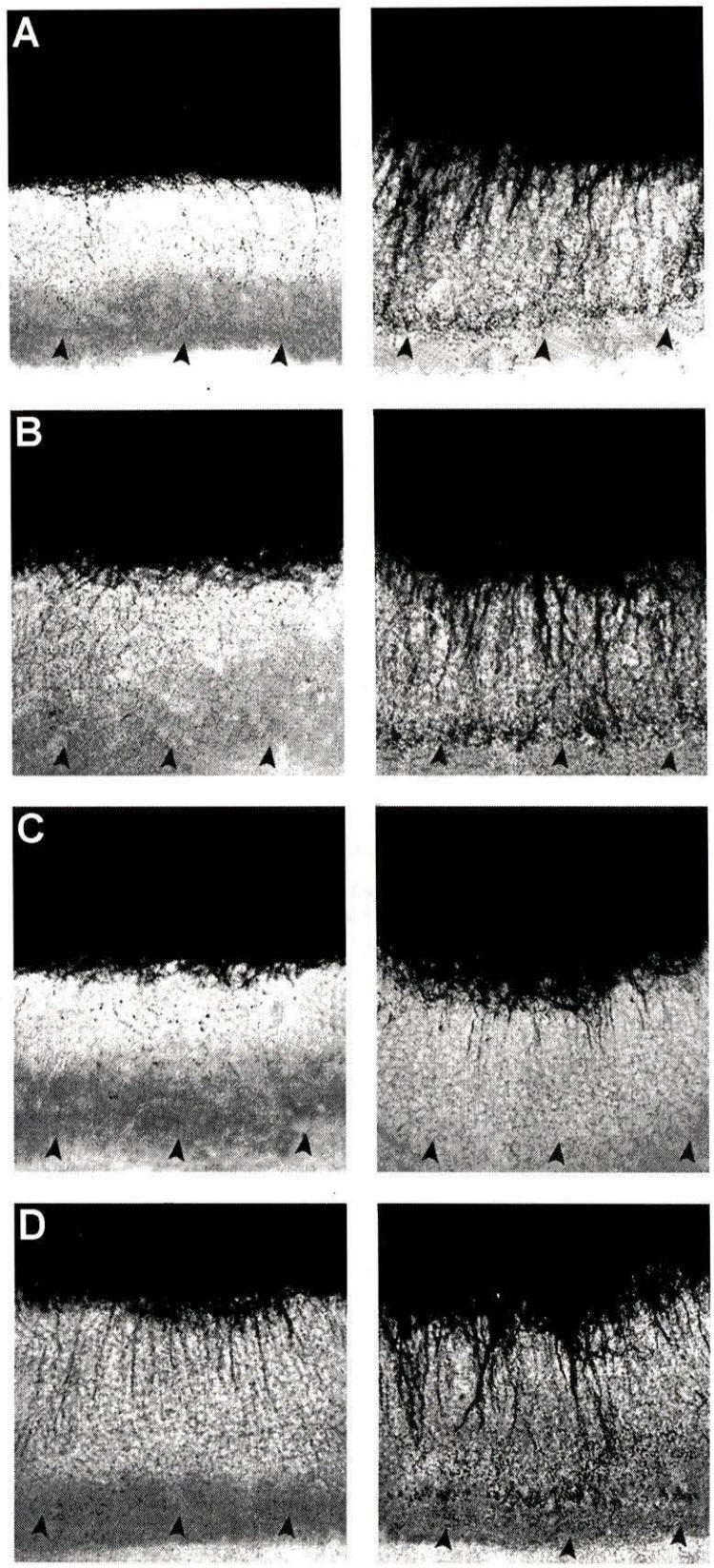
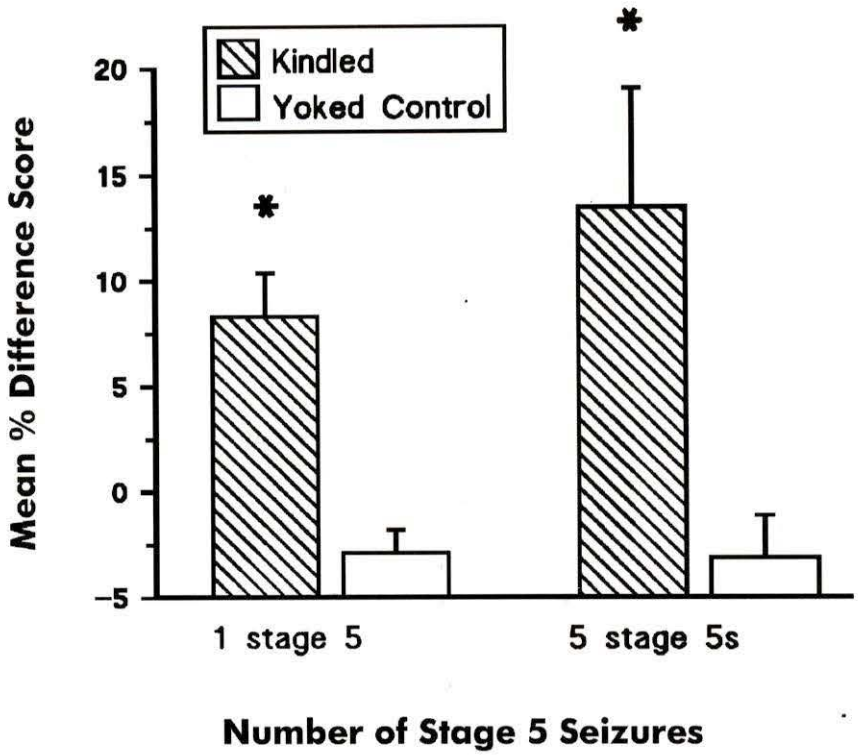

A
B
C
D

Figure Caption

Figure 7. Mossy fiber sprouting subsequent to high-frequency kindling. Mean (± S.E.M.) percentage difference in optical density between the inner and the outer portions of the IML of rats killed 1 day after kindling of 1 (HF1; $n = 6$) or 5 (HF5; $n = 7$) stage 5 seizures with conventional high-frequency stimulation. Note that a negative percent difference score was obtained in some control rats. This occurs when a higher optical density is obtained in the outer portion of the IML (background) than in the inner portion of the IML where ectopic Timm granules typically appear.

* $p < .05$.


Kindled
Yoked Control
Mean % Difference Score
20
15
10
5
0
−5
*
*
1 stage 5
5 stage 5s
Number of Stage 5 Seizures

Mossy Fiber Sprouting Subsequent to Low-Frequency Kindling

No significant difference in optical density was detected between the LF1 ($n = 6$) group and their yoked controls (t (10) = .62, $p > .05$, see Figures 6 and 8), with both the kindled rats and their yoked controls displaying minimal and sparsely distributed Timm granules in the inner molecular layer. In contrast, the low-frequency kindled rats killed 20 days after completion of kindling (LF1-20; $n = 9$) displayed a moderate intensity of sprouting, as indicated by significantly higher percent differences in optical density than in their yoked controls (t (16) = 2.75, $p < .05$; Figure 8). The intensity of sprouting in this group was similar to that seen in tissue from rats sampled 1 day after conventional high-frequency kindling.

Figure Caption

Figure 8. Mossy fiber sprouting subsequent to low-frequency kindling. Mean (± S.E.M.) percentage difference in optical density between the inner and outer portions of the IML of rats perfused 1 (LF1; $n = 6$) or 20 days (LF1-20; $n = 9$) after kindling of one stage 5 seizure with low-frequency stimulation. Note that a negative percentage difference score was obtained in some control rats, which resulted when a higher optical density was obtained in the outer IML (background) than in the inner IML where ectopic Timm granules typically appear. * $p < .05$.

CHAPTER V

DISCUSSION

Mossy Fiber Sprouting Associated with High-Frequency (Conventional) Kindling

The present experiment was designed to determine whether mossy fiber sprouting could be dissociated from kindling. The main objective was to determine whether mossy fiber sprouting could be detected in the inner molecular layer of the dentate gyrus with the Timm stain when seizures are rapidly kindled by application of low-frequency stimulation to the amygdala. For comparison, the density of sprouting that occurs following conventionally kindled seizures by application of high-frequency stimulation to the amygdala was assessed. In agreement with previous investigators (Cavazos et al., 1991), we found that conventional kindling of 1 or 5 generalized stage 5 seizures resulted in significantly more intense sprouting in rats killed 1 day after the last seizure, as compared to controls. Unlike previous investigators (Cavazos et al., 1991), however, we did not detect a significant difference in sprouting between the groups kindled to 1 (HF1) or to 5 (HF5) generalized seizures.

Failure to corroborate previous findings could be due to differences in the techniques used to quantify the intensity of sprouting. In the initial stages of the present experiment all sections were assessed according to the rating scale described by Sutula et al. (1988). However, it quickly became obvious that there was a high degree of subjectivity when using this method. Therefore, to quantify the sprouting more objectively we developed a semi-quantitative computerized scanning technique.

This scanning technique corresponds very closely with the subjective rating scales (personal observations), yet appears to be more sensitive to subtle differences occurring across sections. Regardless of differences in techniques, the possibility that the intensity of sprouting may increase significantly following more than 5 kindled stage 5 seizures can not be ruled out because this was not assessed in this study.

Mossy Fiber Sprouting Associated with Low-Frequency (Rapid) Kindling

Although sprouting was observed in tissue taken from conventionally kindled rats, there was no detection of differences in sprouting, relative to yoked controls, in rats killed 1 day after rapid kindling with low-frequency stimulation of the amygdala. Thus, mossy fiber sprouting does not invariably correlate with kindling of generalized seizures. However, detection of significant amounts of sprouting in rats killed 20 days after completion of low-frequency kindling indicates that mossy fiber sprouting can occur with rapid kindling if there is a sufficiently long elapsed time after kindling. These findings are similar to those of Elmér, Kokaia, Kokaia, Ferencz, & Lindvall (1996), who did not detect significant sprouting until 4 weeks after the induction of rapidly recurring seizures produced within 3 h and 20 min. of stimulation. This suggests that detectable levels of mossy fiber sprouting are not necessary for the induction of kindling but may be a consequence of kindling stimulation or the seizure activity that it triggers. This interpretation does not preclude the possibility that mossy fiber sprouting plays a further role in the maintenance or permanence of kindling.

No sprouting was evident after rapid kindling of one generalized seizure with low-frequency stimulation, which required a mean of 5 days to complete. However, in less than 6 days, detectable levels of sprouting are found following more severe epileptogenic treatments. For example, dense sprouting in the inner molecular layer has been described with the Timm stain within 2 to 4 days of treatment with kainic acid (Cantallops & Routtenberg, 1996; Cronin et al., 1992) or pilocarpine (Mello et al., 1993) suggesting that the mossy fibers are capable of very rapid sprouting that can be detected when the stimulus is sufficiently intense. This finding in turn suggests that the rapid appearance of sprouting may be related to the intensity of the epileptogenic stimuli or to the severity of epileptogenesis. Consistent with this interpretation, Ebert and Löscher (1995) have shown that mossy fiber sprouting can be detected following massed amygdaloid kindling, applied at 30 min interstimulus intervals for 2 days, whereas in their study, mossy fiber sprouting was not evident following conventional kindling that required a mean of 6 days for development of the first generalized seizure. In the massed kindling preparation, the rapid appearance of sprouting is perhaps associated with the intensity of the epileptogenic stimuli, the total number of stimulations applied (see also Wanscher, Kragh, Barry, Bolwig, & Zimmer, 1990), or the duration of secondary afterdischarge (Ebert & Löscher, 1995).

Mossy Fiber Sprouting in the Absence of Fully Generalized Seizures

There is evidence that sprouting occurs in both guinea-pig and rat brains in the absence of fully generalized seizures. In guinea-pigs moderate levels of sprouting can be detected subsequent to single site amygdaloid kindling (Armitage, Mohapel, Thiessen, Gilbert, Teskey, & Corcoran, 1997). Guinea pigs are similar to adult rats in that over the course of kindling they will display a progressive growth in the duration and complexity of afterdischarge as well as an intensification of seizures. However, unlike rats, guinea-pigs manifest uncharacteristic behavioral seizures during kindling and fail to express fully generalized seizures (Teskey, Valentine, Sainsbury, & Trepel, 1995) unless alternate sites are kindled (Teskey, Thiessen, & Gilbert, 1997). Since the most severe seizure evoked was a nongeneralized stage 3 seizure it has become apparent that, in guinea-pigs, reaching a fully kindled state is not necessary for the expression of sprouting. Furthermore, in guinea-pigs, a fully kindled state is not necessary for detection of sprouting that is equal if not denser and more distributed throughout the dorsal-ventral axis than any sprouting reported in the rat brains presented in this study. In rats, a slight but nonsignificant percentage difference in sprouting levels can be detected 20 days after evoking 5 afterdischarges from the amygdala with conventional high-frequency stimulation relative to yoked controls (Armitage, Mohapel, & Corcoran, 1997). However, a patchy and unevenly distributed pattern of sprouting can be detected 18 hr after 15 days of twice daily low-frequency amygdaloid stimulation at intensities that are so low that they do not evoke afterdischarge or behavioral convulsions (Sutula et al., 1988). These findings imply

that mossy fiber sprouting is not associated with kindling per se but rather with electrical stimulation or nonpathological synaptic activation.

In the present study, significant differences in mossy fiber sprouting were not detected in or between unstimulated yoked controls. Although the control rats did not receive any stimulation throughout the experiment, this confirmed that mechanical stimulation of the amygdala due to handling and habitual activity of the rats was not sufficient to induce aberrant sprouting. These findings do not, however, discourage further investigation into the role of mossy fiber sprouting associated with activity-dependent synaptic plasticity or nonepileptiform stimulation (see Mody & Soltesz, 1993). For instance, long-term potentiation is an activity-dependent, nonepileptogenic form of synaptic plasticity that is induced by high-frequency tetanic stimulation (Bliss & Lømo, 1973). It has been speculated that long-term potentiation shares common mechanisms underlying kindling (McNamara, 1986) because both long-term potentiation and kindling involve the application of high-frequency stimulation over a brief period of time (Cain, Boon, & Hargreaves, 1992); both induce potentiation of excitatory hippocampal circuitry (Racine et al., 1983); both are suppressed by NMDA receptor antagonists (Gilbert & Mack, 1990); and both produce enduring electrophysiological alterations (Cain et al., 1992). However, kindling and long-term potentiation have been distinguished as two different forms of synaptic plasticity because there are several prerequisites for induction and maintenance that the two processes do not share. Interestingly, small but significant differences in the density of sprouting have been detected subsequent to induction of long-term potentiation in the

perforant path-dentate gyrus synapse (Armitage, Swayze, Jones, & Corcoran, 1997) and these findings are corroborated with observations of sprouting in area CA3 subsequent to long-term potentiation in the same perforant path-dentate gyrus synapse (Racine, 1995; Racine, Adams, Osehobo, Milgram, & Fahnestock, in press). Despite the fact that the degree of sprouting observed in the long-term potentiation studies is slight relative to the degree of sprouting observed in kindling studies, the fact that differences in the density of mossy fiber sprouting can be detected suggests that sprouting may be associated with activation rather than epileptiform events.

Correlations Between Mossy Fiber Sprouting and Kindling

Associations.

The role of mossy fiber sprouting in kindling thus remains uncertain. Although some studies have reported strong correlations between kindling and mossy fiber sprouting (Cavazos et al., 1991; McNamara, 1992; Rashid, Van der Zee, Ross, Chapman, Stanisz, Riopelle, Racine, & Fahnestock, 1995; Represa et al., 1993; Sutula, Koch, Golarai, Watanabe, & McNamara, 1996; Van Der Zee, Rashid, Khoa, Moore, Stanisz, Diamond, Racine, & Fahnestock, 1995; Watanabe, Johnson, Butler, Binder, Spiegelman, Papaioannou, & McNamara, 1996), other evidence suggests that mossy fiber sprouting is neither necessary nor sufficient for kindling (Clusmann et al., 1992, 1994; Elmér et al., 1996; Elmér, Kokaia, Kokaia, Lindvall & McIntyre, 1997; Haas, Sperber, Benenati, Stanton, & Moshe, in press; Larmet, Reibel, Carnahan, Nawa, Marescaux, & Depaulis, 1995; Racine et al., in press; Sperber, 1984).

Dissociations.

Very rapid amygdaloid kindling seen in rat pups postnatal day 16 occurs in the absence of detectable mossy fiber sprouting, suggesting that mossy fiber sprouting is not a salient prerequisite for epileptogenesis, at least in the immature nervous system (Haas et al., in press; Sperber, 1984). Similarly, baseline levels of Timm staining are higher in a slow-kindling strain of rat than a fast-kindling strain, and neither shows increases in Timm staining after amygdaloid kindling (Elmér et al., 1997). Furthermore, the induction of mossy fiber sproutin in adult rats is unaffected by central infusions of brain-derived neurotrophic factor (BDNF) that retards kindling of generalized seizures (Larmet et al., 1995; Racine et al., in press), suggesting that mossy fiber sprouting is not sufficient for the development of generalized seizures. Similarly, prior destruction of granule cells retarded but did not prevent amygdaloid kindling in Sprague-Dawley rats and did not change the rate of amygdaloid kindling in pprimary or secondary kindling sites within Wistar rats suggesting that the granule cess and their axons, are not necessary for the genesis or maintenance of kindling. Finally, it has been recently demonstrated that prior induction (via transection of the fimbria/fornix) of mossy fiber sprouting (Hannesson, Armitage et al., 1997) does not accelerate kindling (Corcoran, Armitage, Hannesson, Jenkins, & Mohapel, in press; Mohapel, Armitage, Hannesson, & Corcoran, in press), contrary to what would be expected if mossy fiber sprouting were necessary for kindling.

Extending the Research

Notwithstanding the above, these results do not conclusively argue against mossy fiber sprouting as a mechanism of kindling. In recognition that the generality of these results is limited by the restriction of the histological examination to the inner molecular layer of the dentate gyrus, some suggestions for future experiments can be made. For example, mossy fiber sprouting has been described in area CA3 (Ben-Ari & Represa, 1990; Represa et al., 1989; Represa & Ben-Ari, 1992) of the hippocampus following kainic acid (Sundstrom et al., 1993) or conventional kindling treatment. It is not known yet whether mossy fiber sprouting can be detected in this area following rapid kindling. In addition, it remains to be seen whether mossy fiber sprouting within the hippocampus occurs in association with rapid kindling of other structures, like the perirhinal cortex, that can be kindled with high-frequency stimulation within about 5 days (Mohapel et al., 1996; Mohapel & Corcoran, 1995). In this type of experiment it would be possible to double dissociate sprouting with rapid kindling by holding the type of stimulation and the time to reach a generalized seizure constant while altering the site of kindling. Preliminary results suggest that sprouting can be detected 1 and 20 days after the induction of one stage 5 seizure evoked from the perirhinal cortex (Plunet, 1997).

It also remains to be determined whether the density of the Timm stain differs in sites outside the hippocampus after kindling. Areas such as the entorhinal, perirhinal and piriform cortices stain heavily for zinc with the Timm stain and may be worthy of Timm granule density assessment subsequent to kindling. Mossy fiber

terminals are also highly immunoreactive for opioid peptides (particularly dynorphin). In light of this other areas within the neuroaxis that are immunoreactive for opioid peptides (see McGinty, 1985) maybe worth investigation as well. For instance, the caudate-putamen is a prominent structure within the basal ganglia that is capable of synaptic alteration and neuronal degeneration subsequent to neural insult or disease such as Parkinsons'. Similar to the dentate gyrus this structure expresses high levels of the opioid precursor, prodynorphin. In a preliminary assessment of zinc staining in the caudate-putamen we found that there is a reduction in the total Timm stained area after kindling in guinea-pigs. The preliminary findings provide reason to suspect that alterations in zinc content occur in areas outside of the hippocampus following kindling, however, the significance of this decrease in staining will remain undetermined until further data are collected and the degree of seizure-induced cell injury is assessed (see Fujikawa, 1996).

Another way to assess kindling associated changes in morphology would be to use a less specific marker than the Timm stain to label mossy fiber sprouting or sprouting of fiber tracts that would otherwise not be labelled by the Timm stain. For instance, preliminary findings that an immunohistochemical technique that labels the vesicle-associated protein synaptophysin (Honer, Beach, Hu, Berry, Dorovini-Zis, Moor, & Woodhurst, 1994), can be used to determine whether kindling is associated with changes in the density of axon terminals in various sites in the brain (Calakos & Scheller, 1996; Chen, Wong, Banerjee, & Snead III, 1996; Hannesson, Wallace, & Corcoran, 1997). An interesting application of this technique would be to assess the

sprouting that occurs in tissue taken from rats that have had one generalized seizure subsequent to rapid low-frequency kindling. In the present study, there was no evidence of Timm granules one day after a generalized seizure which does not preclude the possibility that another form of detectable sprouting had occurred. In addition, neuronal tracing with high resolution fluorescent dyes (Honig & Hume, 1989) such as fluorescent carbocyanine DiI and reconstruction of cytoarchitecture with biocytin retrograde labels (Lachica, Mavity-Hudson, & Casagrande, 1991; Okazaki et al., 1995) could be used in conjunction with Timm and synaptophysin histochemistry to further clarify the time course, route and targets of collateral sprouting in association with kindling. Thus, until the appropriate experiments are performed, the significance of mossy fiber sprouting in association with epileptogenesis and the therapeutic implications shall remain to be determined.

Summary

In summary, there is a dissociation between the time courses of sprouting and rapid low-frequency kindling. Mossy fiber sprouting followed rather than coincided with the establishment of the kindled state. These results suggest that mossy fiber sprouting is not a cause but rather is a consequence of kindling, although this does not rule out a possible role of mossy fiber sprouting in either the maintenance or the permanence of kindling. Furthermore, these data suggest that mossy fiber sprouting is correlated with conventional kindling because the time courses of sprouting and kindling coincide.

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Appendix A

Classification of the Epilepsies
(Fischer, 1989)

Partial (Local Onset)

Simple partial (no loss of consciousness)
- Motor
- Sensory or somatosensory
- Autonomic
- Psychic

Complex partial (loss of consciousness)
- with or without aura
- with or without automatism

Secondarily generalized

Primary Generalised (No Local Onset)
- loss of consciousness
- tonic-clonic
- tonic
- clonic
- absence (typical or atypical)
- myoclonic
- atonic

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Title of Thesis: Dissociation between mossy fiber sprouting and rapid kindling with low-frequency stimulation of the amygdala

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