DISSERTATION

EPILEPSY AND SYNAPTIC REORGANIZTION IN MODELS OF STATUS EPILEPTICUS AND HYPOXIA-ISCHEMIA

Submitted by

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In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2002

UMI Number: 3053459

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COLORADO STATE UNIVERSITY

March 19, 2002

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY PHILIP A. WILLIAMS ENTITLED "EPILEPSY AND SYNAPTIC REORGANIZATION IN MODELS OF STATUS EPILEPTICUS AND HYPOXIA-ISCHEMIA" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

Epilepsy and synaptic reorganization in models of status epilepticus and hypoxia-ischemia

Synaptic reorganization is a process where new circuits are formed and neuronal populations are abnormally interconnected. These altered circuits are commonly associated with hippocampal sclerosis in humans with temporal lobe epilepsy. This thesis examines the relationship between synaptic reorganization and epilepsy in four different models of brain injury. Chapter 2 attempted to eliminate mossy fiber sprouting (i.e., synaptic reorganization in the dentate gyrus) following pilocarpine induced status epilepticus, by pre-treating rats with a protein synthesis inhibitor, in order to examine the effect that lack of mossy fiber sprouting would have on epileptogenesis following status epilepticus. Chapter 3 examined the hippocampal electrophysiology along the septotemporal axis in kainate-treated epileptic rats, and found a strong association between septotemporal differences in synaptic reorganization and abnormal electrophysiology. Chapters 4 and 5 utilized a hypoxic-ischemic model in young adult and perinatal rats to induce a unilateral lesion that involved the hippocampus for the purpose of examining the potential for inducing mossy fiber sprouting after a hypoxic-ischemic injury. Synaptic reorganization in the dentate gyrus is a model for analyzing the effects that the new abnormal circuits have on

a neuronal population, and the data in this thesis demonstrates that mossy fiber sprouting is a common occurrence following brain injury. This process can occur in other regions (i.e., CA1) and probably commonly occurs at other sites of injury (i.e., focal neocortical infarct). These new circuits may have a restorative function under normal conditions, but may act as a substrate for abnormal behavior in states that range outside the usual. Thus it is the goal of this research to understand how and where synaptic reorganization occurs in models of brain injury, and the physiological conditions that induces these new circuits to express abnormal function.

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CHAPTER 1

Temporal lobe epilepsy

Recurrent seizures occur in approximately 0.65% of the population with the highest incidence of the first seizure occurring in young children and the elderly (Hauser et al., 1993). Temporal lobe epilepsy is the most common epileptic syndrome in adults, but it usually starts during childhood. Both simple seizures (i.e., focal motor and/or somatosensory seizures with out loss consciousness) and complex partial seizures (i.e., may begin as simple seizures and progress into altered consciousness, and/or become generalized seizures) are usually seen in and typically arise from the hippocampal or parahippocampal structures. Approximately 50% of the patients afflicted with temporal lobe epilepsy are resistant to current pharmacotherapies. In those patients in which the lesion can be localized to an amenable region (i.e. a focal area not involving critical regions), resection can abolish seizures in 60-70% of those people. The most common lesion associated with temporal lobe epilepsy is hippocampal sclerosis (Engel, 1989). This is commonly described as neuron loss involving

CA1, CA3, and the hilus, while CA2 and the dentate gyrus appear to be preserved (Margerison and Corsellis, 1966). The hilus is composed of at least 20 morphologically distinct neurons, which are variably susceptible to injury (Amaral, 1978). Mossy cells (excitatory), somatostatin-containing neurons, and neuropeptide-Y-containing neurons (both inhibitory) seem to be the most susceptible neuron populations (Sloviter, 1987; de Lanerolle et al., 1989; Buckmaster and Dudek, 1997), while the parvalbumen-containing basket cells and the calbindin-containing neurons (both inhibitory) seem to be resistant to injury (Sloviter et al., 1991; Buckmaster and Dudek, 1997). Associated with the hippocampal sclerosis are alterations in the pattern of mossy fiber connections. Mossy fibers are the axons of the dentate gyrus granule cells, which contain zinc and can be specifically stained using the Timm silver staining method. Mossy fibers normally synapse on CA3 pyramidal neurons, but in humans with temporal lobe epilepsy, abnormal Timm staining has been found in the inner molecular layer of the dentate gyrus (de Lanerolle et al., 1989; Sutula et al., 1989; Houser, 1990; Babb et al., 1991). It has been hypothesized that this abnormal staining represents new excitatory synaptic connections between granule cells, which are not normally present in this population of neurons (e.g., Tauck and Nadler, 1985; Wuarin and Dudek, 1996; Molnar and Nadler, 1999; Lynch and Sutula, 2000). It is also hypothesized that these new recurrent excitatory synapses form in other areas (e.g., the CA1 pyramidal cells; Esclapez et al., 1999; Smith and Dudek, 2001). Alterations in glutamate and GABA receptors have also been identified (Kraus et al., 1994; Shumate et al., 1998). The glutamate receptor alterations are unclear in that the current results appear to be contradictory and remain to be resolved. The $GABA_A$ receptors appear to have an altered subunit composition that increases the affinity for zinc, which tends to attenuate the inward chloride current. Zinc is contained in high concentrations in the mossy fiber synaptic terminals, and it has been hypothesized that the new synaptic connections in the inner molecular layer may act to depress inhibition under conditions of high activity (seizure) coming from the entorhinal cortex (Buhl et al., 1996; Shumate et al., 1998). This hypothesis has come under critical review due to questions about the bioavailability of zinc under physiologic conditions (Molnar and Nadler, 2001).

In temporal lobe epilepsy the focus of seizure generation is typically not in the hippocampus, but is usually located in the archicortex (i.e., entorhinal cortex), however, as seizures spread from the focus they invariably invade the limbic structures, with the hippocampus being most commonly involved. As the seizures progress through the hippocampus to other structures the seizures appear to become more severe in their character. Under normal conditions the dentate gyrus functions as a gate that blocks large high-frequency input from spreading through the rest of the hippocampus proper (Lothman and Collins, 1981). The granule cells that compose the dentate gyrus have a resting membrane potential relatively more hyperpolarized (-75 to –85 mV) than the pyramidal neurons (-60 to -70 mV). The granule cells are also small neurons and normally have a high input resistance, and the granule cell layer has an extensive inhibitory feedback loop through the basket cells and other polymorphic hilar interneurons. In temporal lobe epilepsy the gating function of the dentate gyrus is compromised by the formation of a positive feedback loop (i.e., mossy fiber sprouting). Several labs have shown that the presence of mossy fiber sprouting can cause the dentate gyrus to display epileptiform bursts under conditions of reduced inhibition and/or increased extracellular potassium. Glutamate transmission mediates these bursts and the mossy fiber sprouting represents new recurrent excitatory connections between granule cells (Cronin et al., 1992; Wuarin and Dudek, 1996; Patrylo and Dudek, 1998; Molnar and Nadler, 1999; Lynch and Sutula, 2000). The formation of new recurrent excitatory synapses is not unique to the dentate gyrus, because synaptic reorganization has also been found in CA1 (Esclapez et al., 1999; Smith and Dudek, 2001). Thus we hypothesize that synaptic reorganization in the from of new recurrent excitatory circuits may commonly occur at sites of brain injury, and this may be an important mechanism that contributes to seizure generation and propagation. These findings have lead to the primary hypothesis underlying chapter 2. This chapter describes an attempt to block mossy fiber sprouting with the protein synthesis inhibitor cycloheximide, and examines the effects of this compound on epileptogenesis, hippocampal sclerosis, and hippocampal electrophysiology.

The development of new recurrent excitatory synapses may also be involved in secondary epileptogenesis. This is the process by which a primary seizure focus appears to generate a secondary seizure focus in the contralateral homotopic cortex (see Morrel et al. 1993 for review). The secondary site can mature and become an independent epileptic focus. Neuron loss at the primary site of injury may cause deafferentation of the contralateral homotopic site and lead to synaptic reorganization, which could potentially contribute to the permanent nature of the secondary site (Dudek and Spitz, 1998). Secondary epileptogenesis appears to primarily involve the neocortical structures and does not appear to be a major feature in temporal lobe epilepsy (Engel, 1989). However, the hippocampus anatomically has extensive commissural input, and although it does not appear to have a strict homotopic arraignment, neuronal loss in one hippocampus would severely deafferentate the other hippocampus. The mossy cells in the hilus are excitatory interneurons that receive input from the granule cells and then synapse back onto the granule cell layer at the region of the inner molecular layer. However, the main input from the mossy cells is not back on to the granule cells within the same lamina of the ipsilateral hippocampus; rather, they appear to provide excitatory drive to the contralateral dentate gyrus (Blackstad, 1970; Frotscher et al., 1981; Frotscher, 1983; Seress and Ribak, 1984). The mossy cells are very sensitive to injury, and are extensively lost in humans and animal models of temporal lobe epilepsy and stroke (Sloviter et al., 1991; Hsu and Buzsaki, 1993). These findings have lead to the hypothesis that is addressed in chapters 4 and 5 in which we study the effects of a unilateral hypoxic-ischemic lesion in 30-day-and 7-day-old rats and examine for anatomic and electrophysiologic alterations in both the ipsilateral and contralateral hippocampi.

The hippocampus is an elongated structure that has distinct functions along the longitudinal axis. In the rat, the septal or dorsal axis is equivalent to the posterior hippocampus in humans, and the temporal or ventral hippocampus of the rat is equivalent to the anterior hippocampus in humans. Functionally, the hippocampus is critically involved in memory consolidation, and it appears that the septal hippocampus is necessary for spatial memory while the temporal hippocampus is not (Moser et al., 1993). Thus, the septal and temporal hippocampus appear to be functionally different. In temporal lobe epilepsy, lesions are more commonly found in the anterior hippocampus, and in rat models of temporal lobe epilepsy anatomic alterations are more prominent in the ventral hippocampus (i.e., neuron loss and mossy fiber sprouting; Magerison and Corsellis, 1966; Buckmaster and Dudek, 1997). The CA3 region of the hippocampus responds differentially along the septal-temporal axis to increased extracellular potassium and depressed inhibition, with the temporal hippocampus being more prone to producing spontaneous bursting (Bragdon et al., 1986). The temporal hippocampus is also more amendable to kindling (a process that utilizes daily evoked seizures to induce an epileptic state in rats)(Racine et al., 1977). Most of the hippocampal slice experiments performed in the Dudek laboratory and many others have utilized the temporal hippocampus. However, in the hypoxic-ischemic rats the septal hippocampus is more severely injured than the ventral hippocampus (i.e., the temporal hippocampus appeared uninjured). Thus the hippocampal recordings from the hypoxic-ischemic rats derive from the septal hippocampus. Therefore we conducted a series of experiments on kainate-induced epileptic rats aimed at comparing the electrophysiology of the septal hippocampus to the temporal hippocampus from the same epileptic animals (see chapter 3).

Stroke

A stroke is defined by the World Health Organization as "rapidly developing clinical signs of focal (at times global) disturbance of cerebral function lasting more than 24 hours or leading to death with no apparent cause other than that of vascular origin". A stroke is caused by either an infarction or hemorrhage. The most common cause is infarction (77% of all strokes) with multiple possible etiologies for the infarct (cardioemboloism, lacunar, intra- and extra-cranial atherosclerosis, and cryptogenic). There are two main types of hemorrhages that cause stroke (intracranial and subarachnoid), with the intracranial hemorrhage being the most common form of the two (for a review see Sacco, 2000). The overall incidence of stroke is 0.2%, and stroke is responsible for 10% of all deaths per year. Most strokes occur in people over 65 years of age, and the most common cause of epilepsy in the elderly is a stroke (Lesser et al., 1985; Ettinger, 1994). The reported incidence rate for epilepsy following a stroke ranges from 2-17% (Pohlman-Eden, 1997; Olsen, 2001).

Animal models of stroke include those that induce global ischemia (four vessel occlusion; Pulsinelli and Brierly, 1979) and focal cortical infarcts (photothrombosis, Watson et al., 1987). The most common model in use is the middle cerebral artery occlusion model (Bederson et al., 1986) that uses a monofilament suture passed up the middle cerebral artery to temporarily occlude the vessel. In the studies reported here, a hypoxic-ischemic model in both 30-day- and 7-day-old rats was used (Rice et al., 1981). This model induces a unilateral lesion that involves the frontoparietal cortex and septal hippocampus, but appears to spare the temporal lobe and temporal hippocampus (Towfighi et al., 1997, 1998). All the above mentioned models are well characterized, but to date spontaneous motor seizures have not been identified. Spontaneous electroencephalographic abnormalities have been seen in both 12-day-old hypoxic-ischemic rats (Romijn et al., 1994) and in 30-month-old rats with photothrombotic lesions in the neocortex (Kelly et al., 2001). It is possible in the 12-day hypoxic-ischemic rats that the animals were not observed long enough after the injury for spontaneous motor seizures to develop.

The common lesion found after a stroke is focal neuronal loss with subsequent gliosis. The middle cerebral artery occlusion model and focal photothrombosis are models for these types of lesions. Cardiac arrest encephalopathy or global ischemia are best modeled by the four-vessel occlusion model. The hypoxic-ischemic model in 30-day old rats (chapter 4) is similar to the four-vessel occlusion model in that blood flow is reduced at a major extracranial artery (carotid artery). However, the hypoxic-ischemic model causes a unilateral lesion ipsilateral to the ligated carotid artery. The neuron loss seen in the four-vessel occlusion model has been characterized, and the typical hippocampal lesion consists of severe neuronal loss of CA1, loss of hilar interneurons, and possibly a small proportion of CA3 neurons (Pulsinelli et al., 1982). Hilar neuronal loss consists of both mossy cells (Hsu and Buzsaki, 1993) and somatostatin containing neurons (Johansen et al., 1987), as well as other types that are not as well characterized. The hypoxic-ischemic lesion in 30-day old rats has been characterized, but not carefully quantified (Towfighi et al., 1997, 1998; see chapter 3), and the pattern of neuronal loss looks similar to what is seen in the four-vessel occlusion model. These findings lead us to hypothesize that the hilar neuronal loss seen in the 30-day hypoxic-ischemic model would lead to mossy fiber sprouting.

Onodera et al. (1990) reported that Timm stain was present in the inner molecular layer of rats that had been subjected to 15 min of global ischemia (four-vessel occlusion). This finding may represent the formation of new recurrent excitatory circuits, but there is currently no data to support this view. We hypothesized that if we also found Timm stain in the inner molecular layer in the 30-day old hypoxic-ischemic model, the hippocampus would show electrophysiologic changes associated with mossy fiber sprouting.

Mody et al. (1994) showed that hippocampal slices taken from rats 3 months after 15 min of global ischemia (four-vessel occlusion) lack hyperexcitability, and the granule cells appear to have normal inhibitory input inspite of the hilar neuronal loss associated with the lesion. However, this study did not attempt to "unmask" new recurrent excitatory circuits with bicuculline and/or increased extracellular potassium. Thus we hypothesized that if mossy fiber sprouting is present, inhibition would have to be reduced and extracellular potassium increased in order to evoke a response consistent with new recurrent excitatory synapses.

Cerebral palsy

Cerebral palsy is defined as a non-progressive motor deficit sustained in the perinatal period. It is believed that perinatal hypoxia-ischemia due to asphyxia or respiratory dysfunction is the most common cause of cerebral palsy. However, several studies indicate that intrapartum factors (intrapartum asphyxia) may be more important contributors to cerebral palsy (Nelson and Ellenberg, 1986; Torfs et al., 1990). At least one-third of all neonatal malformations can be attributed to intrapartum asphyxia. Children with malformations also have a higher incidence of perinatal hypoxia-ischemia.

The incidence of epilepsy associated with cerebral palsy ranges from 15% to 60%, depending on the study (Hadjipanayis et al., 1997; Kwong et al., 1998; Mieszczanek, 2000). The types of seizures are also varied; simple, complex partial, myoclonic, tonic-clonic, and/or generalized (Aneja et al., 2001). Greater than one-third of the people with cerebral palsy associated epilepsy are resistant to pharmacotherapy (Gaggero et al., 2001).

Perinatal hypoxic-ischemic lesions usually injure multiple sites involving the cortex, hippocampus, thalamus, basal ganglia, and cerebellum. In the hippocampus, there is no characteristic lesion since the different hippocampal regions mature at different times. The CA3 and CA2 regions are mature at birth while CA1 will continue to develop until 2 years of age (Friede et al., 1989). Thus any lesion in the perinatal period may be developmental in nature, with a potential for disrupting normal synaptic connections and path finding mechanisms, which could lead to the formation of abnormal connections (i.e., mossy fiber sprouting, mossy fiber to CA1 synapses).

In the literature, cerebral palsy is considered a risk factor for temporal lobe epilepsy (Rocca et al., 1987). However, it is not clear to what degree patients with cerebral palsy and temporal lobe epilepsy have been studied as a subgroup. Thus data on the pathologic changes associated with the combined condition are not available. Therefore, the amount of hippocampal sclerosis and mossy fiber sprouting present in these patients is currently unknown. Based upon the stated 50% increased risk for temporal lobe epilepsy with cerebral palsy, we hypothesized that perinatal rats that received a hypoxic-ischemic injury would develop epilepsy, and that the hippocampus would also have mossy fiber sprouting.

Summary

The common thread in all these experiments is synaptic reorganization, and how it may relate to spontaneous motor seizures and hippocampal electrophysiology. In chapter 2, we attempt to eliminate mossy fiber sprouting and then examine the consequences on epileptogenesis and hippocampal electrophysiology. We found that cycloheximide does not prevent mossy fiber sprouting, in contrast to a previous report. Chapter 3 examines the electrophysiologic changes along the septotemporal axis in epileptic rats, and we found a strong association between the amount of Timm stain in the inner molecular layer and abnormal electrophysiologic responses. In chapters 4 and 5 we determined whether mossy fiber sprouting occurs in models (hypoxia-ischemia) that have not been previously examined, and we assessed the hippocampi for the physiological expression of new recurrent excitatory circuits. We found a strong relationship between the presence of mossy fiber sprouting and spontaneous motor seizures. Our data also supports the hypothesis that the mossy fiber sprouting represents new recurrent excitatory synapses.

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Chapter 2

A REASSESSMENT OF THE EFFECTS OF CYCLOHEXIMIDE ON MOSSY FIBER SPROUTING AND EPILEPTOGENESIS IN THE PILOCARPINE MODEL OF TEMPORAL LOBE EPILEPSY

ABSTRACT

A feature of animal models of temporal lobe epilepsy and the human disorder is hippocampal sclerosis and Timm stain in the inner molecular layer (IML) of the dentate gyrus, which represents synaptic reorganization and may be important in epileptogenesis. We reassessed the hypothesis that pre-treatment with cycloheximide (CHX) prevents Timm staining in the IML following pilocarpine (PILO)-induced status epilepticus (a multifocal model of temporal lobe epilepsy) but allows epileptogenesis (i.e., chronic spontaneous seizures) after a latent period. Hippocampal slices from PILO-treated rats without Timm stain in the IML after CHX treatment were hypothesized to lack the electrophysiological abnormalities suggestive of recurrent excitation. The primary experimental groups were; CHX (1mg/kg) 30-45 min prior to administration of PILO (320 mg/kg, intraperitoneal (IP)), another group received only PILO, and a control group received only saline (0.5 ml, IP). The CHX pre-treatment significantly decreased the number of rats that responded to PILO with status epilepticus compared to rats that received only PILO. Pre-treatment with CHX did not significantly alter the spontaneous motor seizure rate post-treatment compared to treatment with PILO alone in those animals from each group that developed status epilepticus during PILO treatment. Timm stain in the IML was not significantly different between the PILO- and PILO+CHX-treated rats. Using quantitative methods, CHX did not prevent hilar, CA1, or CA3 neuronal loss compared to the PILO-treated rats. Extracellular responses to hilar stimulation in 30 μM bicuculline and 6 mM $[K^+]_o$ demonstrated all-or-none bursting in both the

CHX+PILO- and PILO-treated rats. Whole-cell recordings from granule cells, using glutamate flash photolysis to activate other granule cells, showed that both the CHX+PILO- and PILO-treated rats had excitatory synaptic interactions in the granule cell layer, which were not found after saline treatment. Some rats responded to PILO (with or without CHX-pre-treatment) with only one or a few seizures at treatment, and some of these animals (n=4) demonstrated spontaneous motor seizures within 2 months after treatment. Timm staining and neuron loss in this group were not significantly different from saline-treated rats. These results suggest that in the PILO model, pre-treatment with CHX does not affect mossy fiber sprouting in the IML of epileptic rats, and does not prevent the formation of recurrent excitatory circuits. However, the development of spontaneous motor seizures, in a small number of rats, could occur without detectable hippocampal neuron loss or significant Timm staining in the IML.

INTRODUCTION

Common histopathologic features of temporal lobe epilepsy include hippocampal sclerosis with associated Timm staining in the inner molecular layer (IML) of the dentate gyrus (i.e., mossy fiber sprouting), which are found in both humans and animal models of temporal lobe epilepsy (e.g., Nadler et al., 1980; Ben-Ari, 1985; Sutula et al., 1989; Houser et al., 1990; Babb et al., 1991; Buckmaster and Dudek, 1997a,b). Mossy fiber sprouting in the dentate gyrus (and synaptic reorganization in other temporal lobe circuits) may be a factor in the pathogenesis of temporal lobe epilepsy, and it is hypothesized to be caused by hilar neuron loss and subsequent deafferentation of the inner molecular layer (for review see, Nadler, 1981; McNamara, 1994, 1999; Dudek and Spitz, 1997). Experimental evidence strongly suggests that these aberrant mossy fibers form new recurrent excitatory synapses that may trigger seizures and/or alter the normal gating function of the dentate gyrus (e.g., Tauck and Nadler, 1985; Wuarin and Dudek, 1996; Molnar and Nadler, 1999; Lynch and Sutula, 2000). This new excitatory circuit is normally masked by inhibitory input into the granule cells, but can be revealed under conditions of reduced inhibition and/or high extracellular potassium (Cronin et al., 1992; Wuarin and Dudek, 1996; Patrylo and Dudek, 1998; Molnar and Nadler, 1999; Lynch and Sutula, 2000). Other evidence suggests that the new mossy fiber axons synapse onto basket cells, and that this leads to a form of synaptic reorganization that may be restorative (Sloviter, 1992; Buhl et al., 1996)

Recently, Longo and Mello (1997; 1998) reported that when rats were pre-treated with cyclohexamide (CHX, a potent inhibitor of protein synthesis) prior to receiving pilocarpine (PILO, a muscarinic agonist that causes status epilepticus and multifocal epilepsy (Turski et al. 1983)), Timm staining in the IML is prevented, but the CHX-pre-treated rats still developed spontaneous motor seizures. These data have been used as evidence that mossy fiber sprouting is not necessary for the generation of spontaneous chronic motor seizures.

We tested the hypothesis that CHX would prevent hippocampal neuron loss following PILO induced status epilepticus (SE), and thus prevent mossy fiber sprouting. We further hypothesized that the rats that lack mossy fiber sprouting would be deficient in the electrophysiological events suggestive of new recurrent excitatory synapses in the hippocampal granule cell layer (e.g., see Wuarin and Dudek, 1996, 2001). We used a septo-temporal analysis of Timm staining in the IML, and a quantitative stereological assessment of the hippocampus to address the first hypothesis. The second hypothesis was examined with *in vitro* extracellular and whole-cell patch-clamp recordings in hippocampal slices. We found, however, that pre-treatment with CHX did not prevent hippocampal neuron death nor did it block mossy fiber sprouting in the IML of the dentate gyrus, even though CHX reduced PILO-induced SE and may increase the mortality of PILO-treated rats. Furthermore, when the PILO- and PILO+CHX-treated rats with only a few seizures during treatment (i.e., no SE) were examined, some of these animals had spontaneous motor seizures, but we did not detect histopathologic changes in the hippocampus. Together, these data suggest that 1) pre-treatment with CHX does not block mossy fiber sprouting, but rather affects the likelihood of convulsive SE, and that 2) neuron loss in the hilus, CA3, and CA1, and significant amounts of Timm stain in the IML are not necessary for the development of chronic motor seizures after PILO treatment, and that rats with PILO-induced epilepsy have seizures that possibly arise from other areas of the brain that may have been lesioned, but were not examined in this study (i.e., amygdala, subiculum, entorhinal cortex, and the thalamus).

MATERIALS AND METHODS

Pilocarpine and cyclohexamide treatment

Adult male Sprague-Dawley rats (180-200 g; Harlan) were kept in a standard light/dark cycle (12/12 h) and fed ad libitum. Rats were first injected with CHX (1 mg/kg, subcutaneous). The CHX product number was #-C7698, Sigma lot #-016H1483, and the certificate of analysis is on file at sigma-aldrich.com/techinfo. The CHX was stored at 2-8° C in a dessicator. This product is stable for 5 years and was manufactured in 1996 and used in 1999. Ten minutes after the CHX injection, the rats were administered methyl-scopolamine (1 mg/kg, intraperitoneal). Then, after 30-45 min, the rats were given PILO (320 mg/kg, intraperitoneal). Many of the rats developed SE (defined as 10 or more convulsive seizures in a 90 min period) within 20-30 min following injection of PILO. After 90 min, convulsive seizures were stopped with sodium pentobarbital (17.5 mg/kg, intraperitoneal). Positive control rats were injected with the same protocol as above except CHX was not given. Control animals were also given the same drugs as above, except saline was used instead of PILO. After the rats were treated with pentobarbital, they were allowed to recover, and were monitored for motor seizures until they were used for histological and electrophysiological experiments (approximately 60 days later).

Behavioral monitoring

The rats were coded after status epilepticus so that the observers were unaware of the treatment. This procedure was followed for all experiments so that all observations were made blind. Rats were directly monitored 6 h/wk until euthanized. The animals were also video-monitored for 24-h periods, randomly throughout the 60 days, until used for experiments. The average amount of time each rat was monitored was 50 h of direct monitoring and 110 h of 24-h video-monitoring. The relative latent period was estimated as the time between the injection date and the first observed motor seizure (i.e., direct or video-monitored). A seizure rate was determined by dividing the total number of observed convulsive seizures by the total number of hours the rats were observed. This included both direct- and video-monitored seizures. The relative latent period and seizure rate for each treatment group were averaged, and the treatments were compared to each other using an analysis of variance (ANOVA) with a Tukey's multiple-comparison analysis.

Histology

A modified Timm histological procedure was used to label the zinc-containing axons of the granule cells. The hippocampus that was not used for *in vitro* electrophysiological experiments was dissected from the hemisphere and prefixed in phosphate buffered 0.37 % Na_2S to precipitate the zinc in the mossy fibers. The hippocampus was immersion fixed in phosphate buffered 4% paraformaldehyde (pH=7.2). This same procedure was done on the recorded slices, and the hemisphere that was used for electrophysiology was assigned randomly. The tissue was then saturated with 30% sucrose and sectioned at 35 μm on a sliding microtome. The hippocampus was straightened prior to mounting on the microtome (Buckmaster and Dudek, 1997b). Every sixth section was mounted for Timm stain with cresyl violet counter stain, and the adjacent sections were mounted for cresyl violet staining alone. Every section from the recorded slices was mounted and processed for Timm stain analysis. Timm staining was performed in batches that included sections from saline-, PILO-, and PILO+CHX-treated rats, and the rats with only a few motor seizures at treatment. All the sections were coded and the intensity of the Timm stain in the IML was graded with blind procedures according to the rating scale of Tauck and Nadler (1985). The sections were grouped according to their location along the septotemporal axis starting at the septal end (i.e., 25% is the septal end and 100% is the temporal end). The grades of all the grouped sections were summed and then divided by the total number of sections/group to determine an average Timm score for each region. The Timm score for each region was then averaged for each treatment and rounded to the nearest whole number and a median was determined. The treatments were compared to a hypothetical mean of 0 using the Wilcoxan signed rank test, a non-parametric analysis.

Stereology

Neuronal counts of the hilus, CA1, and CA3 were estimated using the optical fractionator probe (West et al, 1991) in the counting software Stereoinvestigator (MicroBrightfield; Colchester, VT). The cresyl violet-stained sections were used to estimate the total number of neurons, and the sections were coded so that the investigators performing the counts were blind to the treatment. Neurons were defined by their large nuclei with dispersed chromatin and prominent nucleoli. Only those nuclei that came into focus while focusing down through the dissector height were counted. The estimated counts for each section were grouped as described in the Timm stain analysis. Each region contained no less than three sections. Neuronal counts for each region were then derived using the formula from West et al. (1991). The estimated counts from each region were then averaged for each treatment group. Each region for the treatment groups was compared to each other using an ANOVA with a Tukey's multiple-comparisons test.

Slice preparation and recording methods

All rats were anesthetized with pentobarbital sodium (50 mg/kg, I.P.) and their brains were dissected and placed for 30-60 s in oxygenated (95% O_2/5% CO_2), ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 26 $NaHCO_3$, 3 KCl, 1.3 $MgSO_4$, 1.4 NaH_2PO_4, 1.3 $CaCl_2$, and 11 glucose, pH 7.4. The brains were bisected and one-half was glued on the stage of a vibratome (Campden Instruments, Lafayette, IN), and the other half was saved for histology. Four to six 300-400 µm-thick slices, mostly from the middle third of the hippocampus, were cut parallel to the base of the brain (i.e., ventral horizontal sections).

Extracellular Recordings

Slices were trimmed to isolate the hippocampus and were kept in an interface chamber at 32° C, with humidified 95% O_2 and 5% CO_2 blown over the slices. Micropipettes for extracellular recordings were pulled from borosilicate glass capillaries (1B100F-4, World Precision Instruments, Sarasota, FL) to an open resistance of 2-30 MΩ, and filled with 1 M NaCl. Extracellular recordings were amplified with an AC coupled Axoprobe-1A amplifier at a gain of 100. Recording and analysis of all the electrophysiological data was done without knowledge of the treatment. The recording pipette was placed into the granule cell layer, and the bipolar stimulating electrode (platinum/iridium Teflon-coated) was placed in the hilus to evoke population spikes. Recordings were initially obtained in control solution (ACSF). Three stimulus intensities based upon the minimum current needed to evoke a potential (minimum, 2X-minimum, 4X-minimum) were used to assess population spike responses. Slices that did not produce population spikes of 5 mV or greater with hilar stimulation were not included in the analysis. The hippocampal slices were then bathed in 30 μM bicuculline and the stimulation protocol was repeated, as established in the control solution. While still in 30 μM bicuculline, the extracellular potassium was raised from 3 mM to 6 mM, and the stimulation protocol was again repeated. The glutamate receptor antagonists 2-amino-5-phosphonovaleric acid (AP-5, Sigma) at 50 μM and 6,7-dinitroquinoxaline-2, 3,(1H,4H)-dione (DNQX, Sigma) at 50 μM, were used to assess the role of glutamate receptors in the generation of population spike bursts. After the completion of the recordings, the slices were individually fixed for Timm stain analysis. A linear regression was performed to compare burst duration and Timm stain grade.

Whole-cell patch clamp recordings

For the whole-cell recordings, two to four 300 μm-thick slices were kept for 1-2 h in oxygenated ACSF at 32° C before being transferred to the recording chamber where recordings were obtained at room temperature (21-23° C). Perfusion solution (10 ml) containing caged glutamate (L-glutamic acid, γ-(α-carboxy-2-nitrobenzyl) ester (250 μM), Molecular Probes, Eugene OR) was oxygenated and recirculated at 2 ml/min. Pipettes for whole-cell recordings were made from borosilicate glass capillaries (KG-33, Garner Glass, Claremont, CA) with a horizontal pipette puller (P-87 Flaming-Brown pipette puller, Sutter

Instruments, Novato, CA) to an open resistance of 2-4 MΩ. Pipette solution contained (in mM) 140 K-gluconate, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), and 4 magnesium ATP, pH 7.2. The data were not used if the resting membrane potential was less than -70 mV when the whole whole-cell configuration was obtained. Whole-cell currents were amplified with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA), filtered (2 kHz), digitized (44 kHz) and stored on video tapes (Neuro-Corder, Neurodata Instruments, New York, NY). Data analysis was done off line with sampling rates 5-10 kHz (pClamp 6, Axon Instruments). The detection and measurement of spontaneous EPSCs was done with Mini Analysis (Synaptosoft, Leonia, NJ). Every detected event was examined and only EPSCs, characterized by a typical fast rising phase and slow decay phase, were included. Recording and analysis of the electrophysiological data was done without knowledge of the treatment. Cumulative amplitude distributions were compared with the Kolmogorov-Smirnov two-sample test. The significance of the maximum difference between distributions was determined using a χ^2 distribution (Siegel, 1956). The χ^2 test was also used to test for differences in the effects of the flash photolysis of caged glutamate between the different treatment groups. Data are expressed as mean ± SEM.

Flash photolysis of caged glutamate

Uncaging of glutamate (Callaway and Katz, 1993) was obtained with a xenon flash lamp (Chadwick-Helmuth, El Monte, CA). The flash of UV light was transmitted through a high-numerical-aperture, oil-immersion objective (X40, Nikon) mounted underneath the bottom of the recording chamber (coverslip) and focused approximately 200 μm into the tissue. An HeNe laser aimed directly through the objective into the tissue was used to determine the location of the photostimulation. A monochrome CCD camera (Cohu, San Diego, CA) and video monitor were used to determine the location of, and the distance between, the recorded cells and the spot of laser light. Flashes with intensities 50-100 mJ combined to a concentration of caged glutamate of 250 μM, produced a spatial resolution of approximately 100 μm. Consequently, photostimulations were applied in the granule cell layer at sites 150 μm apart.

RESULTS

Morbidity and mortality during status epilepticus

The morbidity and mortality rates during status epilepticus were analyzed to provide an external physiologic measure of the systemic effect of CHX. The saline-injected animals were not included in this analysis. The morbidity rate (i.e., rats having at least one seizure by approximately 30 min after PILO administration, Fig. 1A) was significantly lower in the PILO+CHX rats (57%; a total of 35 animals were injected, and 17 rats had status epilepticus, 3 rats $\leq$ 5 seizures, and 15 rats had no motor seizures) compared to PILO treatment alone (90%; a total of 29 rats were injected, and 20 rats had status epilepticus, 6 rats $\leq$ 5 seizures, and 3 rats had no motor seizures, p=0.01, χ^2). In those animals that did have motor seizures during treatment, both groups behaved in a similar manner during status epilepticus, but the mortality rate (Fig. 1B) was three-times higher in the PILO+CHX-treated rats (35%, 7 out of 20) compared to the PILO-treated animals (11%, 3 out of 26); however, this difference was not significant (p=0.06, Fisher exact test). If these ratios existed for a larger number of replications (i.e., 30 per group), the mortality rate would have been significantly different between these two groups. The rats that did not have seizures (n=18) or had 5 or fewer seizures during treatment (n=9) were kept for behavioral observation to assess the occurrence of spontaneous motor seizures. All animals in these groups survived treatment. Data from the rats that did not have any seizures during treatment are not included in any part of this study. These data suggest that CHX acted systemically to depress the occurrence of PILO-induced status epilepticus in a significant proportion of animals.

Fig. 1

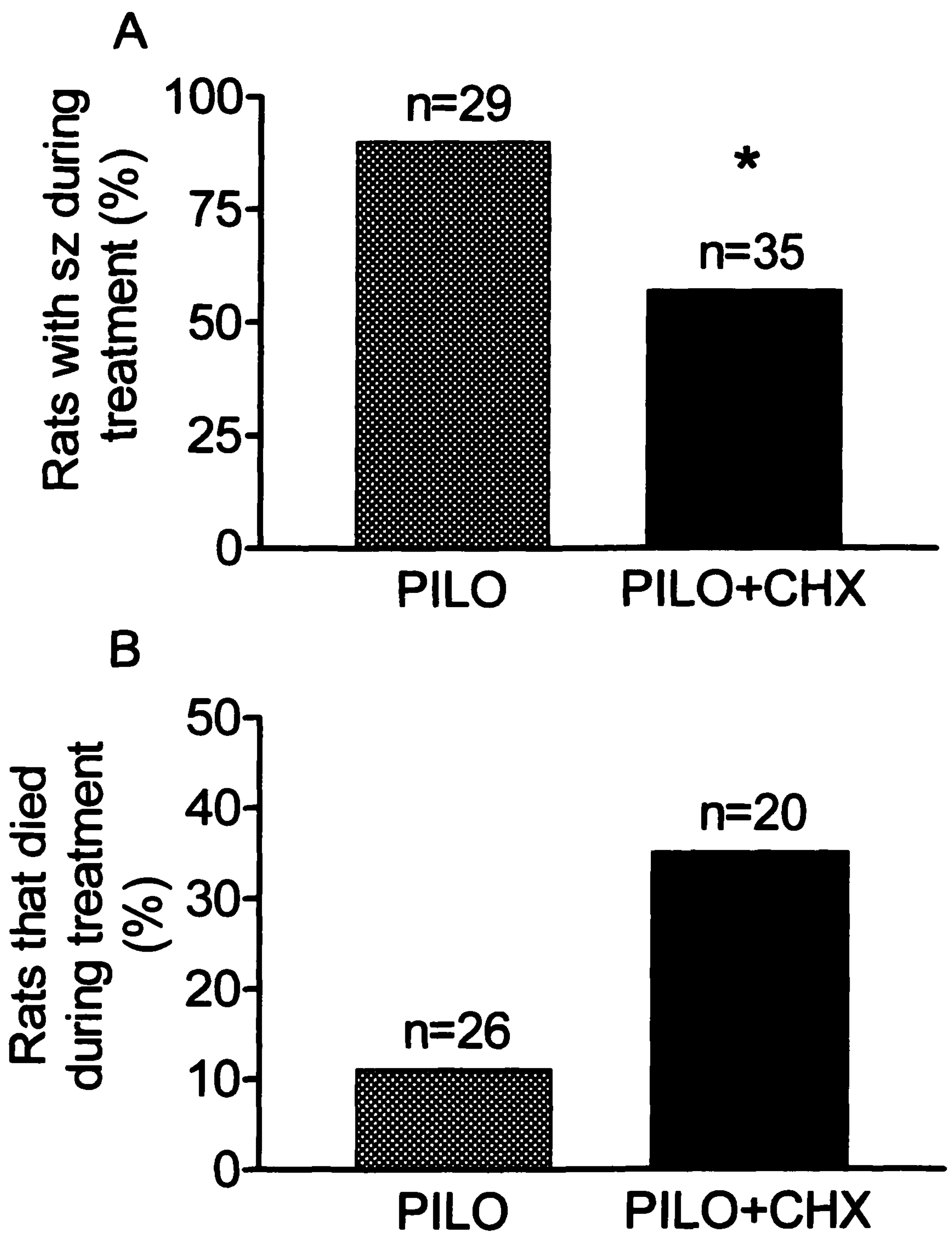

A
Rats with sz during treatment (%)
100
75
50
25
0
n=29
*
n=35
PILO
PILO+CHX
B
Rats that died during treatment (%)
50
40
30
20
10
0
n=20
n=26
PILO
PILO+CHX

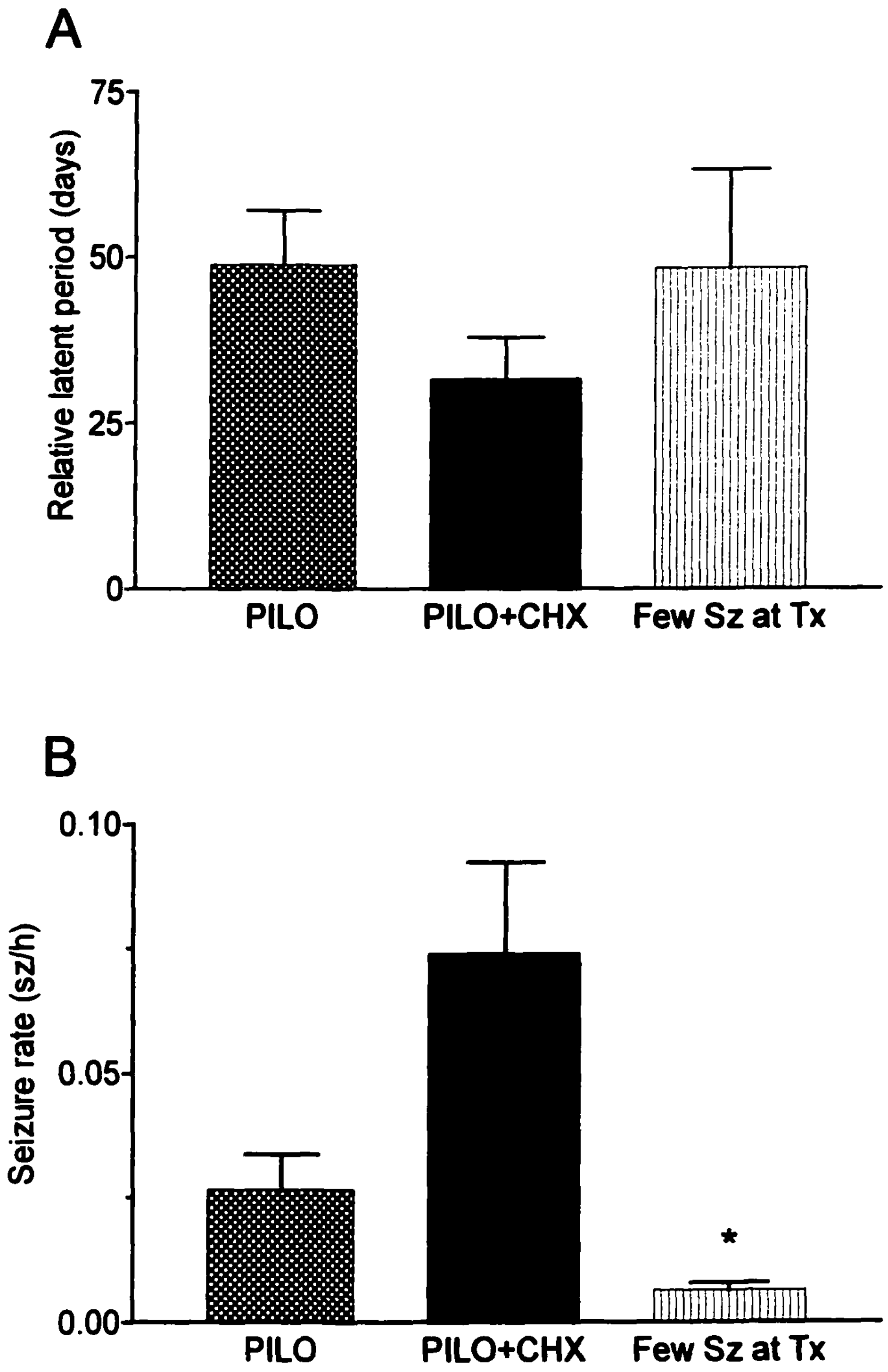
Figure 1. Morbidity and mortality rate during pilocarpine treatment. A) Morbidity rate was defined as the number of rats that responded to treatment with at least one motor seizure. The morbidity rate was significantly lower if the rats were pre-treated with CHX (57% for PILO+CHX and 90% for PILO, $p=0.01, \chi^2$). B) Mortality rate is defined as the total number of rats with acute motor seizures that died within a 24-h period after administration of PILO. The mortality rate was 35% in PILO+ CHX rats (n=20), and 17% in rats treated with only PILO (n=26). Although the mortality rate was three-fold higher for the CHX-pre-treated rats, the difference was not significant between these two groups ($p=0.06, \chi^2$). Rats that did not respond with SE, but experienced a few seizures during treatment showed no mortality (not shown).

Estimated latent period and seizure rate

In order to determine if the CHX treatment had an effect on PILO-induced epileptogenesis, the relative latent period (i.e., the interval between PILO treatment and the observation of the first motor seizure) and the seizure rate were determined by both direct observation and 24-h video-monitoring. The average duration of observation of each animal was 160 h (50 h of direct observation and 110 h of video-monitoring). The saline-treated animals were observed with the other two groups, but not included in this analysis because they were never seen to have seizures. The relative latent period is considered to be an estimate, since we did not video-monitor for 24 h per day, 7 days per week. The relative latent period (Fig. 2A) was not significantly different between the PILO+CHX rats (31.5 $\pm$ 8 days), PILO-treated animals (49 $\pm$ 6 days), and the rats that had only a few seizures at treatment (48 $\pm$ 14 days, $p > 0.05$, ANOVA). The seizure rate (i.e., the number of observed motor seizures per total hours of observation, Fig. 2B) was not significantly different between PILO+CHX (0.074 $\pm$ 0.02 SEM, n=10) and PILO-treated rats (0.027 $\pm$ 0.007 SEM, n=10, $p > 0.05$, ANOVA, Tukey). The seizure rate for the PILO-treated animals was also not significantly different from the animals that had only a few seizures during treatment (0.0064 $\pm$ 0.002 SEM, n=4 of 9, $p > 0.05$, ANOVA, Tukey). Only the data from the four animals that had $\leq$ 5 seizures at treatment (i.e., no status epilepticus), and later had spontaneous seizures, were used in the rest of this study. However, the PILO+CHX-treated rats had a significantly higher seizure rate than the animals with only a few seizures at treatment ($p < 0.05$, ANOVA, Tukey). These results support the hypothesis that CHX pre-treatment does not block or otherwise depress the course of epileptogenesis after PILO-induced status epilepticus.

Figure 2. Relative latent period and seizure rate. A) The relative latent period was based on an average of 160 h of both direct observation and 24-h video-monitoring. The average time to the first observed seizure was not significantly different between the PILO+CHX-, PILO-treated rats and rats with only a few seizures during treatment ($p > 0.05$, ANOVA). B) The spontaneous chronic motor seizure rate for each animal was calculated using the same data as was used for calculating the relative latent period. The seizure rate was defined as the total number of class III, IV, and V seizures (Racine scale, 1972) observed during the total hours of observation. The PILO+CHX rats had a significantly higher seizure rate than the rats that had only a few seizures during treatment ($p \leq 0.05$, ANOVA, Tukey). There was no significant difference between the PILO+CHX- and PILO-treated rats, nor was there a statistical difference between the PILO-treated animals and rats with only a few seizures at treatment.

Fig. 2


A
75
50
25
0
Relative latent period (days)
PILO
PILO+CHX
Few Sz at Tx
B
0.10
0.05
0.00
Seizure rate (sz/h)
*
PILO
PILO+CHX
Few Sz at Tx

Timm stain in the IML

Timm stain in the IML of the dentate gyrus was analyzed throughout the extent of the septotemporal axis using the Tauck and Nadler scale (1985) to assess the hypothesis that treatment with CHX prior to PILO-induced status epilepticus prevents the development of mossy fiber sprouting. Timm stain in the IML was not significantly different anywhere along the septotemporal axis between the CHX-pretreated rats and the PILO-only animals (Fig. 3 and 4) and both were significantly different from a score of 0 ($p<0.05$, Wilcoxan signed rank test, no differences in Timm staining were seen between the recorded hippocampal slices and the intact hippocampus for individual rats). Both the saline-treated and the rats with only a few seizures at treatment did not have Timm scores significantly different than a score of 0 ($p>0.05$, Wilcoxan signed rank test). However, the range for the rats with only a few seizures at treatment increased from 0-1 in the regions up to and including the 75% septotemporal distance to 0-2 in the 100% region. These data indicate that CHX had no detectable effect on mossy fiber sprouting in the IML. However, the data from those animals that had only a few seizures during PILO treatment suggested that significant amounts of mossy fiber sprouting in the IML were not necessary for the generation of spontaneous motor seizures.

Fig.3

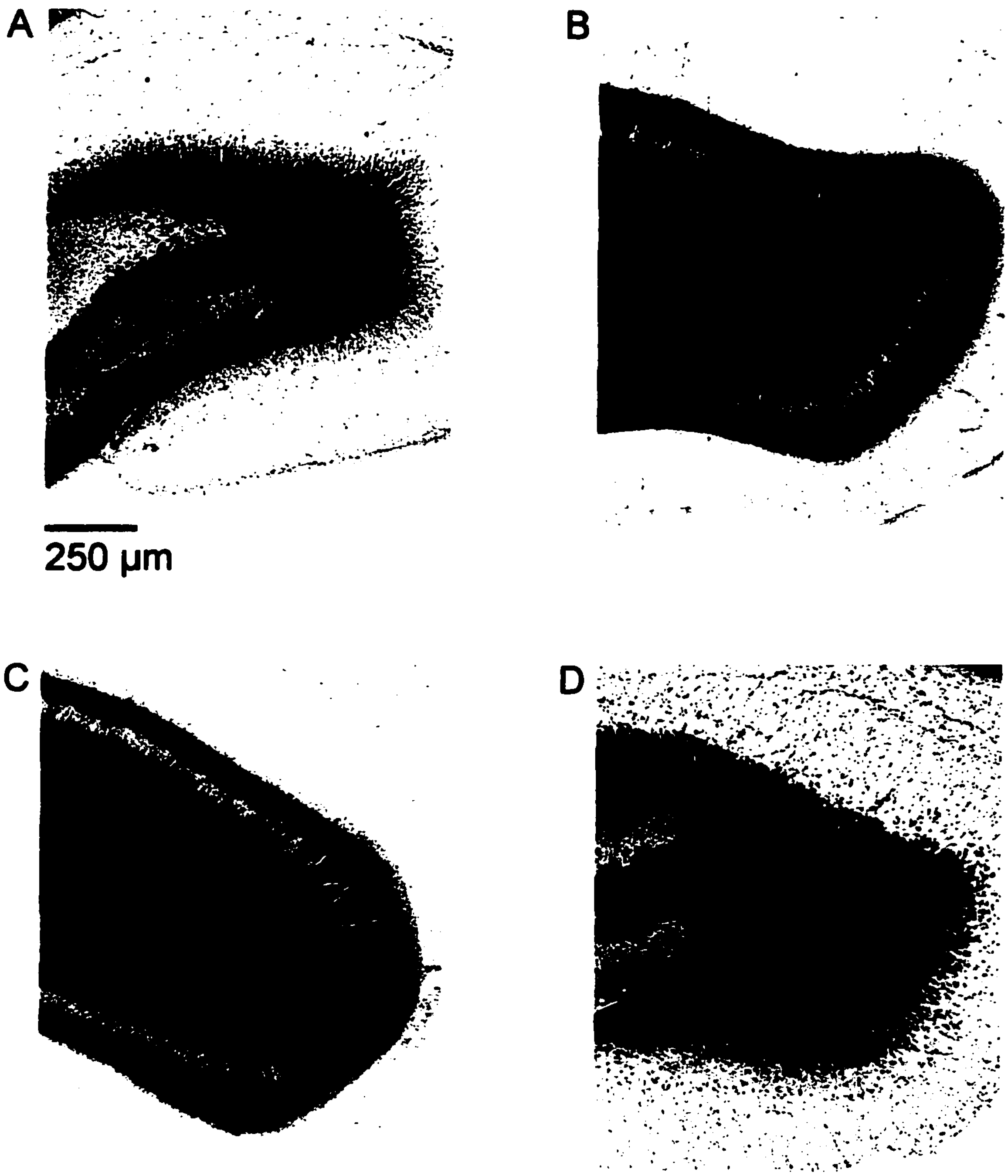


Fig. 4

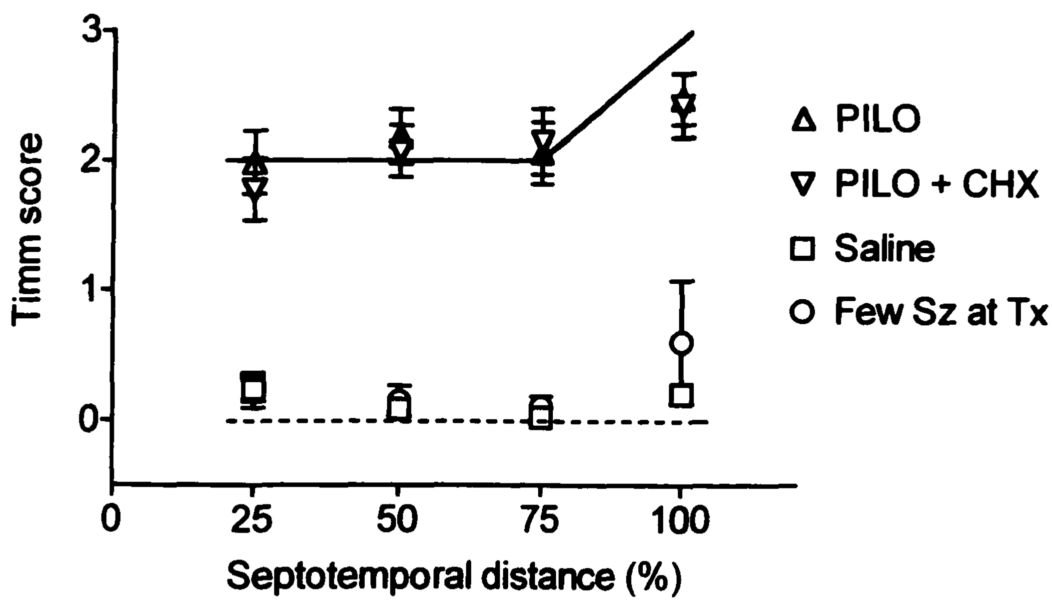

3
2
1
0
Timm score
0
25
50
75
100
Septotemporal distance (%)
PILO
PILO + CHX
Saline
Few Sz at Tx

Figure 3. Timm stain in the inner molecular layer (IML). Panel A shows Timm stain in the IML of the dentate gyrus from a saline-treated rat (scale bars = 250 µm), and this was scored as a 0 on the Tauck and Nadler scale (1985). Panel B shows Timm stain from a PILO-treated animal. Panel C is from a PILO+CHX-treated rat, and panel D is from a rat that had only a few seizures during treatment. All sections are from approximately the same level of the septotemporal axis. For both the PILO- and PILO+CHX- treated animals, this amount of Timm stain in the IML was scored as a 3 using the Tauck and Nadler scale.

Figure 4. Timm stain in the IML along the septotemporal axis. The septal end is defined as 25% and the temporal end as 100%. Every sixth section was sampled along the septotemporal axis. At no point throughout the extent of the hippocampus was there a significant difference in the average Timm score between the PILO+CHX rats and the PILO-treated animals, although both groups had significantly elevated Timm scores compared to the saline-treated rats at all points along the septotemporal axis. Rats that had only a few seizures during treatment did not have significant Timm stain in the IML compared to the saline-treated animals. Each point represents an average score and SEM for the region from each treatment group. The upper line represents the median scores for both the PILO+CHX and PILO animals which were identical to each other, and the lower line represents the median scores for both the saline rats and the 4 rats with only a few seizures at treatment that had spontaneous motor seizures, which were also identical to each other. The range of scores for all regions in the PILO+CHX and PILO treated rats is 1 to 3. The range of scores for all regions in the saline and up to 75% septotemporal distance in the rats with few seizures at treatment is 0 to 1. The range in the 100% septotemporal region for the rats with few seizures at treatment is 0 to 2. Each point and the error bars represent an average and SEM this was done in order to compare this data to previous work by the authors.

Stereologic neuron counts

In order to address the hypothesis that pre-treatment with CHX prevents neuron loss during PILO-induced status epilepticus, we performed a stereologic estimate of neuronal populations in the hilus, CA1 and CA3 along the septotemporal axis. Hilar neuron loss was not significantly different between the PILO+CHX- and the PILO-treated animals. However, both the PILO+CHX- and

PILO-treated rats had significant neuronal loss at the temporal end of the hippocampus (100 %) compared to the saline-treated animals (Fig. 5, 6A, $p < 0.05$, Tukey). The PILO+CHX-treated animals did not have significantly different neuron counts at 75% along the septotemporal axis compared to the saline-treated and PILO-treated rats, but the PILO-treated animals did have significant neuron loss in this region compared to the saline-treated animals. The PILO- and PILO+CHX-treated animals had significant neuronal loss in CA3 in the temporal region when compared to the saline-treated rats (Fig. 5, 6C, $p < 0.05$, Tukey). The CA1 pyramidal cell layer showed significant neuron loss in both the PILO+CHX- and PILO-treated animals throughout the extent of the septotemporal axis compared to the saline-treated rats, and no significant difference was detected between the PILO+CHX and PILO treatments (Fig. 5, 6B, $\alpha = 0.05$, Tukey). The rats with few seizures at treatment were not significantly different from the saline-treated rats in any of the areas examined (i.e., hilus, CA3, and CA1). These data show that CHX had little or no detectable effect in preventing neuronal loss in the hilus (i.e., the 75% region of the hilus), and was not effective in areas CA3 and CA1 of the hippocampus. These data also suggest that detectable neuron loss (by the optical fractionator method) in the hilus, CA3, and CA1 is not required for the appearance of spontaneous motor seizures.

Fig. 5

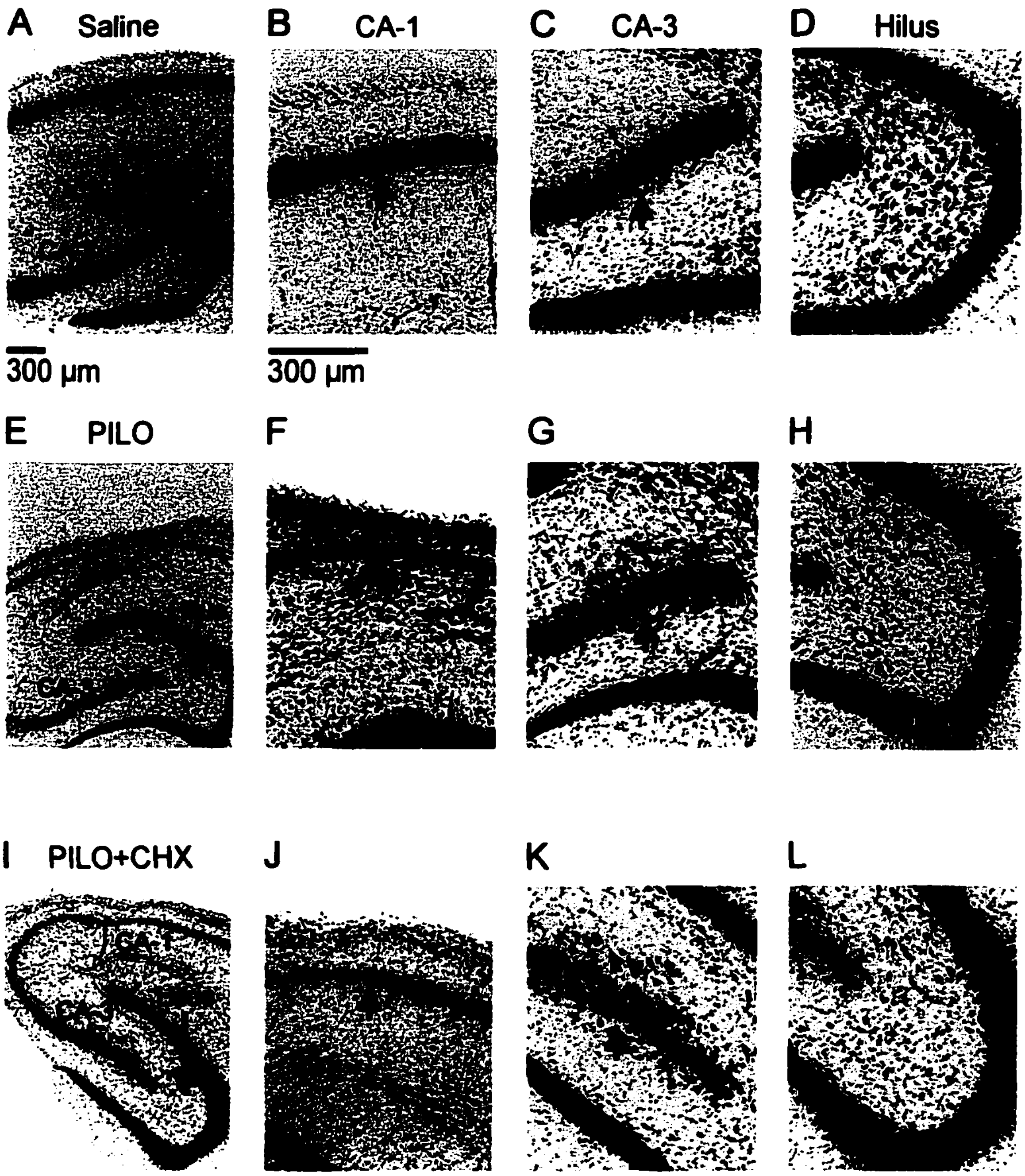
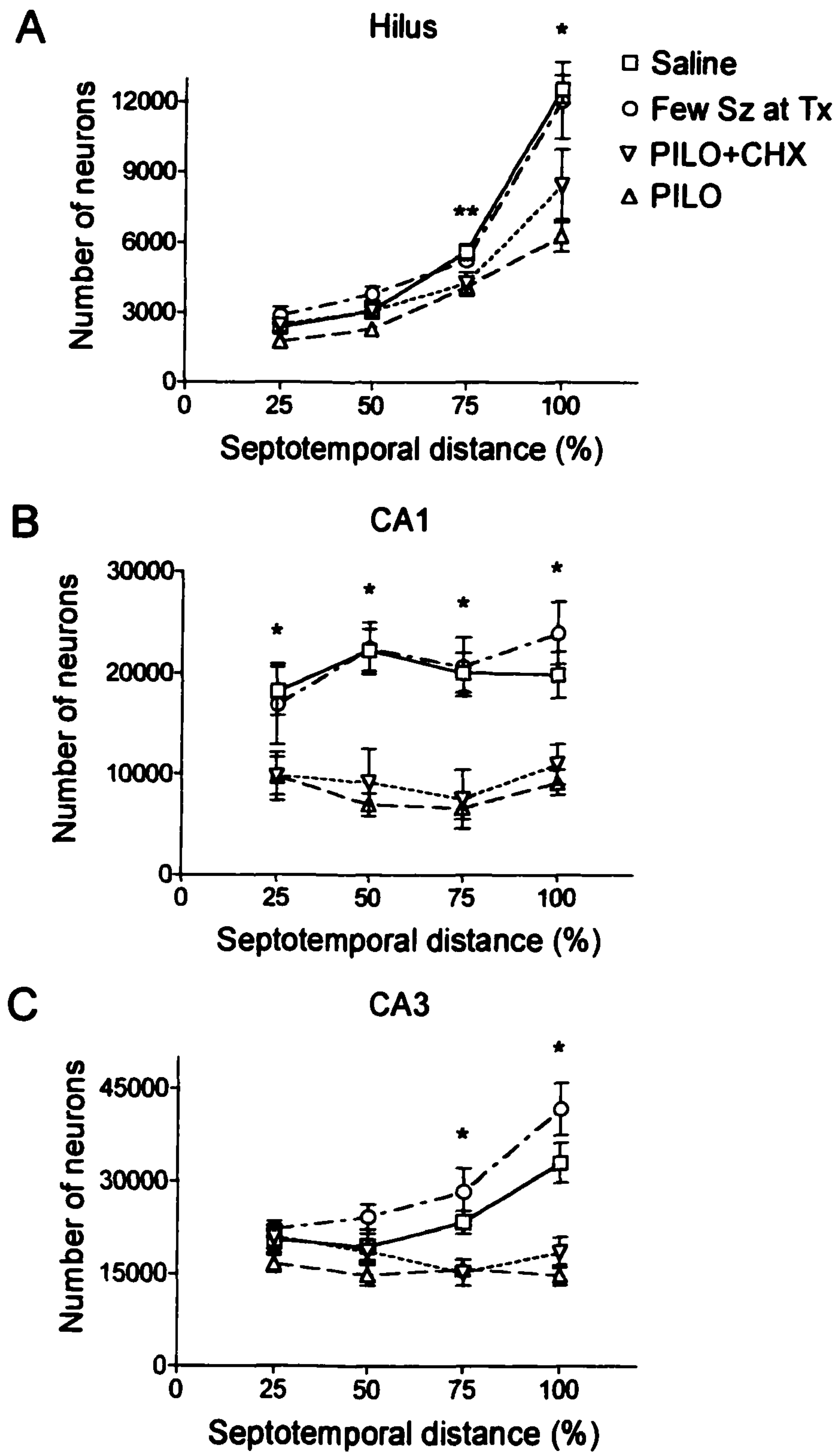

A Saline
B CA-1
C CA-3
D Hilus
300 μm
300 μm
E PILO
F
G
H
I PILO+CHX
J
K
L

Fig. 6



A
Hilus
Number of neurons
12000
9000
6000
3000
0
Septotemporal distance (%)
0
25
50
75
100
**
*
Saline
Few Sz at Tx
PILO+CHX
PILO
B
CA1
30000
20000
10000
*
C
CA3
45000
30000
15000

Figure 5. Cresyl violet stain and neuronal loss. Panels A-D show cresyl violet staining from a saline-treated rat (both scale bars = 300 μm; A, E, and I are low magnification, all others are high magnification). Panels E-H are sections from a rat treated with PILO, and panels I-L are from a PILO+CHX-treated animal. Panels F and J show neuronal loss in CA1 for both the PILO- and PILO+CHX-treated rats compared to the saline-treated rats in panel B. Panels G and K demonstrate slight neuronal loss in CA3 in both the PILO and PILO+CHX treatments when compared to CA3 from the saline-treated rat in panel C. Hilar neuronal loss in both the PILO- and PILO+CHX-treated rats is shown in panel H and L respectively, and the hilus from the saline-treated animal is represented in panel D. CHX pretreatment did not appear to prevent neuronal loss in the hilus, CA1, or CA3.

Figure 6. Neuron counts along the septotemporal axis. The septal-most end was defined as 25% and the temporal pole as 100%. Graphs of neuronal counts revealed no significant difference at any point along the septotemporal axis between the PILO+CHX-treated and the PILO-treated animals in the hilus (A), CA1 (B), and CA3 (C). Both the PILO+CHX- and PILO-treated animals had significant neuronal loss in the temporal area of the hilus and CA3 compared to the saline-treated rats (the • symbol denotes significant difference). In the 75% region of the hilus (••), the PILO+CHX-treated rats were not significantly different from either the PILO- or saline-treated animals, but the rats treated with PILO were significantly different from the saline-treated rats. CA1 for both the PILO+CHX and the PILO treatments had significant neuronal loss compared to the saline treatment throughout the extent of the septotemporal axis. Rats that had only a few seizures during treatment did not have significant neuron loss as compared to the saline-treated animals in any of the hippocampal areas examined.

Field-potential recordings from the dentate gyrus

The purpose these studies was to test the hypothesis that new recurrent excitatory circuits would not be formed after CHX pre-treatment prevented mossy fiber sprouting in the IML. Therefore, the dentate gyrus would hypothetically not show epileptiform activity under conditions of reduced inhibition and increased extracellular potassium with hilar (i.e., antidromic) stimulation. Abnormal network activity was defined as the presence of repetitive firing of population spikes, with a distinct threshold for burst discharge (i.e., all-or-none, Fig. 7). Often the initial burst was followed, after a variable latent period, with additional bursts (i.e., afterdischarges). Control responses consisted of a single population spike or multiple population spikes with a graded response (i.e., the number of population spikes increased with higher stimulus intensity, and no threshold could be determined, Fig. 8A-E). In control solution, only a single population spike could be elicited from all the treatment groups. In 30 μM bicuculline, all of the hippocampal slices from saline-treated animals and from rats that had only a few seizures during treatment responded with single population spikes. However, 4 out of 9 PILO+CHX-treated rats and 5 out of 9 PILO-treated animals had hippocampal slices that showed bursts of population spikes when bathed in 30 μM bicuculline. When the extracellular potassium was raised from 3 mM to 6 mM while still in 30 μM bicuculline, the hippocampal slices from saline-treated animals still did not demonstrate bursts of population spikes to hilar stimulation, but slices from 2 out of 10 saline-treated rats did show a graded response to increasing stimulus intensity. In 2 out of 4 of the rats that had only a few seizures during treatment, some of the hippocampal slices responded with a mixture of a graded response and small afterdischarges (Fig. 8F). In this same bathing solution, 8 out of 9 of the PILO+CHX- (Fig. 9D) and 7 out of 9 of the PILO-treated rats had hippocampal slices with bursts of population spikes. When bursts of population spikes were found, 50 μM AP-5 with 50 μM DNQX were added to the bath to block NMDA and AMPA receptors respectively. In all cases (n=11) where AP-5 and DNQX were added to the bath, population spike bursts were blocked, and the responses recovered when AP-5 and DNQX were washed from the slices (Fig. 9E, F, G). The hippocampal slices were processed for Timm stain after the recording, and the average Timm score was obtained in the same fashion as described in the Materials and Methods section (n for recorded slices=23 for PILO+CHX; n=27 for PILO; n=29 for saline). The duration of the population spike bursts was determined from responses recorded in 30 μM bicuculline and 6 mM $[K^+]_o$ by measuring from the stimulus artifact to the last 0.5 mV population spike, and the duration from each slice was averaged for each animal. All graded responses were eliminated from this analysis. The average burst duration was transformed into the natural log to reduce variance, and then a correlation of average burst duration and median IML Timm-stain grade for each animal was performed. Burst duration correlated with Timm stain in the IML (r^2=0.6820, p=0.0006, Fig. 10). Timm-stained sections from the recorded hippocampi for both the PILO+CHX- and the PILO-treated rats were significantly different from a hypothetical mean of 0 (p<0.004), Wilcoxan signed rank test), while recorded sections from the control rats were not significantly different from 0. These data suggest that CHX did not prevent the formation of new recurrent excitatory circuits in the dentate gyrus, and that as the Timm stain increased in the IML, so did the propensity to generate an epileptiform burst.

Fig.. 7

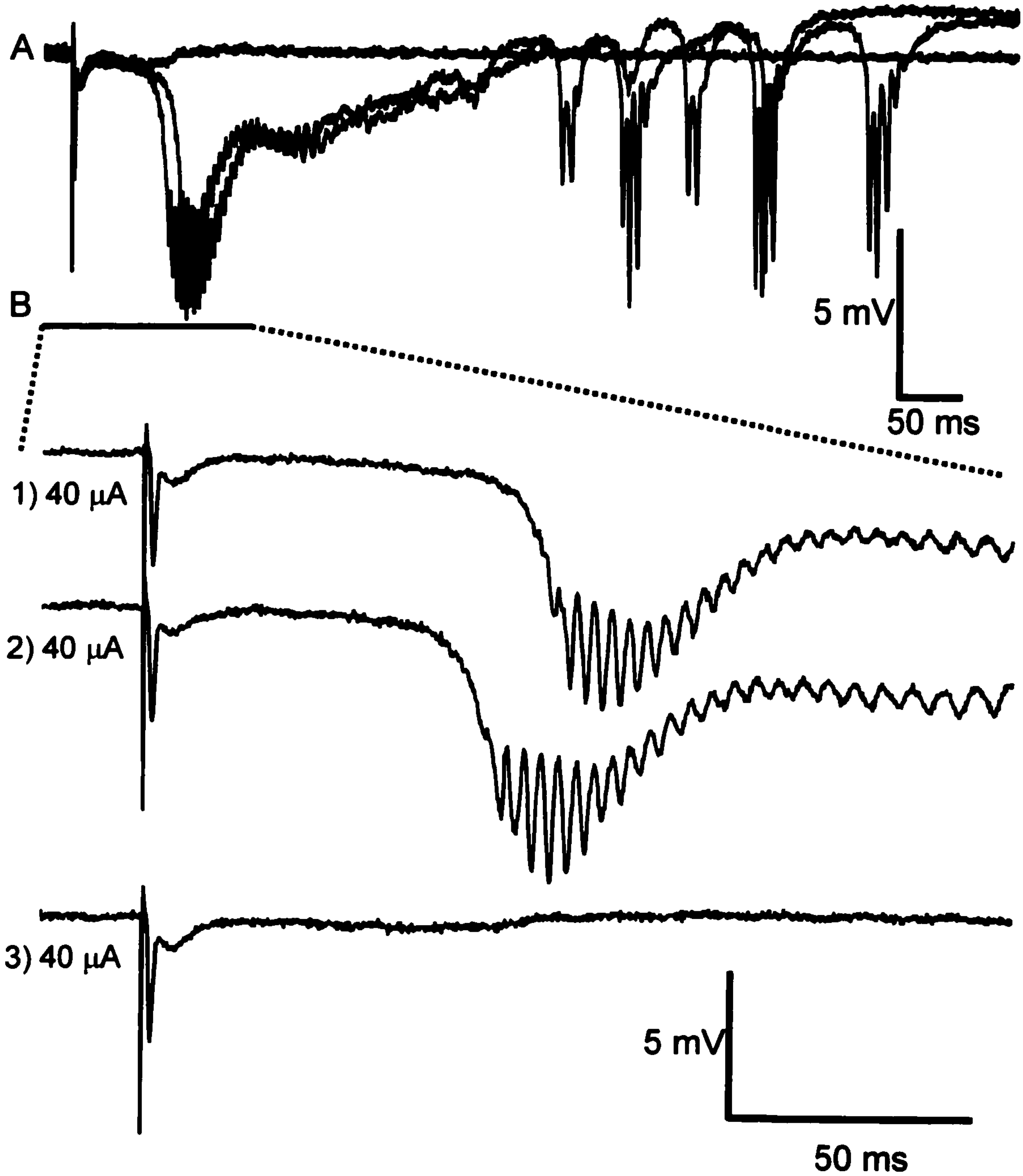


Fig. 8

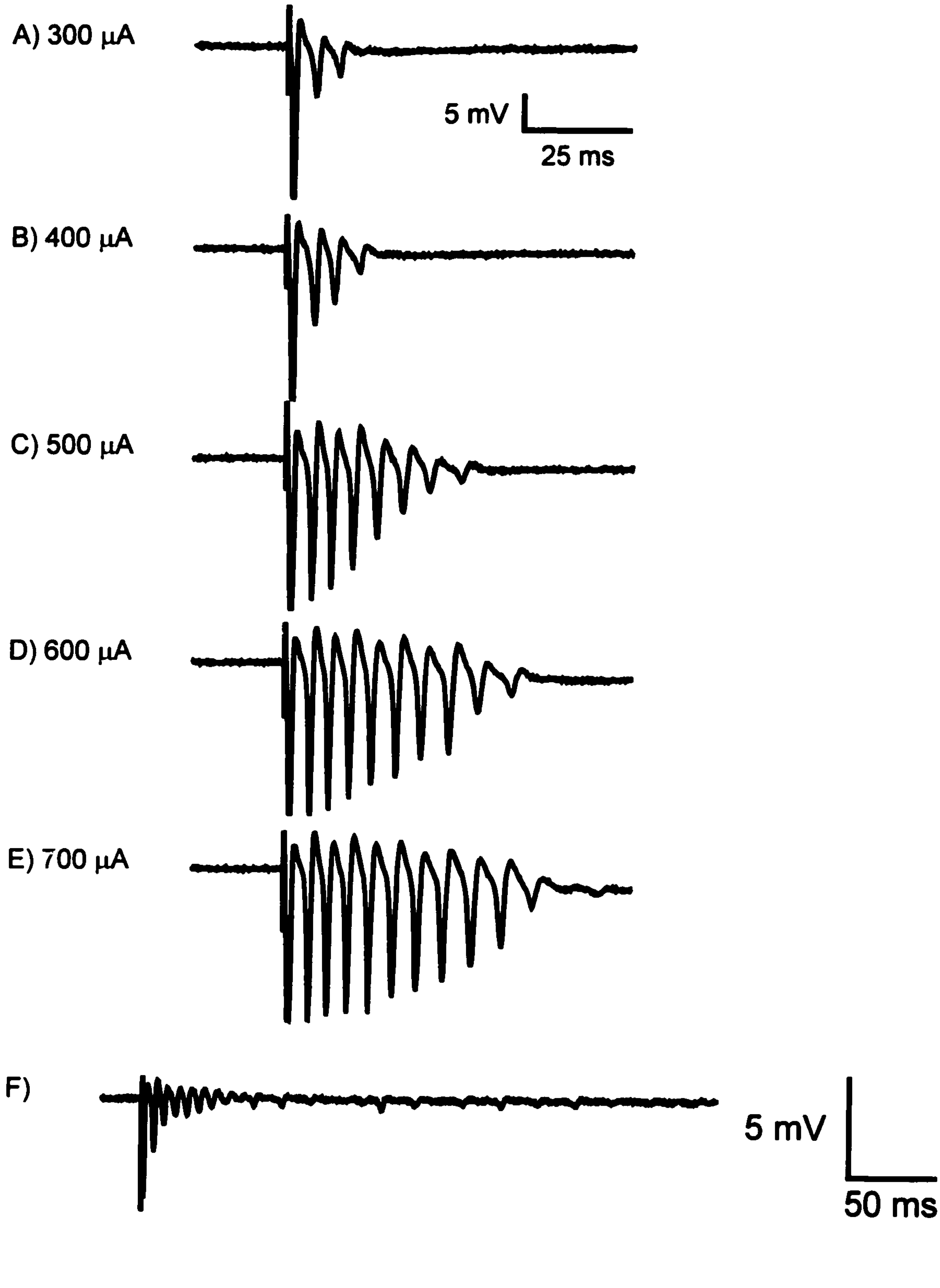


Fig. 9

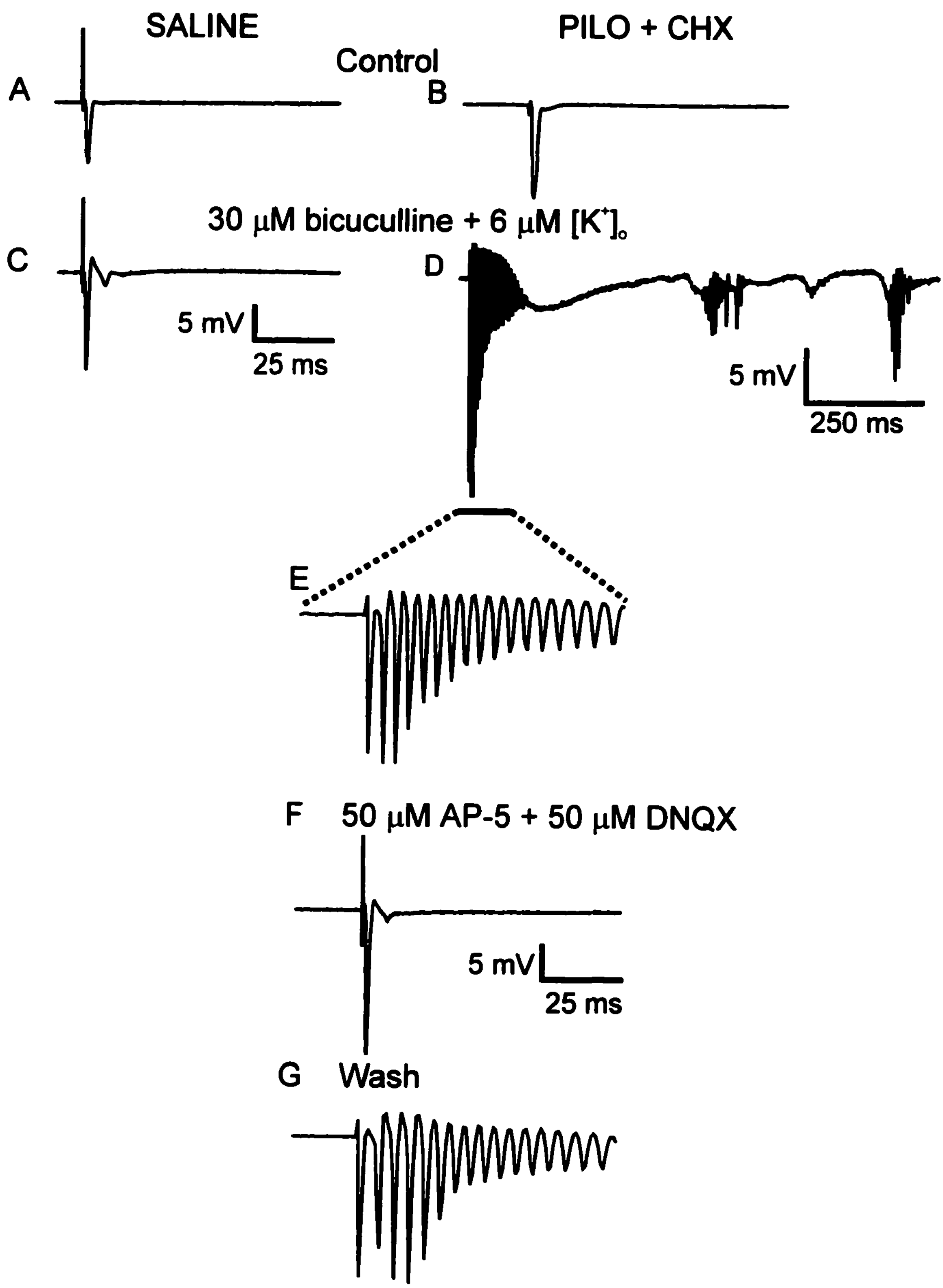

SALINE
PILO + CHX
Control
A
B
30 μM bicuculline + 6 μM [K+]o
C
D
5 mV
25 ms
5 mV
250 ms
E
F 50 μM AP-5 + 50 μM DNQX
5 mV
25 ms
G Wash

Fig. 10

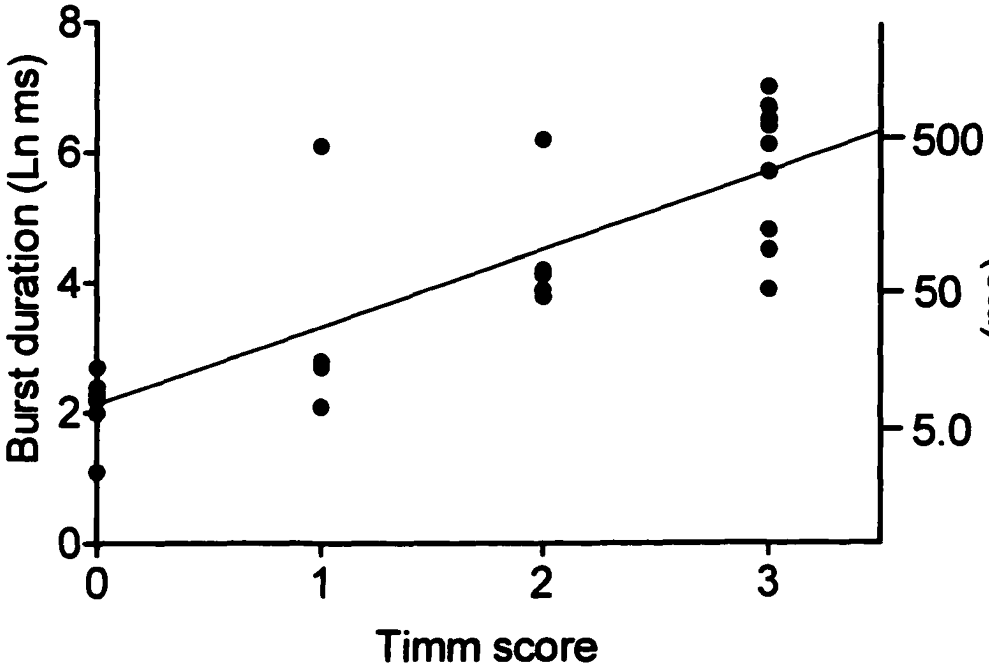


Figure 7. Burst discharges in the dentate gyrus. Recordings in hippocampal slices from the granule cell layer of a PILO-treated rat with antidromic stimulation at 40 μA. Stimulation frequency was 0.2 Hz. The slice was bathed in 30 μM bicuculline and 6 mM $[K^+]_o$. (A) The initial burst during prolonged afterdischarges had a variable onset latency. (B) The expanded traces demonstrate the variable latency of burst onset (B1 and B2), plus occasional failure of the bursts even though the initial population spike was unchanged (B3). Identical responses were recorded in slices from PILO+CHX rats.

Figure 8. An example of a graded response and a response with small afterdischarges from a rat with only a few seizures at treatment that subsequently had spontaneous motor seizures. Panels A-E demonstrate a graded response in 30 μM bicuculline and 6 mM $[K^+]_o$. Note that as the stimulus amplitude increases so does the number of population spikes. This response is seen in control animals and in the rats with only a few seizures at treatment. Saline-treated animals with this response typically can only be induced to have 5-6 population spikes at the highest stimulus intensity. Panel F shows a response from an animal with only a few seizures at treatment that demonstrates small afterdischarges occurring after the initial burst of population spikes, which are graded with stimulus intensity.

Figure 9. Glutamate-mediated network bursts in a hippocampal slice from a PILO+CHX-treated rat. All the traces shown are extracellular recordings from the granule cell layer with antidromic stimulation at the maximum stimulus intensity (i.e., 400 μA). Panels A and B show an average of five responses from saline and PILO+CHX-treated rats respectively in control solution. Panel C is an average of five responses from a saline-treated rat bathed in 30 μM bicuculline and 6 mM $[K^+]_o$, and panel D shows a single response from a PILO+CHX-treated rat in the same solution. Panel E is an expanded version of D, and panels F and G are from the same slice as in E. Panel F demonstrates blockade of the bursts with 50 μM AP-5 and 50 μM DNQX, and washout of the block is shown in panel G.

Figure 10. Linear regression plot of burst duration vs Timm stain in the IML. The burst duration (the time from the stimulus artifact to the last 0.5-mV population spike) was plotted against the median Timm score from the recorded slices. This plot includes data from all three groups (i.e., saline-, PILO+CHX-, and PILO-treated rats, but not rats that only had a few seizures at treatment). All responses were to antidromic stimulation at the maximum stimulus. Hippocampal slices that produced a graded response (i.e., the number of population spikes increased as the stimulus intensity was increased, and multiple population spikes were not evoked in an all-or-none manner) to hilar stimulation were not included in the analysis. The right side of the graph shows the transformed numbers in the original format. Linear regression analysis shows $r^2 = 0.6820$ and a $p = 0.0006$. Each point is the median Timm score of the hippocampal slices recorded from one rat and the average burst duration evoked from those slices. The line

represents the linear regression. This graph indicates that as the average Timm score increased, the duration of the network bursts also increased.

Changes in spontaneous EPSC amplitude and frequency

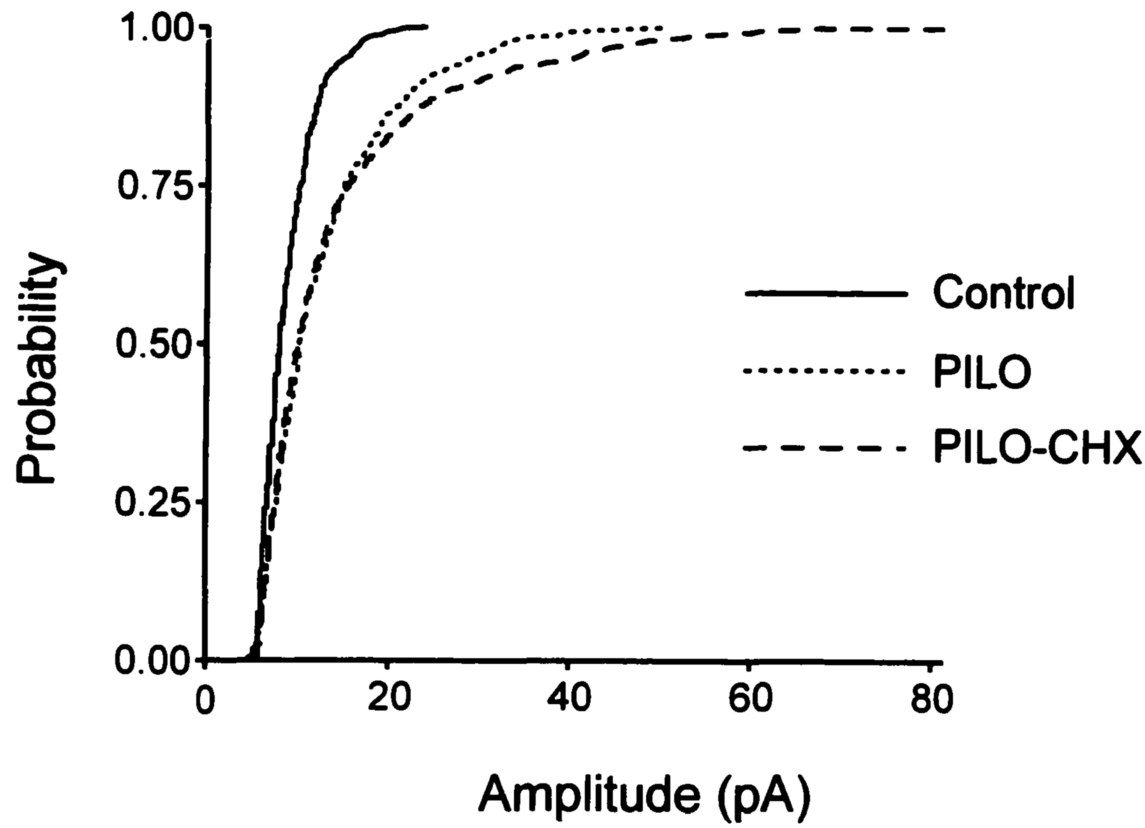
The following electrophysiological experiments were aimed at testing two hypotheses: 1) PILO treatment results in an increase of the amplitude and the frequency of spontaneous EPSCs (sEPSCs) in granule cells, and 2) pretreatment with CHX blocks the PILO-induced increase in amplitude and frequency of sEPSCs. A whole-cell patch-clamp analysis of the granule cells from rats with only a few seizures at treatment was not performed. Whole-cell recordings were obtained from granule cells at their resting membrane potential, and the amplitude and frequency of sEPSCs was measured during one 120-s period for each cell (for representative examples of these types of recordings see: Wuarin and Dudek, 2001). The results from PILO-treated rats (n = 15 granule cells, 8 rats) were compared to the results obtained from PILO+CHX-treated animals (n = 10 granule cells, 5 rats) and to the results from saline-injected animals (n = 16 granule cells, 12 rats).

Cumulative amplitude distributions were constructed for each group and compared using the Kolmogorov-Smirnov (K-S) test. When compared with controls, the cumulative amplitude distribution for spontaneous EPSCs in granule cells from PILO-treated animals revealed a highly significant increase in the EPSC amplitudes ($p<0.001$, 1-tailed, Fig. 11). Similarly, comparison between PILO+CHX-treated rats and controls showed significantly larger amplitudes in the PILO+CHX group ($p<0.001$, 1-tailed, Fig. 11). However the K-S test showed no significant difference between the PILO group and the PILO+CHX group (p=0.19, 2-tailed, Fig. 11). This result suggests that EPSC amplitudes were larger in the PILO-treated animals than in saline-injected controls, and that this increase was not blocked by pre-treatment with CHX.

The mean EPSC frequency for each treatment group was saline = 0.26 $\pm$ 0.02, PILO = 0.43 $\pm$ 0.23, and PILO+CHX = 1.21 $\pm$ 3.70 (EPSC/sec). The trend shown here was that the PILO and PILO+CHX groups had a higher frequency of EPSCs as compared to the saline-treated animals, but the three groups were not significantly different from each other (p=0.06, ANOVA).

Figure 11. Cumulative probability plots of spontaneous EPSCs in granule cells. Pre-treatment with CHX did not prevent the increase in amplitude of spontaneous EPSCs after PILO treatment. Spontaneous EPSCs (n = 507) were pooled from 5 saline-treated rats, 5 PILO-treated rats (n = 714), and from 5 PILO-CHX-treated rats (n = 1453). An example of these EPSCs in control and hippocampi from rats with kainate-induced epilepsy can be seen in Wuarin and Dudek (2001). The Kolmogorov-Smirnov test revealed no significant difference between the PILO and PILO+CHX group, but both groups were different from controls (see text for statistical analysis).

Fig. 11


1.00
0.75
0.50
0.25
0.00
Probability
0
20
40
60
80
Amplitude (pA)
Control
PILO
PILO-CHX

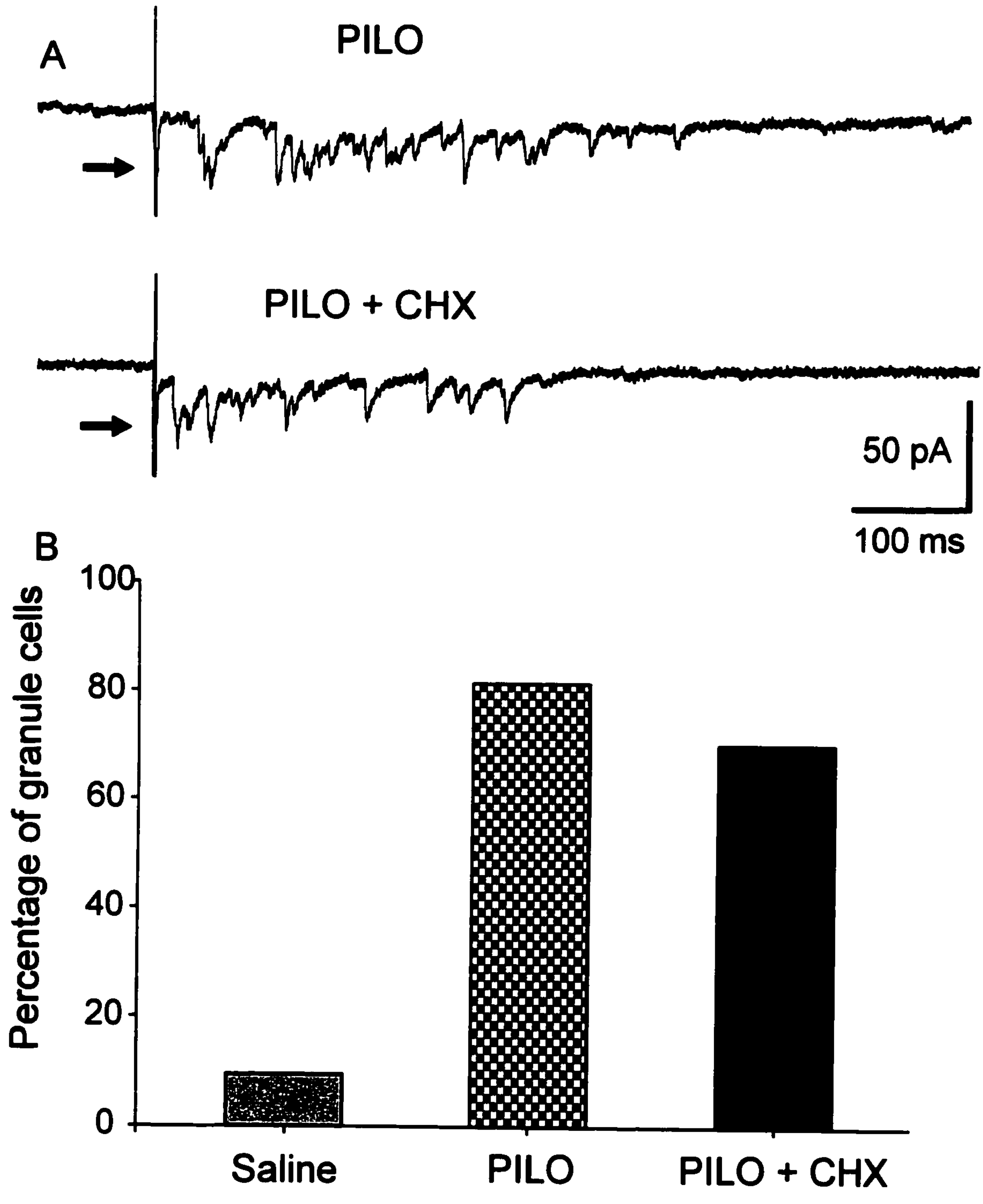
Flash photolysis

Local stimulation with application of glutamate microdrops and photoactivation of caged glutamate has been shown to evoke repetitive EPSPs and EPSCs in hippocampal slices from kainate-treated rats with Timm stain in the IML (Wuarin and Dudek, 1996; Molnar and Nadler, 1999, Wuarin and Dudek, 2001). With flash photolysis of caged glutamate, we tested the hypotheses that 1) PILO treatment increases the number of granule cells showing repetitive EPSCs, when compared to controls, and 2) pretreatment with CHX prevents this increase.

Photostimulations were first applied directly on the recorded granule cell to assure that the flash of UV light was uncaging glutamate and producing action potentials. The flash was then moved away from the recorded cells in 150 μm steps, first on one side of the recorded cell and then on the other side, until reaching the tip of the granule cell layer. Each location was stimulated several times (>3 stimulations per location) and the presence or absence of repetitive EPSCs was assessed with whole-cell recordings at resting membrane potential. The response to photostimulation was consistent for a given location (i.e., photostimulation evoked repetitive EPSCs every time or it never evoked repetitive EPSCs). Thus, for each stimulation location, the response was positive every time or negative every time (Fig. 12)(for a control response see figure 8a in Wuarin and Dudek (2001)). Two out of 21 granule cells tested in slices from saline-injected animals (9.5%) showed repetitive EPSCs in response to photostimulation. In contrast, photostimulation evoked repetitive EPSCs in a significantly larger proportion of granule cells tested from PILO- (13/16 granule cells, 81%, $p<0.002$, ANOVA) and PILO+CHX-treated rats (7/10 granule cells, 70%, $p<0.002$, ANOVA). There was no significant difference in the proportion of the granule cells responding to photostimulation with repetitive EPSCs between PILO- and PILO+CHX-treated animals ($p=0.6$, ANOVA).

Figure 12. Effect of flash photolysis of caged glutamate applied in the granule cell layer. A: Examples are shown of photostimulation-evoked repetitive EPSCs in a granule cell from a PILO-treated rat and in a granule cell from a PILO+CHX-treated rat. Both traces show whole-cell patch-clamp recordings at resting membrane potential (PILO = -72 mV, PILO+CHX = -69 mV, corrected for the liquid junction potential). The granule cell from the PILO-treated animal was located at the tip of the outer blade, and photostimulation was applied in the outer blade at a distance of approximately 300 μm. The granule cell from the PILO+CHX-treated animal was located in the apex, and the flash was applied approximately 450 μm away in the outer blade. Arrows show the artifact produced by the flash. B: plot of the percentage of granule cells showing repetitive EPSCs in response to photostimulation for each treatment group.

Fig. 12


A
PILO
PILO + CHX
50 pA
100 ms
B
Percentage of granule cells
100
80
60
40
20
0
Saline
PILO
PILO + CHX

DISCUSSION

The present study provides evidence that in PILO-treated rats, single injection pre-treatment with CHX does not: 1) inhibit mossy fiber sprouting, 2) prevent neuronal loss in the hilus, CA1, or CA3, or 3) alter the abnormal electrophysiological responses commonly observed months after SE and associated with mossy fiber sprouting. However, the data also suggest that CHX interferes with the development of SE after PILO treatment, and that CHX may also increase the mortality rate associated with PILO treatment. This study provided evidence that, independent of any effects of CHX, significant amounts mossy fiber sprouting and detectable neuron loss in the hilus, CA3, and CA1 are not necessary for the generation of spontaneous motor seizures after PILO-induced SE.

Morbidity and mortality rate

One of the difficult aspects of this study was that a significant number of rats, when pre-treated with CHX, appeared to be resistant to the convulsant effect of PILO. Similarly Williams and Jope (1994) reported that both CHX and anisomycin attenuate seizure induction in lithium/pilocarpine-induced status epilepticus. Their paper reports that the induction of the first spike train and status epilepticus are delayed by pre-treatment with protein synthesis inhibitors, and that in 20% of the rats pre-treated with CHX, neither spike trains nor status epilepticus were seen. When this effect was coupled with a three-fold increase in the mortality rate for the PILO+CHX rats, it made the establishment of a suitable number of replications difficult. One possible explanation for the resistance to PILO treatment may be found in the effect that CHX has on the inositol triphosphate (IP3) signaling pathway, which appears to be the main route of seizure induction for PILO (for a review on this topic, see Savolainen and Hirvonen, 1992). The rate-limiting steps in the formation of IP3 appear to occur during the phosphorylation of inositol-monophosphate via the two enzymes phosphotidylinositol kinase (PI kinase) and phosphotidylinositol phosphate kinase (PIP kinase), the latter of these two enzymes being the most limiting in the process (Majerus et al., 1984; Lundberg et al., 1985; Bazenet, et al., 1990; Singhal et al., 1994). A study by Weber et al. (1996) showed that the activity of PI kinase and PIP kinase was reduced to 15% and 12% of normal respectively in rat bone marrow 30 min after the animals were injected with CHX, and also that the IP3 levels were reduced to 50% of normal at this time period. The PI kinase and PIP kinase activity remained at or below these levels for at least 6 h, and the IP3 levels also remained at 50% of normal for at least 6 h. In this model, PILO was given approximately 40 min after CHX injection; thus, in these pretreated rats, the IP kinase and PIP kinase activity and IP3 levels were probably reduced prior to PILO injection, and threshold levels of these substances may be needed for PILO induction of seizures. The data presented here suggest this possibility, but further studies would be necessary for a rigorous analysis of this question. These data do indicate that the CHX did have a systemic effect on the animals, and that a reduction of protein synthesis (specifically PI kinase and PIP kinase) probably mediated the effect. Both of these enzymes have a high turn-over rate, which would account for their reduced activity after CHX treatment (Weber, et al., 1996). Cycloheximide, or other protein synthesis inhibitors, should probably be avoided in the PILO model because of the high potential for interfering with the IP3 signaling pathway. The overall effect of a three-fold increase in mortality and a significant decrease in the induction of SE in the animals pre-treated with CHX may mean that these two epileptic groups were not equivalent from the outset in these experiments.

Latent period and seizure rate

The observation that the relative latent period and seizure rate were not significantly different for the PILO+CHX and the PILO treatment groups supports the hypothesis that CHX has no effect on epileptogenesis. A sub-population of rats responded to PILO treatment with only a few motor seizures (i.e., 1-2), regardless of whether they were pre-treated with CHX. One of the more interesting findings in this study was that when this sub-population of rats was subsequently monitored, some of them had spontaneous motor seizures several weeks after treatment. The seizure rate in these rats was low, but not significantly different from the PILO-treated rats, which was probably due to the small number of animals in the group that had only a few seizures during treatment but later also had spontaneous motor seizures (n = 4). Another factor was that the PILO-treated rats had not reached their maximum seizure rate by the end of the 2-month observation period. Kainate-treated rats, for example, generally require several months to attain a maximum seizure rate (Hellier et al., 1998). Therefore, if the rats had been used at 120 days and not 60 days after treatment, a high and stable seizure rate may have been reached and the seizure rates in the PILO-treated rats that experienced SE versus those that had a few seizures at treatment may have been different.

Mossy fiber sprouting

Our finding that Timm stain is present in the IML of rats pre-treated with CHX is in contrast to what was found by Longo and Mello (1997). In their study the majority of rats pre-treated with CHX did not demonstrate Timm stain in the IML. At least three potentially significant factors are relevant to the differences between these two studies: (1) We assessed Timm staining in every sixth section (i.e., at 210 μm intervals with 35-μm thickness for each section). The variation across sections was noted to be as great as one full grade in the Timm's scale. The effect of this variation can be reduced by increasing the number of sections sampled. The level of tissue sampling in the Longo and Mello (1997) study was not reported. (2) We used a different strain of rat (i.e., Sprague-Dawley, as opposed to Wistar) in order to compare our electrophysiological data from the PILO model to results with the kainate model. Strain differences exist in the magnitude of inositol phosphate (IP) metabolism during PILO administration (for review see Savolainen and Hirvonen, 1992). The seizure response appears to be identical in the two strains, but the accumulation of inositol-1-phosphate (one metabolic measure of the signaling pathway) can be five times higher in the

Sprague-Dawley rats as compared to Wistar rats. Two strains of mice can have different responses in the amount of Timm staining in the IML after kainate treatment (Schauwecker et al, 2000), with one strain apparently showing no Timm stain in the IML following kainate-induced SE. Thus, strain differences can affect the response to SE and subsequent injury. Both our experiments and the study of Long and Mello (1997) did demonstrate Timm stain in the IML of the PILO-treated rat; therefore, any possible strain differences in the response to PILO-treatment are insufficient to offset the end result of mossy fiber sprouting and chronic signs after PILO-induced SE. It remains possible that the differences in Timm stain in the IML found in the two studies could be because Sprague-Dawley rats may not respond to CHX pre-treatment in the same fashion as Wistar rats. (3) In our study, a saline control group was included to provide a baseline for Timm stain in the IML, which meant that a more rigorous multiple-comparisons test was the appropriate statistical analysis. However, when the saline group was not included, and a t-test was performed on the data, the CHX-pre-treated rats were still not significantly different from the PILO-treated rats. One technical aspect of Timm staining that could also account for some of the differences seen between these two studies is the possibility of false negatives with Timm-stain in the IML.

In order for CHX to block mossy fiber sprouting, two assumptions must be made. The first assumption is that the signals for mossy fiber sprouting occur in the first 24-h after status epilepticus. Gap-43 is a potential signal for mossy fiber sprouting that increases after PILO-induced status epilepticus, and CHX can prevent the rise of GAP-43 mRNA, which occurs after kainate treatment, as shown by Bendotti et al. (1996). However, it should not be assumed that the effect of a single dose of CHX on protein synthesis lasts any longer than 8 h. Jonec and Wasterlain (1979) showed that protein synthesis was inhibited by 75% about 2 h after injection of CHX at 1.5 mg/kg. Another study reported that 15 mg/kg CHX (a ten times higher dose) reduced the expression of c-fos and c-jun protein after kainate injection up to 4 h after treatment, but that protein expression of both c-jun and c-fos were both increased above control levels by 8 h, although not to the levels of the kainate-treated animals (Won et al., 1997). This short-term suppression of protein synthesis on signaling pathways and regulation of mRNA levels can be longer lasting (Bendotti et al. 1996), because 2 mg/kg of CHX prior to kainate treatment can reduce the surge of GAP-43 mRNA for 24 h. Also, c-fos mRNA levels were reduced for 16 h in kainate-treated animals if they were pre-treated with 2 mg/kg CHX (Schreiber et al., 1993). In both of these studies, the actual GAP-43 and c-fos protein levels at these time points was not reported. In the studies presented above, none of the data suggest that the direct effect of CHX lasts any longer than 12 h, and the indirect effects last no longer than 24 h. These reports also indicate that even at higher doses of CHX, protein synthesis is not completely blocked. The second assumption that is made is that CHX will block synthesis of a specific signaling protein. In *in vitro* synaptoneuronal preparations, low levels of CHX could reduce total protein synthesis, but synthesis of αCamII was actually increased at the same time (Scheetz et al., 2000). Thus it is not always the case that CHX blocks increases in the concentration of all proteins. Therefore, based on this earlier research, this protocol would not be expected to block protein synthesis completely, and the blockade would be short-lived. Furthermore, it may not specifically block the increase in a signaling protein; therefore, the experimental and conceptual basis for proposing that CHX would block mossy fiber sprouting is only practical if a critical signal for sprouting occurs only in the 24-h period after SE.

Neuronal loss

The finding that pre-treatment with CHX did not prevent neuronal loss in the hilus (although it may have had a minimal protective effect in the hilus at 75% along the septotemporal axis), CA1, or CA3 was similar, but not identical to what was found by Longo and Mello (1997). They reported no significant difference between the PILO+CHX and PILO treatments in hilar and CA1 loss, but CA3 was significantly preserved in the PILO+CHX rats. This is in contrast to our finding in which CA3 loss was not significantly different between the two treatment groups. There are two potential reasons for this difference: 1) The method of neuronal counting and the sampling frequency could have been different, and 2) This study includes baseline data from saline-injected animals. It is unknown what method was used by Longo and Mello (1997) to estimate neuronal counts, nor is it clear how frequently the septotemporal axis was sampled. The present study used the 3-D optical fractionator method to derive neuronal counts (West et al., 1991; West, 1999), which was not utilized by other studies that have reported

CHX prior to kainate treatment may prevent neuronal loss (Schreiber et al., 1993). We also sampled more extensively than Buckmaster and Dudek (1997b) in order to reduce the inter-sectional variance for more accurate counts. The use of saline controls was not reported by Longo and Mello (1997), and this lack of baseline data to compare neuronal counts could influence the overall statistical analysis. An example of this can be seen in our data. When the saline data are removed and a t-test is utilized to compare the PILO+CHX and the PILO data, they are significantly different in the septal 50% of the hilus and septal 25% in CA3 ($p=0.05$ and 0.04 respectively). When the multiple comparison test was used, which incorporates the baseline data from the saline rats, the differences for these two regions are not significant. This indicates that if saline controls would have been used in the Longo and Mello (1997) study, then their results might not have been significant.

An interesting, but preliminary, finding of this study is that neuronal loss was not significantly different from the saline controls in any of the areas examined in those animals that had only a few seizures during treatment and that subsequently developed spontaneous seizures. This implies that animals that experience a few motor seizures during PILO treatment, and that do not have obvious hippocampal injury and subsequent mossy fiber sprouting, may still potentially become epileptic. The optical fractionator method seeks to reduce the inherent error in neuronal counting by utilizing unbiased techniques (for a review see: West, 1999). However, it is possible that this technique is not sensitive enough, as applied to the relatively small sample of rats used in this part of the study, to detect small differences in neuronal populations that may be present in the rats pre-treated with CHX or in the rats with only a few seizures at treatment. These findings also do not preclude the possibility that certain subpopulations of neurons in the hilus (i.e., mossy cells) or in CA3 and CA1 were lost but not detected by the quantitiative methods employed (i.e., we did not do immunocytochemistry to detect these subpopulations). Systemic PILO injection likely damaged other areas of the brain (multifocal epilepsy; amygdala, subiculum, entorrhinal cortex, and the thalamus), but we did not detect other possible lesions because we only examined the hippocampus. It is also possible that basilar dendrites of granule cells have formed and are contributing to synaptic reorganization (Spigelman et al., 1998; Buckmaster and Dudek, 1999; Ribak et al., 2001), but we would not be able to detect these by the methods employed in this work.

Electrophysiology

The premise behind these experiments was that pre-treatment with CHX would prevent mossy fiber sprouting, and therefore, the granule cell layer under conditions of reduced inhibition and elevated extracellular potassium would have responses to antidromic stimulation that were similar to saline controls. This would have supported the hypothesis that the bursting found under these conditions in the PILO- and kainate-treated rats (Wuarin and Dudek, 1996; Patrylo and Dudek, 1998) was due to the mossy fiber sprouting. However, CHX did not prevent mossy fiber sprouting, and we found that the PILO+CHX-treated rats had responses that could not be distinguished from the PILO-treated rats.

These results in the PILO model of temporal lobe epilepsy thus support the previous findings from our laboratory in the kainate model. Furthermore, burst duration was strongly correlated with Timm stain in the IML, which has not been shown before. One of the possible reasons for this finding is due to our using the rats at a fairly early time point after PILO-induced SE. Wuarin and Dudek (2001) found that the temporal progression of Timm stain in the IML, which appears to reflect axon growth in the dentate gyrus, was associated with new recurrent excitatory synapses beginning within the second week after kainate-induced SE, and that this progression proceeded for several months after kainate treatment. Thus, we may have been sampling epileptic rats that were at different levels of synaptic reorganization, which implies that as granule cell interconnections increase, so does the ability of the network to sustain a burst under conditions of reduced inhibition and elevated potassium.

In the hippocampal slices from 2 of the 4 rats that had only a few seizures during treatment, but had spontaneous seizures later, electrophysiological recordings revealed mildly hyperexcitable slices. This might have been due to a small but not significant increase in mossy fiber sprouting, but may have been associated with enough new excitatory synapses to cause mild hyperexcitability. Other mechanisms, like formation of basilar dendrites or glutamate receptor alterations, could also potentially explain these findings.

The whole-cell patch-clamp recordings demonstrated that slices from both the PILO- and PILO+CHX-treated rats had increased EPSC amplitudes as compared to the slices from the saline-treated animals. This finding is consistent with what has been found by Simmons et al. (1997) and Wuarin and Dudek (2001), where the amplitude and frequency of the EPSCs were found to increase with the density of Timm stain in the IML. The present study did not find a significant increase in the frequency of EPSCs in granule cells from either PILO- or PILO+CHX-treated rats, but there was a general trend towards a higher frequency in these two groups. This general trend may have been significant if more neurons were sampled for this study. These data suggest that excitatory drive onto the granule cells in the PILO- and PILO+CHX-treated animals was increased. This increased excitatory drive could be from new recurrent excitatory circuits or from other neurons in the slice. Postsynaptic changes in glutamate receptor type and density could also account for these changes in the EPSC amplitude.

Focal flash photolysis of caged glutamate has been used to map local neuronal circuitry (Callaway and Katz, 1993; Katz and Dalva, 1994; Dalva and Katz, 1994). It has also been shown that glutamate microdrops and minimal lasar photostimulation of the granule cell layer could evoke EPSCs in recorded granule cells in tissue from kainate- and PILO-treated rats (Wuarin and Dudek, 1996; Molnar and Nadler, 1999; Lynch and Sutula, 2000). The results reported here from a blind study support these earlier findings, and further suggest the formation of new recurrent excitatory circuits between granule cells.

CONCLUSION

The main conclusions of this study are: (1) CHX pre-treatment has considerable complicating effects that appear to invalidate an analysis of synaptic reorganization and hippocampal histopathology, and their relation to chronic epileptogenesis; (2) CHX did not block mossy fiber sprouting, and it had virtually no effect on hippocampal histopathology; (3) Although a preliminary result, it appears that chronic seizures can occur, albeit infrequently, after treatment with PILO (with or without CHX) independent of any obvious alterations in hippocampal histopathology. It is conceivable that significant changes did occur in the hippocampus, but they were not detected by cell counts or Timm stain, although the preliminary electrophysiological results suggested subtle changes in the granule cell network.

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Chapter 3

Epileptiform activity in septal and temporal hippocampal slices in the kainate model of temporal lobe epilepsy

ABSTRACT

In human temporal lobe epilepsy the anterior hippocampus is commonly identified as the focus or epileptogenic zone, and often surgical resection of this area can resolve or dramatically reduce the incidence of seizures. We hypothesized that in the kainate rat model of temporal lobe epilepsy the temporal region would demonstrate more robust epileptiform activity as compared to the septal area.

Male Sprague-Dawley rats (200 gm) were treated with kainic acid (5 mg/kg, IP) hourly until motor seizures were induced. The animals were used for electrophysiologic experiments 6-12 mo post kainate treatment, and all the animals were observed to have chronic spontaneous motor seizures prior to euthanasia. Temporal and septal hippocampal slices were obtained from opposite hemispheres, and the slices were placed into the same interface chamber for extracellular recordings from the dentate gyrus with hilar stimulation (antidromic). After the recordings, the slices were processed for Timm staining to evaluate mossy fiber sprouting in the inner molecular layer (IML) of the dentate gyrus.

In control solution, no slices were seen to produce all-or-none bursts of population spikes. Significantly more temporal slices produced all-or-none bursting than the septal slices when bathed in bicuculline and when the extracellular potassium was concurrently raised to 6 mM. When the extracellular potassium was raised to 8 mM, no difference in the proportion of slices with all-or-none bursting was found. The temporal slices also had significantly longer

bursts of population spikes when compared to the septal slices for the different recording solutions. The septal slices had a significantly higher threshold for bursting in 6 mM potassium. Timm stain of the recorded slices showed that the average score of Timm stain in the IML of the temporal slices was significantly higher compared to the septal slices. This finding may account for or contribute to the differences between the epileptiform responses of the temporal and septal hippocampus, although other extrinsic or intrinsic differences between the septal and ventral hippocampus could contribute to the disparity in the electrophysiologic responses.

INTRODUCTION

The septal (or dorsal) hippocampus is functionally, anatomically, and electrophysiologically different from the temporal (or ventral) hippocampus (Nadel, 1968; Brazier, 1970; Koreli, 1977; Gaarskjaer, 1978; Seress and Pokorny, 1981, Cavazos et al., 1992, Moser et al., 1993, Jung et al., 1994). This septal-temporal difference applies to both the normal function and pathologic conditions (Elul, 1964; Ashton et al., 1989, Lee et al., 1990; Williamson et al., 1991), and is directly relevant to temporal lobe epilepsy. Many studies that utilize *in vivo* recording, implant the hippocampal electrodes into the septal hippocampus for technical ease of electrode placement. Conversely, many *in vitro* hippocampal slice studies utilize temporal slices. Thus, it is unclear if data collected from these two regions are similar.

Studies by Racine et al (1977) showed that significantly more stimulations were required to achieve kindling in the septal hippocampus compared to the temporal hippocampus. Once kindling was achieved, however, no differences were detected between the two regions. The frequency of spontaneous bursts generated from CA3 in 7 mM potassium was significantly higher in temporal slices from normal rats compared to septal slices (Bragdon et al., 1986). Another study utilizing hippocampal slices from normal animals (Gilbert et al., 1985) showed that the ventral CA1 had a greater tendency to produce othordromic bursting in normal solution and under conditions of increased extracellular potassium ($[K^+]_o$). After kindling, the induction of mossy fiber sprouting in to the inner molecular layer (IML) can vary along the septotemporal axis, based upon the site of stimulation (Cavazos et al., 1992).

This study compares the septal hippocampus to the temporal hippocampus in kainate-treated epileptic rats only. No control rats are used in this study. This study utilized extracellular recordings from the granule cell layer with antidromic stimulation in septal and temporal hippocampal slices, which were prepared from the opposite hemispheres of the same kainate-treated rat. We examined the evoked responses in control solution, and compared these responses to those in solutions known to "unmask" new excitatory circuits commonly found after kainate treatment (Wuarin and Dudek, 1996; Patrylo and Dudek, 1998). The hypothesis of this study was that in hippocampal slices from kainate-treated rats with spontaneous recurrent motor seizures, the temporal hippocampus has more robust electrophysiologic alterations compared to the septal hippocampus.

MATERIALS AND METHODS

Treatment

Adult male Sprague-Dawley rats (180-200 g; Harlan) were kept in a standard light/dark cycle (12/12 h) and fed ad libitum. Rats were injected with kainate (5 mg/kg, intraperitoneal; Sigma, St Louis, MO) diluted in sterile 0.9% saline at a rate of 5 mg/ml every hour, and the rats were constantly monitored for motor seizures. The first seizure usually appeared after the third or fourth injection. A modified Racine scale (Racine, 1972; Ben-Ari, 1985) was used to characterize motor seizures during treatment. Class I and II seizures (staring, head nodding, and wet dog shakes) were not considered in this protocol, but Class III (lordotic posture with forelimb clonus), Class IV (forelimb clonus with rearing back onto the hindlimbs), and Class V seizures (a class IV seizure that fell over) were all considered to be motor seizures. Animals with $\geq$10 class III-V seizures per hour received a half dose of the kainate, and the seizures were "titrated" in this way until the rats experienced a minimum of 3 h of approximately 10 seizures per hour. The animals were treated with 3-6 ml of lactated ringers subcutaneously following treatment.

Behavioral Monitoring

The rats were coded so that the observers were unaware of the treatment. This procedure was followed for all experiments so that all observations were made blind. Rats were directly monitored 6 h/wk until euthanized. All rats used in these experiments had demonstrated multiple spontaneous motor seizures prior to euthanasia.

Histology

A modified Timm histological procedure was used to label the zinc-containing axons of the granule cells. After the electrophysiological recordings, slices were placed into individual containers to maintain the identity of each slice and pre-fixed in phosphate buffered 0.37 % Na_2S to precipitate the zinc in the mossy fibers. The slices were emersion fixed in phosphate buffered 4% paraformaldehyde (pH=7.2). The tissue was then saturated with 30% sucrose and sectioned at 35 μm on a sliding microtome. Every section from the recorded slices were mounted and processed for Timm stain. All of the sections were coded and the intensity of the Timm stain in the IML was graded with blind procedures according to the rating scale of Tauck and Nadler (1985). The grades of all the grouped sections were summed and then divided by the total number of sections per group to determine an average Timm score for each region. The median score for each recorded slice was also determined. The Timm staining between the two regions was compared using the Wilcoxan ranked sum procedure.

Slice preparation and recording methods

All rats were anesthetized with pentobarbital sodium (100 mg/kg, i.p.), and their brains were dissected and placed for 30-60 s in oxygenated (95% O_2/5% CO_2), ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 26 $NaHCO_3$, 3 KCl, 1.3 $MgSO_4$, 1.4 NaH_2PO_4, 1.3 $CaCl_2$, and 11 glucose, pH 7.4. The brains were bisected and one-half was glued on the stage of a vibratome (Campden Instruments, Lafayette, IN), and the other half was stored in the ice-cold ACSF. One hemisphere was coronally sectioned from rostral to caudal to obtain septal slices, while the other hemisphere was horizontally sectioned from ventral to dorsal to obtain temporal slices. Septal and temporal slices were randomly obtained from the two hemispheres, and septal or temporal slices were randomly sectioned first. Two 400-μm slices from both the septal and temporal hippocampus (a total of 4 slices) were then transferred to the recording chamber, and allowed to recover for a minimum of 1.5 h prior to recording.

Extracellular Recordings

Slices were trimmed to isolate the hippocampus and were kept in an interface chamber at 32° C, with humidified 95% O_2 and 5% CO_2 blown over the slices. Micropipettes for extracellular recordings were pulled from borosilicate glass capillaries (1B100F-4, World Precision Instruments, Sarasota, FL) to an open resistance of 2-30 MΩ, and filled with 1 M NaCl. Extracellular recordings were amplified with an Axoprobe-1A amplifier (DC-coupled) at a gain of 100x. The recording pipette was placed into the granule cell layer, and the bipolar stimulating electrode (platinum/iridium Teflon-coated) was placed in the hilus to evoke population spikes (i.e., antidromic stimulus). Recordings were initially obtained in control solution (ACSF). Three stimulus intensities based upon the minimum current needed to evoke a 0.5 mV population spike (minimum, 2X-minimum, 4X-minimum) were used to assess population spike responses. Slices that did not produce population spikes of 5 mV or greater with hilar stimulation were not included in the analysis. The hippocampal slices were then bathed in 30 μM bicuculline methiodide (a $GABA_A$ antagonist) and the stimulation protocol was repeated, as established in the control solution. While still in 30 μM bicuculline, the extracellular potassium was raised from 3 mM to 6 mM and then to 8 mM, and the stimulation protocol was again repeated for each solution. After the completion of the recordings, the slices were individually fixed for Timm stain analysis.

Analysis

Extracellular bursts were only analyzed if they were all-or-none (i.e., the bursts did not significantly increase in length with an increase in stimulus strength (see fig. 3D; fig 2B)). Slices that demonstrated a graded response (i.e., burst length increases with stimulus strength; see fig. 2A), were considered burst failures. Burst duration was measured from the stimulus artifact to the last 0.5 mV population spike. Threshold to bursting was measured as the minimum stimulus intensity required to evoke an all-or-none burst. A Fisher's exact test was used to compare the percentage of slices with all-or-none bursting. A Wilcoxan signed rank test was used to compare Timm staining in the IML between the septal and temporal hippocampal regions. An analysis of variance (ANOVA) with a Newman-Keuls multiple comparison test was used to compare the burst duration and threshold data.

RESULTS

Burst duration

We examined the burst length generated by the septal and temporal hippocampal slices. We have found that burst length positively correlates with the Timm stain score in the IML (Williams et al., submitted), and may be a relative measure of the amount of new excitatory synapses formed between granule cells. We found that the temporal hippocampal slices had a significantly longer duration of all-or-none bursts compared to the septal slices when bathed in bicuculline alone, bicuculline with 6 mM or 8mM $[K^+]_o$ ($p < 0.01$, ANOVA, Newman-Keuls, fig. 1, fig. 3A,). This result suggests that the temporal hippocampus in kainate-treated rats has a greater amount of new recurrent excitatory synapses compared to the septal hippocampus, and the temporal hippocampus is more susceptible to ictal-like activity compared to the septal hippocampus.

All-or-none bursting

In order to determine if there was a difference between the septal and temporal slices, we examined their potential for generating all-or-none bursts (figure 3B). An example of an all-or-none burst versus a graded response is shown in figure 2A and 2B. We found in this study that hippocampal slices from kainate-treated rats, regardless of their septal-temporal origin, did not show all-or-none bursting in normal ACSF (fig. 1). However, when $GABA_A$ inhibition was blocked with 30 μM bicuculline, a significant proportion (58%, $p < 0.004$, Fisher's exact test, n=12 per group) of the temporal slices began to produce all-or-none bursting as compared to the septal slices. When the $[K^+]_o$ was raised to 6 mM, all (100%) of the temporal hippocampal slices showed all-or-none bursting, which was significantly more than the number of the septal slices that had bursting (42%, $p < 0.004$, Fisher's exact test, n=12 per group). The $[K^+]_o$ was then raised to 8 mM which increased the percent of septal slices that showed all-or-none bursting to 75%, and the difference between the number of septal versus temporal slices that were bursting was no longer significant (fig. 1, fig. 3B). This indicates that the septal hippocampus is capable of producing network bursts in response to an antidromic stimulus, but that it requires a higher extracellular potassium to induce this response in a majority of the slices as compared to the temporal hippocampus.

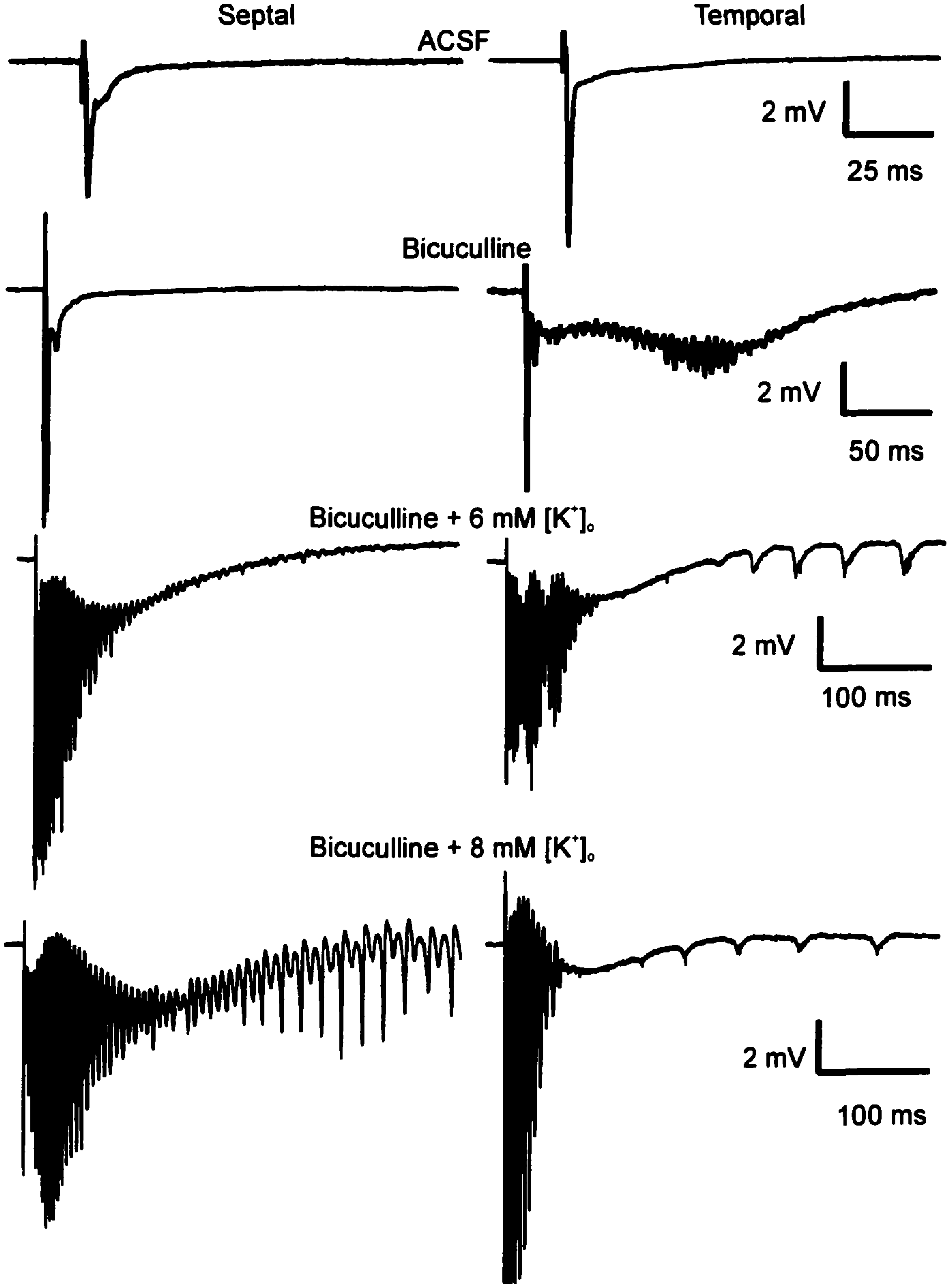
Bursting threshold

The minimum stimulus intensity required to evoke an all-or-none burst was measured to assess the burst threshold for each different recording condition (fig. 1C). The burst threshold is a measure of the ease of which a burst can be induced, and is probably determined by multiple factors (e.g., amount of new excitatory synapses, intrinsic membrane properties, glutamate receptor subtypes). We found that the septal hippocampal slices had a significantly higher threshold for bursting in bicuculline and 6 mM $[K^+]_o$ compared to the temporal slices and compared to septal slices in bicuculline and 8 mM $[K^+]_o$ ($p < 0.05$, ANOVA, Newman-Keuls). Since the septal hippocampal slices in only bicuculline did not produce all-or-none bursting it is assumed that the threshold under these conditions were exceedingly high. However, when the $[K^+]_o$ was raised to 8 mM, there was no difference between the septal and temporal hippocampus. These data show that the temporal hippocampus in kainate-treated rats has a significantly lower threshold to bursting, but this effect can be negated by increasing the $[K^+]_o$ to 8 mM.

Stimulus intensity

The definition of a graded burst response is an increase in the number of population spikes, and thus an increase in burst duration, as the stimulus intensity is increased (Smith and Dudek, 2001), while all-or-none bursting should not significantly change in burst duration as the stimulus intensity is increased. Burst duration for all the different recording conditions was compared using the average burst duration evoked by the minimum and the maximum stimuli. The septal hippocampus had a small but not significant increase in burst duration as the stimulus intensity increased (Fig. 1D). The temporal hippocampus showed a decrease in burst duration as the stimulus intensity increased, thus indicating that with the increased stimulus intensity we induced a mild burst suppression in the temporal hippocampal slices. This data also implies that it is not necessary or even advantageous to use a maximum stimulus intensity to evoke all-or-none bursting in epileptic hippocampal slices.

Fig. 1



Septal
Temporal
ACSF
2 mV
25 ms
Bicuculline
2 mV
50 ms
Bicuculline + 6 mM [K⁺]ₒ
2 mV
100 ms
Bicuculline + 8 mM [K⁺]ₒ
2 mV
100 ms

Fig. 2

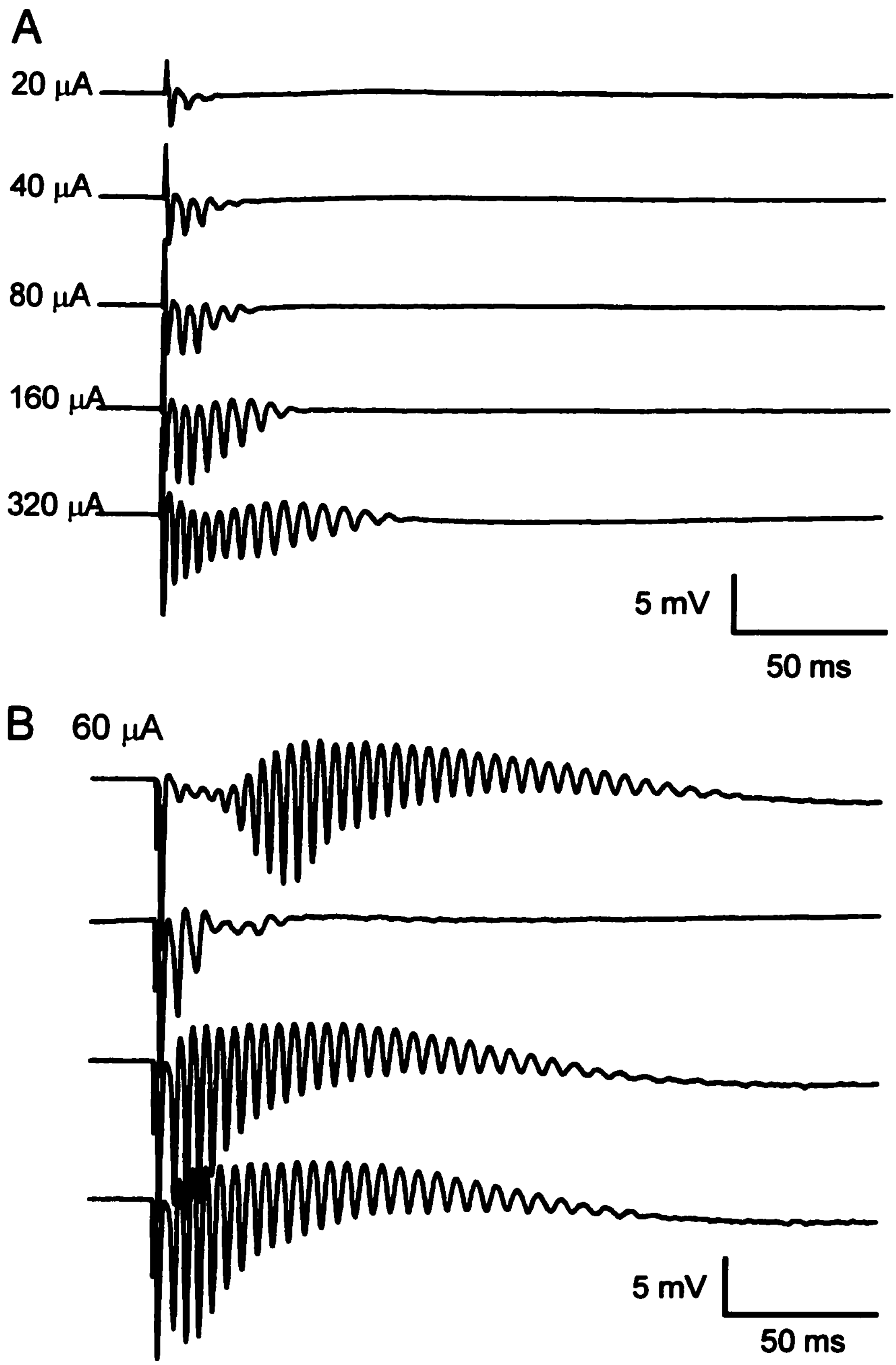
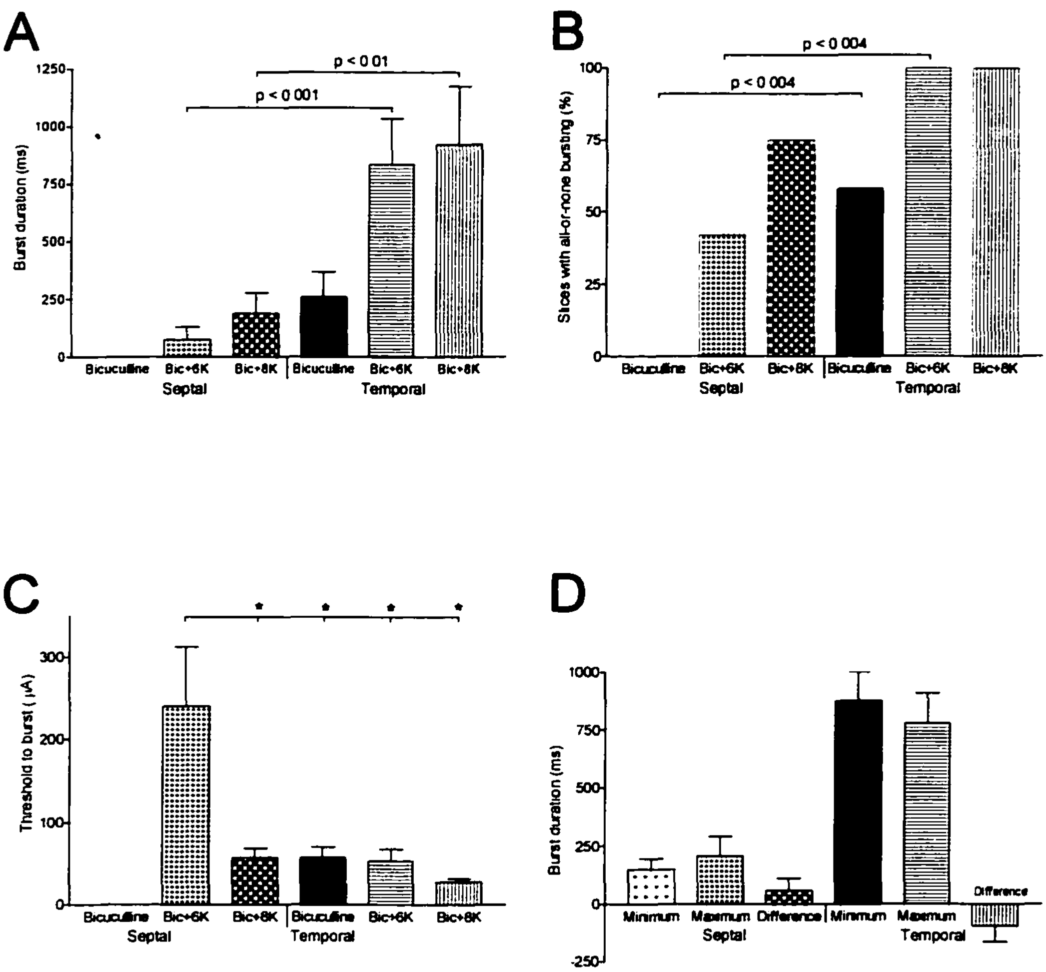


Fig. 3



A
p < 0.01
p < 0.001
Burst duration (ms)
Bicuculline
Bic+6K
Bic+8K
Septal
Temporal
B
p < 0.004
p < 0.004
Slices with all-or-none bursting (%)
C
Threshold to burst (µA)
D
Burst duration (ms)
Minimum
Maximum
Difference

Figure 1 The change in burst duration for the septal and temporal slices as a function of the different recording solutions. These recordings are from the same rat and hippocampal slice (i.e., either septal or temporal). In ACSF only single population spikes could be evoked with hilar stimulation. In 30 μM bicuculline alone the septal slice produced only a single population spike, while the temporal slice produced a small burst of population spikes. In 30 μM bicuculline and 6 mM $[K^+]_o$ the septal slice began to burst, and the temporal slice showed bursting with afterdischarges. While still in 30 μM bicuculline and with the $[K^+]_o$ increased to 8 mM both the septal and temporal slices began to show long bursts of population spikes.

Figure 2 A) Graded burst from a septal slice in bicuculline and 6 mM $[K^+]_o$. More population spikes were evoked as the stimulus intensity was increased progressively from 20 μA to 320 μA. Graded bursts were not included in any of the data analysis. B) All-or-none bursting from a temporal slice in bicuculline and 6 mM $[K^+]_o$ at threshold (60 μA stimulus intesity).

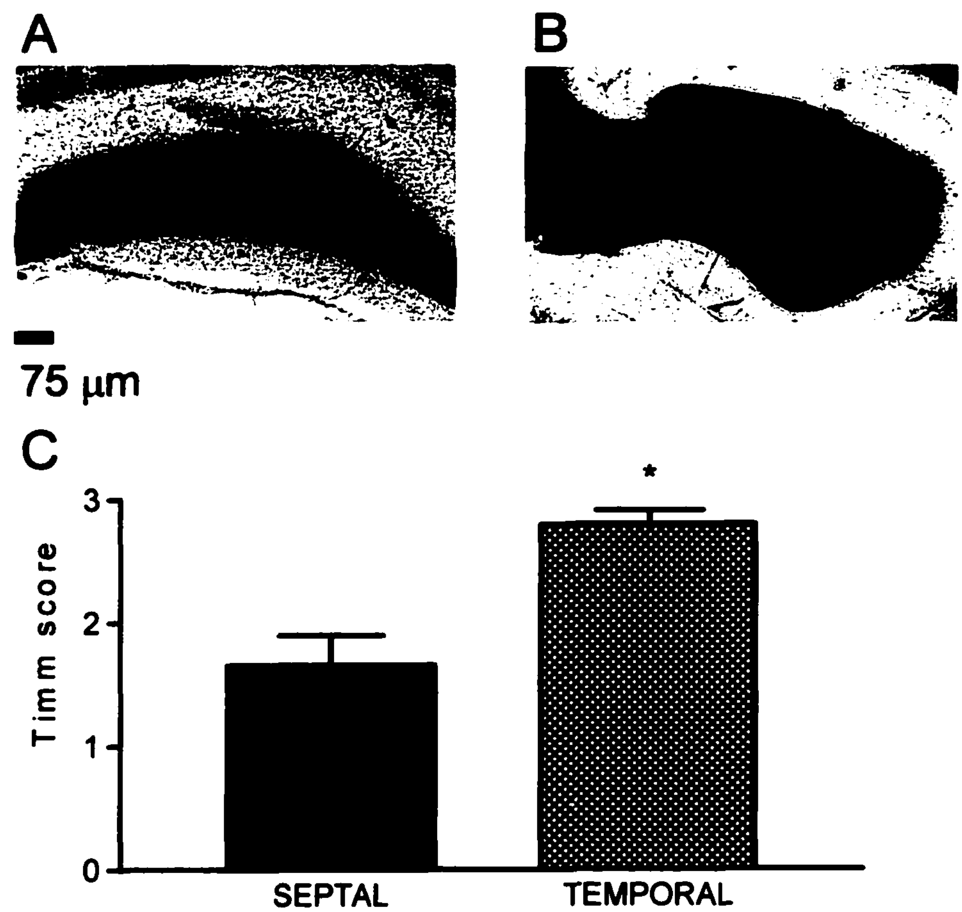
Figure 3 Summary data on the characteristics of the all-or-none bursting in hippocampal slices from kainate-treated rats. A) Duration of the all-or-none burst for septal and temporal slices. The septal slices did not burst in bicuculline alone so no comparison can be made to the temporal slices. The average burst duration for the septal slices in bicuculline + 6 mM $[K^+]_o$ was significantly shorter than the average duration for the temporal slices ($p<0.001$, ANOVA, Newman-Keuls). The average burst duration for the septal slices in bicuculline and 8 mM $[K^+]_o$ was also significantly shorter than the average burst duration for the temporal slices ($p<0.01$, ANOVA, Newman-Keuls).
B) Percent of hippocampal slices with all-or-none bursting. In bicuculline alone none of the septal hippocampal slices had all-or-none bursts (n=12), while 58% of the temporal slices (n=12) had all-or-none bursting ($p<0.004$, Fishers exact test). When the $[K^+]_o$ was raised to 6 mM while still in bicuculline, 42% of the septal slices had all-or-none bursts, and 100% of the temporal slices were bursting ($p<0.04$, Fishers exact test). When the $[K^+]_o$ was raised to 8 mM, 75% of the septal slices (n=8) had all-or-none bursting, and all of the temporal slices (n=8) continued to generate bursts to hilar stimulus. C) The threshold to all-or-none bursting in the septal and temporal hippocampal slices. Since the septal slices in bicuculline alone did not burst, the "threshold" for burst generation would be considered to be infinite. The septal slices in bicuculline + 6 mM $[K^+]_o$ had an average threshold to bursting which was significantly elevated above all the other recording conditions ($p<0.01$, ANOVA, Newman-Keuls). 3D) Burst duration as a function of the minimum stimulus versus the maximum stimulus (4X-minimum). Neither the septal nor the temporal hippocampal slices showed a significant increase in burst duration as the stimulus intensity increased ($p>0.05$, paired t-test), which is to be expected from all-or-none bursting.

Timm stain analysis

One potential explanation for the difference in the septal versus temporal hippocampal slices and their response to the antidromic stimulus could be that the amount of Timm stain in the IML at the two regions was substantially different. Thus the amount of new recurrent excitatory synapses could be increased in the region with more Timm stain in the IML. The Timm stain in the IML from the recorded hippocampal slices (fig. 4) showed that the temporal hippocampus had a significantly higher Timm score as compared to the septal hippocampus ($p < 0.05$, Wilcoxan signed rank test). Thus, one potential explanation for the electrophysiological results is that the temporal hippocampus has more new excitatory synapses than the septal hippocampus.

Figure 4 Timm stain in the IML of septal and temporal hippocampal slices. A) Timm stain in a septal hippocampal slice which was scored a 1 by the Tauck and Nadler scale (1985). B) Timm stain in a temporal hippocampal slice which was scored as a 3. C) The average Timm score for the septal hippocampus was 1.8 ± 0.3 and the median score was 1.5, and the average score for the temporal hippocampus was 2.8 ± 0.1 and the median score was 3. The score for the septal hippocampus was significantly different from the temporal hippocampus ($p=0.004$, Wilcoxan signed rank test).

Fig. 4



DISCUSSION

These experiments indicate that the septal hippocampus was not equivalent to the temporal hippocampus in kainate-treated rats with recurrent seizures in regard to the extracellular network properties and Timm stain in the IML. The temporal hippocampus has a lower threshold to evoke all-or-none bursts, the bursts generated were substantially longer, the threshold for bursting was lower, and the Timm stain in the IML was significantly increased compared to the septal hippocampus. One implication of these data is that when experiments are conducted on the hippocampi of kainate-treated rats, both *in vitro* and *in vivo*, care must be taken in the analysis and interpretation of the results obtained from these experiments with regard to what region of the hippocampus is being examined. This data also implies that the temporal hippocampus, as is seen in human temporal lobe epilepsy, may play a more prominent role in seizure generation and/or propagation than the septal hippocampus.

Differences in mossy fiber sprouting and neuron loss along the septotemporal axis of the hippocampus have been reported for both the kindling and kainate models of temporal lobe epilepsy (Cavazos et al., 1992; Buckmaster and Dudek, 1997). In the kindling model, differences along the septotemporal axis depended upon the site of stimulation and the number of seizures the animals had experienced. The kainate-treated rats showed a clear septotemporal difference, because mossy fiber sprouting was lower in the septal region as compared to the temporal end. Hilar neuron loss was significantly greater in the temporal end compared to the septal end. These results may be partially explained by the study of Lothman and Collins (1981) which showed that the ventral (temporal) hippocampus was more activated during kainate-induced seizures than the dorsal (septal) hippocampus. It is possible that these findings are model dependent, because it was shown by Ferraro et al., (2000) that mossy fiber sprouting in the pilocarpine-treated model of temporal lobe epilepsy was equally elevated throughout the septotemporal axis. Thus, in order to dissect out the relative contribution that the differences in septotemporal mossy fiber sprouting may contribute to our findings, this study should be repeated in the pilocarpine model.

New recurrent excitatory circuits may potentially be formed in the dentate gyrus by mechanisms other than mossy fiber sprouting. Several investigators have shown that granule cells form basal dendrites in several models of temporal lobe epilepsy (Spigelman et al., 1998; Buckmaster and Dudek, 1999; Riback et al., 2000), it is unknown if these newly formed dendrites have a differential distribution along the septotemporal axis or their relative contribution to the new recurrent excitatory circuit following status epilepticus.

In the normal hippocampus, a differential allocation of glutamate receptors is present along the septotemporal axis (Martens et al., 1998), which could contribute to regional differences in seizure susceptibility. A decreasing gradient of MK-801 binding (NMDA) was seen along the septotemporal axis, and an increasing gradient for AMPA binding was seen in the septal to temporal direction. It is unknown if this gradient changes after status epilepticus. It has been reported that glutamate receptor subunits appear to be differentially regulated in humans with temporal lobe epilepsy and in rat models of temporal lobe epilepsy (Kraus et al., 1994; Grigorenko et al., 1997; Rafiki et al., 1998; de Lanerolle et al., 1998; Mathern et al., 1998). However, it is not reported if these changes in glutamate subunit expression occur throughout the septotemporal axis. If a septotemporal gradient existed for glutamate subunit expression that was altered after the causative insult (i.e., kainate-induced status epilepticus) then the resulting changes could be another possible mechanism that could cause the electrophysiologic differences between the septal and temporal regions shown in this study.

Previous studies have shown that when $[K^+]_o$ was increased in hippocampal slices from kainate-treated rats bursting can be evoked from the granule cell layer (Wuarin and Dudek, 1996; Patrylo and Dudek, 1999), and that as the $[K^+]_o$ was increased so did the efficacy for inducing bursts. These studies were carried out in temporal hippocampal slices. Thus, it was unknown if the septal hippocampus would have a different response in elevated $[K^+]_o$. The present study showed that the septal hippocampus required elevated $[K^+]_o$ in order for all-or-none bursting to be seen, which was not the case for the temporal hippocampus. It also required the $[K^+]_o$ be elevated to 8 mM before bursting became consistent in the septal hippocampus. It was observed that the threshold for burst generation was significantly increased in the septal hippocampus until $[K^+]_o$ raised to 8 mM. Increased $[K^+]_o$ has multiple effects on neurons: it will tend to depolarize the neurons (for a review see Somjen, 1979), depress inhibitory post synaptic potentials (Korn et al., 1987), and potentially induce ephaptic mechanisms (Schweitzer et al, 1992). It is unknown if the septal hippocampus responds to high $[K^+]_o$ in a differential fashion compared to the temporal hippocampus (i.e., neurons are not as depolarized, inhibitory post synaptic potentials are not as depressed, or K^+-current properties are different). A further analysis of these potential mechanisms needs to be pursued.

Available evidence from human studies and from animal models of temporal lobe epilepsy suggest that the temporal lobe (anterior in humans) is more sensitive to injury than the septal hippocampus, and is more likely to be involved in seizure generation and propagation (for a discussion of the human studies see Wieser et al., 1993; Lothman and Collins, 1981; Williamson et al., 1991; Buckmaster and Dudek, 1997). The septal hippocampus has some of the common alterations often associated with temporal lobe epilepsy (i.e., mossy fiber sprouting), it is not typically altered to the same degree of the temporal hippocampus (i.e., mossy fiber sprouting, neuron loss, and electrophysiology). By investigating the differences between the septal and temporal hippocampus, with both *in vivo* and *in vitro* studies, it may be possible to dissect out the relevant mechanisms involved in seizure generation and propagation in temporal lobe epilepsy.

SUMMARY AND CONCLUSION

This study provides evidence that the electrophysiologic properties associated with new recurrent excitatory circuits in kainate-treated epileptic rats are differentially expressed in the septal and temporal hippocampus. This may be due to differences in the amount of mossy fiber sprouting found in the two regions, but other potential mechanisms probably are also involved. We conclude from this study that when studying models of temporal lobe epilepsy, it is important to take into account the hippocampal region that is being studied, and if possible the experiments should be designed to asses both regions. We also propose that the differences in the septal and temporal hippocampus may represent important mechanisms in seizure generation and propagation in human temporal lobe epilepsy.

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Chapter 4

NEURON LOSS AND SYNAPTIC REORGANIZATION IN YOUNG ADULT RATS IN A HYPOXIC-ISCHEMIC MODEL OF STROKE

ABSTRACT

Neuron loss and synaptic reorganization in the hippocampus has been associated with the induction of epilepsy after an injury, as is seen in both animal models and in humans with temporal lobe epilepsy. One of the potential chronic consequences of a stroke is the development of epilepsy. We tested the hypothesis that hilar neuronal loss caused by a unilateral hypoxic-ischemic insult will induce synaptic reorganization in the dentate gyrus, and that these new circuits will lead to abnormal epileptiform events.

The right common carotid artery of 30-day old rats was ligated. The rats were placed for 30 min into a chamber (37°C and 90%) filled with 8% oxygen. Ipsilateral or contralateral hippocampal slices from the septal region were taken from rats for extracellular recordings from the granule cell layer of the dentate gyrus. The other hippocampus was processed for Timm staining and cresyl violet staining. The cresyl violet stained tissue was used for an unbiased quantitative stereologic analysis of the entire hippocampus to evaluate hilar and CA1 neuronal loss.

This treatment induced a lesion in the right (ipsilateral) frontoparietal neocortex and septal hippocampus; the left (contralateral) hippocampus was not grossly injured. Three of 20 (15%) lesioned animals were observed to have spontaneous motor seizures. The ipsilateral hippocampus had significant hilar and CA1 neuronal loss compared to the contralateral and sham-control hippocampi, which were not significantly different from each other. The septal

region of the ipsilateral and contralateral hippocampus had small but significantly increased amounts of Timm staining in the inner molecular layer compared to the sham-control hippocampi, and the amount of Timm staining was greatly increased if the rats were seen to have spontaneous motor seizures. Ipsilateral hippocampal slices displayed all-or-none bursts of population spikes in response to hilar stimulation when bathed in 30 μM bicuculline and 6 mM $[K^+]_o$. Under identical conditions, a small percentage of contralateral hippocampal slices demonstrated all-or-none bursts of population spikes with a variable latent period. This study suggests hypoxia-ischemia is associated with synaptic reorganization in the lesioned region of the hippocampus, and these new circuits can predispose the hippocampus to abnormal electrophysiological activity. They also suggest that increased amounts of sprouting in the IML of the dentate gyrus may be associated with an increased probability of the occurrence spontaneous motor seizures.

INTRODUCTION

Cerebrovascular disease is often followed by chronic epileptic seizures in humans, and is the leading cause of epilepsy in the elderly (Lesser et al., 1985; Ettinger, 1994). With the incidence of epilepsy following a stroke ranging from 2-17% (Pohlman-Eden, 1997; Olsen, 2001).

Probably the central feature of human temporal-lobe epilepsy is Ammon's horn sclerosis, where neurons in the dentate hilus and in the CA3 and/or CA1 areas have degenerated (Babb and Brown, 1987; Franck and Roberts, 1990). In the rat four-vessel-occlusion model of forebrain ischemia, the 30-day hypoxic-ischemic model (H-I) and in the human hippocampus after an ischemic injury, a qualitatively similar pattern of neuronal death occurs in the hilus of the dentate gyrus and pyramidal cells of the hippocampus (see Pulsinelli and Brierly, 1979; Pulsinelli et al., 1982; Petito et al., 1987; Onodera et al., 1990; Franck, 1993; Towfighi et al, 1997, 1998).

In human temporal-lobe epilepsy (de Lanerolle et al., 1989; Sutula et al., 1989; Houser, 1990; Babb et al., 1991) and both the kainate and pilocarpine models (e.g., Nadler et al., 1980, Mello et al., 1993), the Timm's stain and other anatomical techniques have shown that the mossy fibers of the dentate granule cells appear to innervate the inner molecular layer (IML) of the dentate gyrus, a condition that is not usually present in normal animals or humans. It was shown by Onodera et al, (1990) that the four-vessel occlusion model of global forebrain ischemia can induce a small amount of Timm stain in the IML.

The hypothesis that enhanced recurrent excitation could underlie epileptogenesis was advanced by the experiments of Tauck and Nadler (1985) when they showed that mossy fiber sprouting in kainate-treated rats was associated with paired-pulse potentiation and multiple population spikes to hilar stimulation, which would be expected to activate exclusively the mossy fiber axons of the dentate granule cells. Subsequent experiments (Cronin et al., 1992) revealed that in most preparations from kainate-treated rats, the responses to hilar and perforant path stimulation were relatively normal; when $GABA_A$-mediated inhibition was blocked with bicuculline, however, some preparations with mossy fiber sprouting showed bursts to hilar stimulation. Some of these bursts had long and variable latencies when evoked at low stimulus intensities, a characteristic expected of circuits with recurrent excitation (Traub and Wong, 1982; Miles and Wong, 1986,1987; Christian and Dudek; 1988a,b). Mody et al. (1995) reported that inhibition was intact 3 months after 15 min of global ischemia, and that hyperexcitability was not present. If the reorganization is similar to the kainate model, then it may be necessary to block or depress inhibition to reveal the new recurrent excitatory circuit.

The goal of this study was to test the hypothesis that a hypoxic-ischemic injury at 30-days of age would induce neuronal loss and synaptic reorganization in the hippocampus, and that these new circuits would lead to hyperexcitability in the dentate gyrus. We used a quantitative stereological assessment of the hippocampus, and a septo-temporal analysis of Timm staining in the IML to address the first portion of this hypothesis. The second part of the hypothesis was examined with *in vitro* extracellular and whole-cell patch-clamp recordings in hippocampal slices. We found the ipsilateral hippocampus to have significant hilar and CA1 neuron loss, while the contralateral hippocampus appeared uninjured. In animals with a hypoxic-ischemic injury, Timm stain in the IML of both the ipsilateral and contralateral hippocampus was significantly increased above sham control rats. We found that a small percentage of these animals went on to develop spontaneous motor seizures, and those animals that displayed spontaneous motor seizures had significantly increased Timm stain in the IML in both the ipsilateral and contralateral hippocampus compared to the lesioned rats that were not seen to have seizures. We also found that the ipsilateral hippocampus from the hypoxic-ischemic rats showed abnormal electrophysiologic responses. These results suggest that hilar neuronal loss induced by hypoxia-ischemia was associated with synaptic reorganization in the lesioned region of the hippocampus, and these new circuits can predispose the hippocampus to abnormal electrophysiological activity. They also suggest that increased amounts of sprouting in the IML of the dentate gyrus may be associated with an increased probability of spontaneous motor seizures, and that the contralateral hippocampus may have synaptic reorganization with out detectable neuronal loss.

MATERIALS AND METHODS

Hypoxia-ischemia

A modified version of the Levine preparation as described by Rice et al., (1980) was used to create a H-I injury in the right hemisphere. Sprague-Dawley rats male and female 30 days of age were anesthetized using a 2% Isoflurane/oxygen mixture. The ventral midline of the neck was surgically prepared and infused with bupivicaine (0.5%, 0.5 ml). A 1 cm ventral midline incision was made and the right common carotid artery exposed and double ligated with 4-0 dexon. The skin was closed with 4-0 dermalon, and the rats were allowed to recover in a heated cage. Sham controls had the carotid artery exposed but not ligated. After 2 hr of recovery the ligated rats were placed in an airtight chamber where the temperature and humidity were maintained at 37^0 C and 90% respectively. The chamber was then filled with an 8% oxygen and 92% nitrogen mixture. The oxygen content of the chamber was monitored with an oxygen sensitive electrode (Microelectrode, Inc). The rats were exposed to 8% oxygen for 30 min and then allowed to recover in room air. The rats were then observed the next day for seizures, and placed in the vivarium for chronic seizure observation. Any rats that had seizures in the 24-h period following hypoxia-ischemia were placed into a separate group for data analysis.

Behavioral monitoring

Rats were directly monitored by a trained observer that is blind to the animals treatment for an average of 6 h a week until the rats are used for electrophysiological experiments. Only grade 3 seizures or higher in the Racine scale were recorded.

Slice preparation and in vitro electrophysiological studies

Rats were anesthetized (sodium pentobarbital 100 mg/kg I.P.) and euthanized by decapitation. The brain was rapidly removed and stored for 1 min in ice-cold physiological solution (composition in mM: 124 NaCl, 3 KCl, 1.3 $CaCl_2$, 26 $NaHCO_3$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 11 glucose equilibrated with 95% O_2, 5% CO_2, pH 7.2-7.4). The brain was bisected along the mid-line, the occipital pole blocked and glued to the chuck of a vibroslicer in order to cut 400 □m-thick coronal slices of the hippocampus. The slices placed into the recording chamber and allowed to recover for 1.5 h prior to recording. The opposite hippocampus was used for anatomical studies. Slices were studied electrophysiologically in recording chambers that allow multiple stimulating and recording electrodes.

Histology

A modified Timm histological procedure was used to label the zinc-containing axons of the granule cells. The hippocampus that was not used for *in vitro* electrophysiological experiments was dissected from the hemisphere and prefixed in phosphate buffered 0.37 % Na_2S to precipitate the zinc in the mossy fibers. The hippocampus was emersion fixed in phosphate buffered 4% paraformaldehyde (pH=7.2). This same procedure was done on the recorded slices. The tissue was then saturated with 30% sucrose and sectioned at 35 μm on a sliding microtome. The hippocampus was straightened prior to mounting on the microtome (Buckmaster and Dudek, 1997). Every sixth section was mounted for Timm stain with cresyl violet counter stain, and the adjacent sections were mounted for cresyl violet staining. Every section from the recorded slices was mounted and processed for Timm stain analysis. All the sections were coded and the intensity of the Timm stain in the IML was graded with blind procedures according to the rating scale of Tauck and Nadler (1985). The sections were grouped according to their location along the septotemporal axis starting at the septal end (i.e., 25% is the septal end and 100% is the temporal end). The grades of all the grouped sections were summed and then divided by the total number of sections/group to determine an average Timm score for each region. The Timm score for each region was then averaged for each treatment, and the treatments were compared using the ANOVA procedure with a student-Newman-Kuels multiple-comparison analysis.

Stereology

Neuronal counts of the hilus and CA1 were estimated using the optical fractionator probe (West et al, 1991) in the counting software Stereoinvestigator (MicroBrightfield; Colchester, VT). The cresyl violet stained sections were used to estimate the total number of neurons, and the sections were coded so that the investigators performing the counts were blind to the treatment. Neurons were defined by their large nuclei with dispersed chromatin and prominent nucleoli. Only those nuclei that came into focus while focusing down through the dissector height were counted. The estimated counts for each section were grouped as described in the Timm stain analysis. Each region contained no less than three sections. Neuronal counts for each region were then derived using the formula from West et al. (1991). The estimated counts from each region were then averaged for each treatment group. Each region for the treatment groups was compared to each other using an ANOVA with a student-Newman-Kuels multiple-comparisons test.

Extracellular Recordings

Extracellular electrodes were filled with 1 M NaCl (2-20 MΩ). Bipolar stimulating electrodes were made of two tightly wound Teflon-insulated platinum-iridium wires (75 μm diameter, SIU-SC100, Winston Electronics, Millbrae, CA; Grass S88, Quincy, MA)). The recording pipette was placed into the granule cell layer, and the bipolar stimulating electrode was alternately placed in the hilus and the perforant path to evoke population spikes and field post-synaptic potentials respectively. Recordings were initially obtained in control solution (ACSF). Three stimulus intensities based upon the minimum current needed to evoke a 0.5 mV population spike (minimum, 2X-minimum, 4X-minimum) were used to assess population spike and field post-synaptic potential responses. Slices that could not produce population spikes, with hilar stimulation, of 5 mV or greater were not included in the analysis. The hippocampal slices were then bathed in 30 μM bicuculline and the stimulation protocol was repeated as was established in the control solution. While still in 30 μM bicuculline the extracellular potassium was raised from 3 mM to 6 mM, and the stimulation protocol was again repeated. Hippocampal slices that produced a graded response (i.e., as the stimulus intensity increased the number of population spikes increased, and no threshold for the multiple population spikes could be determined) to hilar stimulation were not included in the analysis. The glutamate receptor antagonists 2-amino-5-phosphonovaleric acid (AP-5, Sigma) 50 μM and 6,7-dinitroquinoxaline-2,3,(1H,4H)-dione (DNQX, Sigma) 50 μM, were used to assess the role of glutamate in the generation of population spike bursts . After the completion of the recordings the slices were individually fixed for Timm stain analysis.

RESULTS

Neuron Loss

In order to assess the extent of the H-I injury to the hippocampus neurons were quantified in the hilus and CA1 region of the hippocampus through the septotemporal axis using the optical fractionator method (West et al., 1991). A qualitative assessment of the hippocampus shows that the H-I lesion appeared to be limited to the septal half of the ipsilateral hippocampus, with a severe loss of CA1 and an apparent loss of hilar neurons, while CA3 appeared relatively intact (fig 1). A quantitative assessment of hilar and CA1 (CA3 was not quantified in this study) neurons revealed that hilar neuron loss in the ipsilateral hippocampus was significant throughout the extent of the septotemporal axis (fig. 2A; $p<0.01$, ANOVA, SNK) when compared to the contralateral and sham control hippocampi, which did not have significantly different neuron counts in either the hilus or CA1. However, the temporal (100%) ipsilateral hilus was not significantly different from the contralateral temporal hilus, but it was significantly different from the sham control at the temporal end. The CA1 region of the ipsilateral hippocampus demonstrated significant neuron loss (fig. 2B, $p<0.001$, ANOVA, SNK) up to the temporal end where CA1 neuron counts were not significantly different from either the contralateral or sham control hippocampi. Hilar neuron loss was positively correlated with CA1 neuron loss (fig.5A, $r^2=0.71$, $p=0.0001$), indicating that the severity of CA1 neuron loss was closely matched by hilar neuron loss. These data indicate that the H-I lesion as it effects the hippocampus was most severe in the septal hippocampus, but the injury does to a less extent involve the temporal end. This data also suggests that the contralateral hippocampus is not injured by unilateral ligation and global hypoxia.

Fig. 1

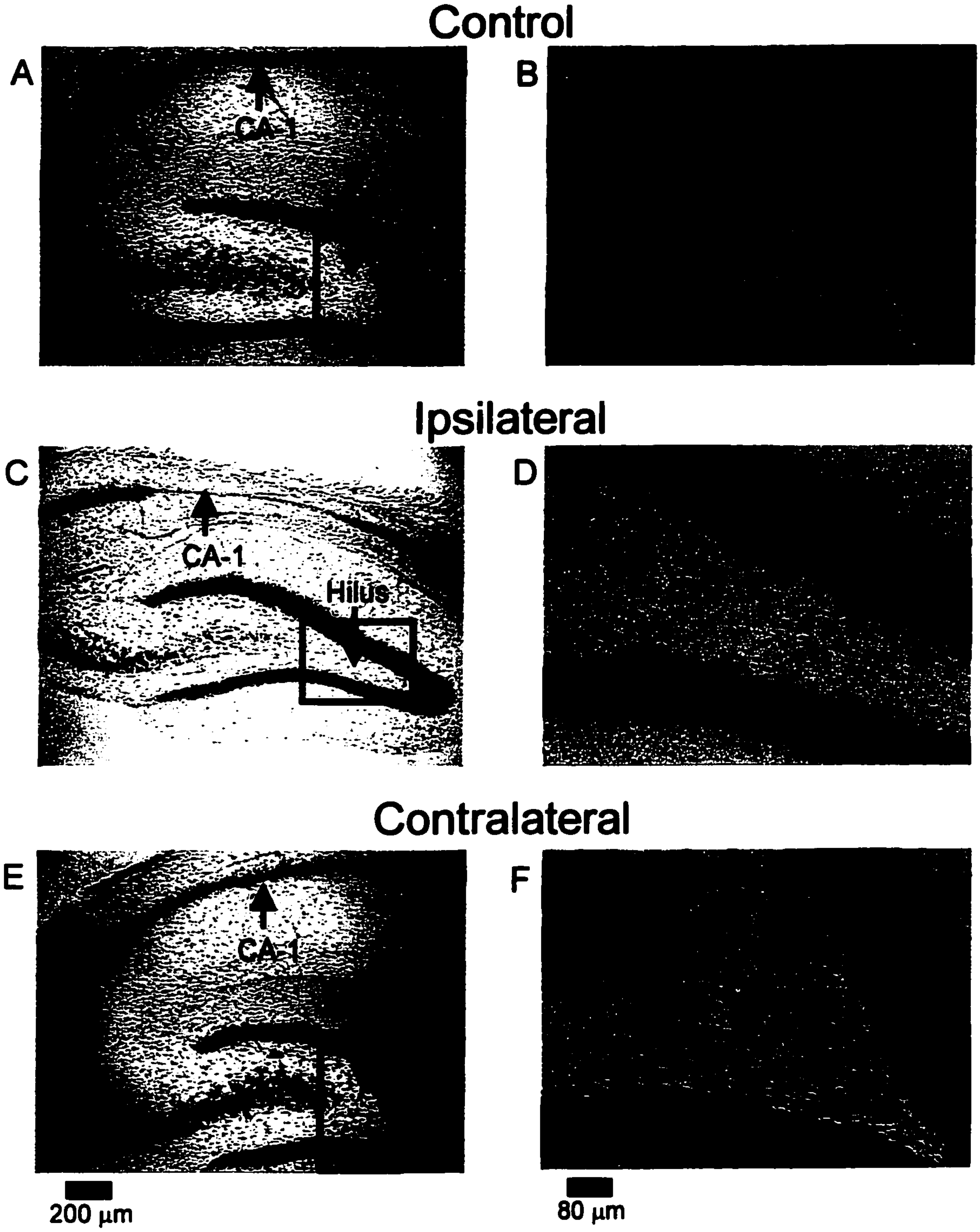
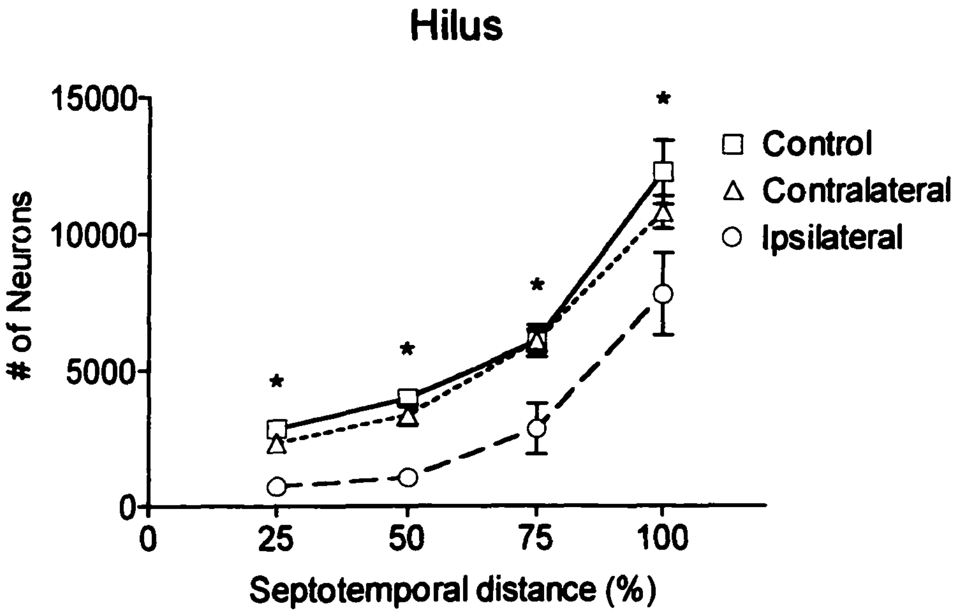

Control
A
CA-1
B
Ipsilateral
C
CA-1
Hilus
D
Contralateral
E
CA-1
F
200 μm
80 μm

Fig. 2

A


Hilus
15000
10000
5000
0
# of Neurons
0
25
50
75
100
Septotemporal distance (%)
*
Control
Contralateral
Ipsilateral

B

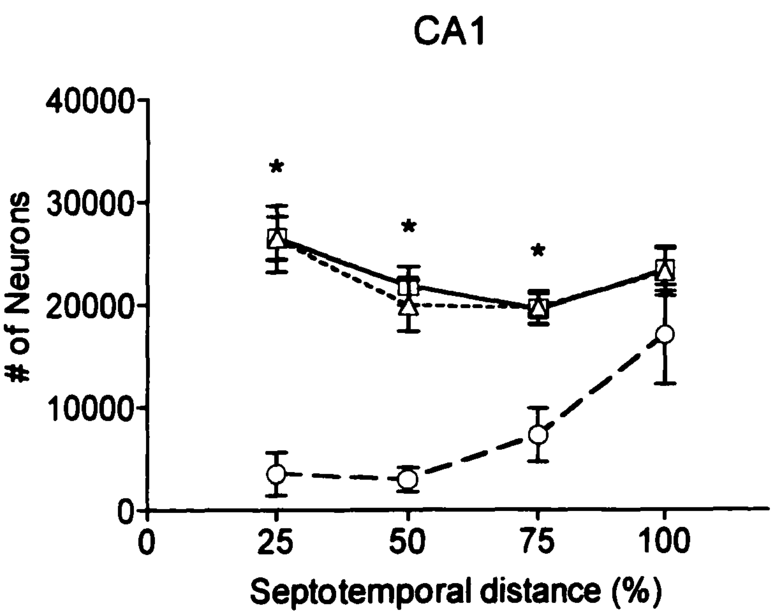

CA1
40000
30000
20000
10000
0
# of Neurons
0
25
50
75
100
Septotemporal distance (%)
*

Figure 1 Hippocampal neuron loss after a H-I injury in the septal hippocampus. The hilus and CA1 of the ipsilateral hippocampus were most susceptible to hypoxic-ischemic injury (panels C and D). Both the contralateral and sham control hippocampi did not demonstrate obvious neuron loss (panels E, F and A, B respectively).

Figure 2 A septotemporal quantitative analysis of neuron loss after a H-I injury. The ipsilateral hippocampus had significant neuron loss in the hilus (fig. 5A) throughout the septotemporal axis compared to both the sham control and the contralateral hippocampus ($p<0.01$. ANOVA, SNK), except at the temporal end (100%) where the ipsilateral hippocampus was significantly different from the sham control ($p<0.05$, ANOVA, SNK), but not the contralateral hippocampus ($p>0.05$, ANOVA, SNK). The contralateral hippocampus did not have significant hilar or CA1 neuron loss compared to the sham control hippocampus ($p>0.05$, ANOVA, SNK). Severe pyramidal cell loss in CA1 occurred in the septal region (25% and 50%), and neuron loss was significantly different ($p<0.001$, ANOVA, SNK) from both the sham control and contralateral hippocampus up to the temporal end (100%) where neuron loss in the ipsilateral hippocampus was not significantly different from either the sham control or the contralateral hippocampus ($p>0.05$, ANOVA, SNK).

Mossy fiber sprouting

Both recorded and whole hippocampi from the ipsilateral and contralateral hemispheres were examined for Timm stain in the IML of the dentate gyrus to assess for the presence of mossy fiber sprouting. A small but significant amount of Timm stain was found in both the ipsilateral and contralateral septal hippocampi from the H-I injured rats as compared to the sham control animals (fig. 3 and 4; $p<0.05$, ANOVA, SNK). The temporal hippocampus did not show an increase in Timm stain in the IML for either the ipsilateral or contralateral hippocampi. This finding is similar to what was shown by Onodera et al., (1990) in the four-vessel occlusion model. Mossy fiber sprouting and hilar neuron loss were weakly negatively correlated with each other (i.e., Timm stain scores increased with a decrease in hilar neurons) in the middle region of the hippocampus (i.e., 50% and 75% septotemporal axis; r^2=-0.41, p=0.0002; r^2= -0.14, p=0.047 respectively; fig. 5B and 5C). Timm staining in the IML in the septal and temporal ends did not correlate with neuron loss (r^2=0.06 and r^2=0.02 respectively, data not shown). Implying that the small amount of mossy fiber sprouting that was induced by H-I injury was related to the amount of hilar neuronal loss that occurred.

Figure 3 Timm stain in the IML was assessed in both the ipsilateral and contralateral hippocampi of the H-I lesioned rats. No Timm stain is seen in the IML of the control hippocampi (A, B). Both the ipsilateral (C, D) and contralateral (E, F) hippocampi from a H-I lesioned rat contained a small amount of Timm stain in the IML (these would be scored a 2 and a 1 respectively by the Tauck and Nadler scale).

Figure 4 Septotemporal analysis of Timm stain in the IML. The 25% region was the septal end and the 100% region was the temporal end, and each data point is an average Timm score. Timm stain in the IML of both the ipsilateral and contralateral hippocampi from lesioned animals was small, but significantly elevated in the septal hippocampus (i.e., the 25% and 50% region of the hippocampus) as compared to the sham controls ($p<0.05$, ANOVA, SNK; Fig. 3A). While Timm stain in the IML of the ipsilateral hippocampi was not significantly elevated from Timm stain in the IML of the contralateral hippocampi from the lesioned animals.

Figure 5 Neuron loss and mossy fiber sprouting. In panel A hilar neuron loss in the ipsilateral hippocampus significantly correlated to CA1 neuron loss in the ipsilateral hippocampus (r^2=0.71, p=0.0001). Hilar neuron loss in the 50% (panel B) and in the 75% region of the hippocampus (panel C) significantly correlated with Timm stain in the IML (r^2=-0.41, p=0.0002; r^2=-0.14, p=0.047 respectively).

Fig. 3

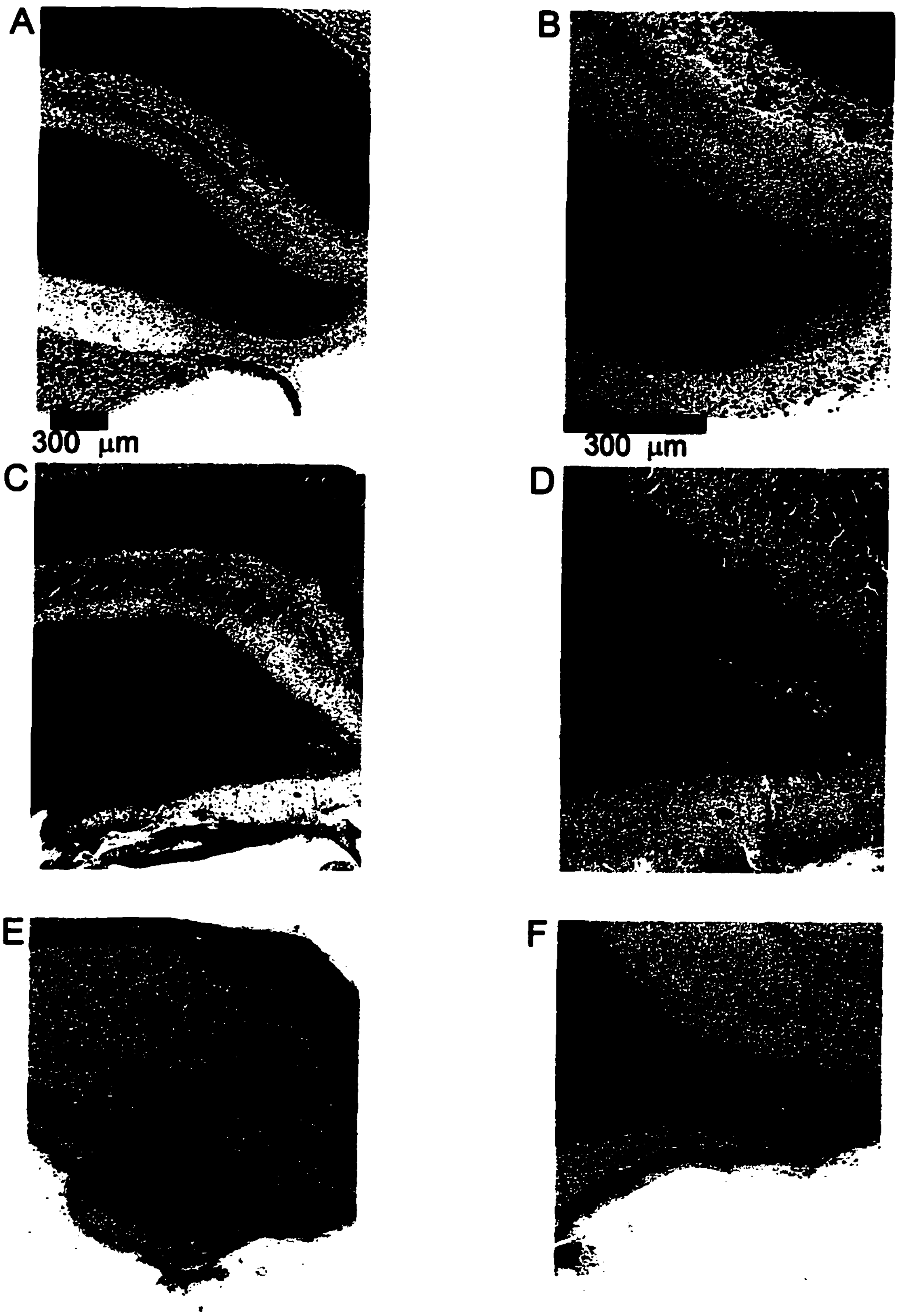


Fig. 4

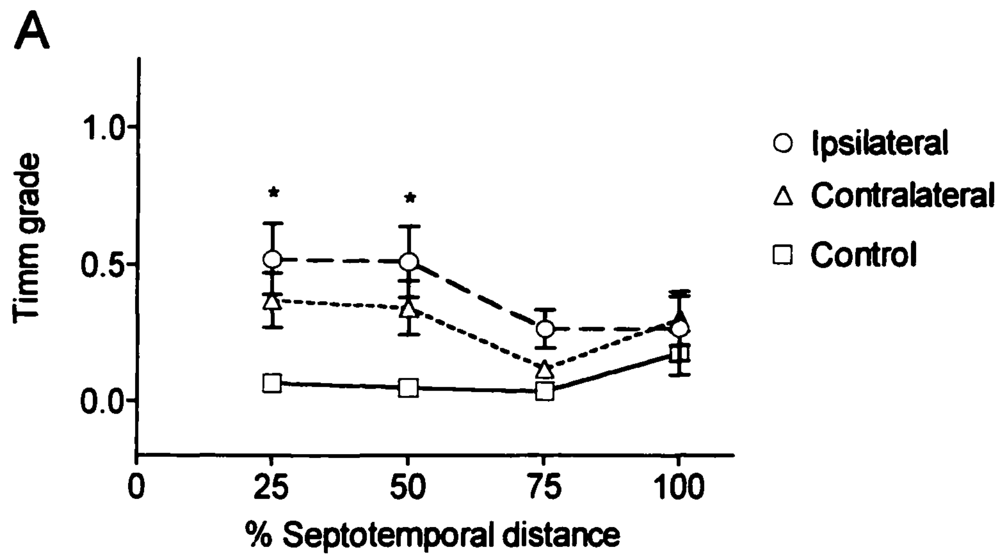
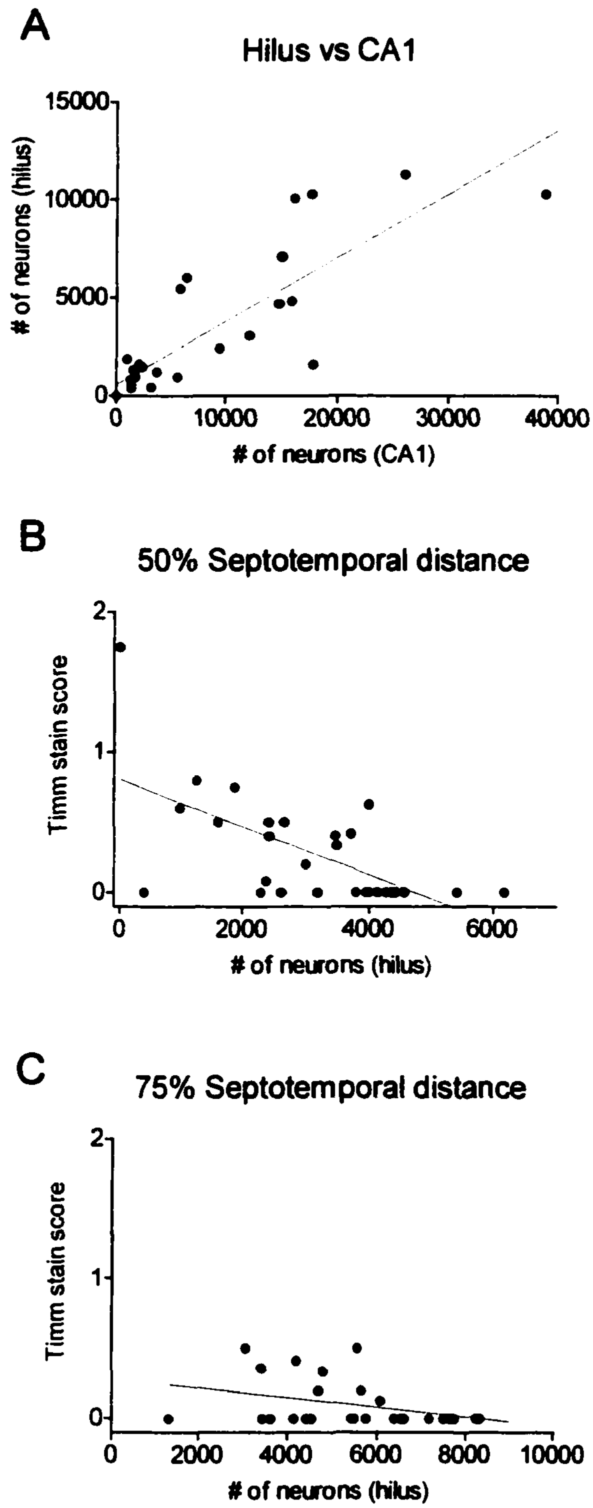

A
Timm grade
1.0
0.5
0.0
*
*
0
25
50
75
100
% Septotemporal distance
Ipsilateral
Contralateral
Control

Fig. 5



A
Hilus vs CA1
15000
10000
5000
0
# of neurons (hilus)
0
10000
20000
30000
40000
# of neurons (CA1)
B
50% Septotemporal distance
2
1
0
Timm stain score
0
2000
4000
6000
# of neurons (hilus)
C
75% Septotemporal distance
2
1
0
Timm stain score
0
2000
4000
6000
8000
10000
# of neurons (hilus)

Extracellular electrophysiology

The septal hippocampi from the H-I injured animals were assessed for electrophysiological evidence of new recurrent excitatory synapses in the granule cells of the dentate gyrus as defined by the presence of all-or-none bursts of population spikes. This was done in hippocampal slices by recording with extracellular electrodes placed into the granule cell layer, while a bipolar stimulating electrode was placed alternatively into the perforant path (orthodromic stimulation) or into the hilus (antidromic stimulation). All-or-none bursts of population spikes were defined as bursts of multiple population spikes with a threshold (fig 7) evoked by antidromic stimulation. Slices that produced multiple population spikes, but had graded response (i.e., as stimulus intensity increased so did the number of population spikes) were considered a control response (see below). All-or-none bursts of population spikes were never seen in the sham control animals (fig. 6E), but graded responses were elicited when control slices were bathed in 6 mM $[K^+]_o$ and 30 μM bicuculline (100% of the control animals), nor were all-or-none bursts of population spikes generated in the ipsilateral or contralateral slices when bathed in control solution (Fig. 6C and 6D) or in 30 μM bicuculline. However, when the slices from the ipsilateral and contralateral hippocampi (fig. 6F) were bathed in 30 μM bicuculline and 6 mM $[K^+]_o$ 80% of the H-I rats had ipsilateral hippocampi that showed all-or-none bursting, and 37% of the lesioned animals had contralateral hippocampi that showed all-or-none bursts of population spikes. The number of rats with that had all-or-none bursting in ipsilateral hippocampi was significantly different from the control animals (p=0.0007, Fisher's exact test). Orthodromic stimulation did not produce any abnormal responses (i.e., bursts of population spikes) in any of the animals (fig. 6A and 6B) in control solution, and orthodromic stimulation was not done when the slices were bathed in 30 μM bicuculline or 6 mM $[K^+]_o$ and 30 μM bicuculline. Paired-pulse stimuli also showed normal inhibitory responses (data not shown) in all the animals tested. In all the slices that demonstrated all-or-none bursting the bursts of population spikes could be blocked by the application of 50 μM AP-5 and 50 μM DNQX (fig. 8B), and after several hours of wash out the response could be recovered (fig. 8C). This data implies that the hippocampi following a H-I injury can have abnormal electrophysiologic responses that look like epileptiform bursts of population spikes, and that these responses primarily occur in the injured hippocampi. These abnormal responses are glutamatergicaly mediated, and imply that new excitatory synapses have formed in the granule cell layer.

Figure 6 Hippocampal slice response in both control solution and in 30 μM bicuculline and 6 mM $[K^+]_o$. Panels A (control) and B (ipsilateral) are extracellular recordings from the granule cell layer with orthodromic stimulus of the perforant path in control solution. Panels C (control) and D (ipsilateral) are antidromic (hilar stimulus) responses from the same recording site in the granule cell layer while bathed in control solution. Panels E (control) and F (ipsilateral) are antidromic responses from the same recording site in 30 μM bicuculline and 6 mM $[K^+]_o$. Note the large burst of population spikes that occurs in the ipsilateral lesioned hippocampus, this bursting was only seen in 30 μM bicuculline and 6 mM $[K^+]_o$. All recordings are averages of 5 responses except for panel F which is a single trace. Stimulus artifacts have been reduced for cosmetic purposes, and the initial response of the burst in panel F has been clipped.

Figure 7 All-or-none bursting in a hippocampal slice from an ipsilateral hippocampus of a lesioned rat. Panel A is a composite of responses to 3 stimuli at 400 μA and 0.2 Hz. Panels B, C, and D are the responses in A. Note the failure, in panel C, for the slice to generate a long burst of population spikes that are seen prior to (panel B) and following (panel D) this burst failure. This recording was made in 30 μM bicuculline and 6 mM $[K^+]_o$. Panel E shows a graded response to hilar stimulation in 30 μM bicuculline and 6 mM $[K^+]_o$. The number of population spikes progressively increases as the stimulus intensity increases from 100 μA to 200 μA to 400 μA.

Figure 8 The network bursts from the ipsilateral lesioned hippocampi were mediated by glutamatergic synaptic responses. Panel A is a burst of population spikes from an ipsilateral lesioned hippocampus in 30 μM bicuculline and 6 mM $[K^+]_o$ with antidromic stimuli. Panel B is the same slice while being bathed in the same solution above with the addition of 50 μM AP-5 and 50 μM DNQX. Note the blockade of the burst of population spikes from panel A. Panel C is the response from the same slice after 120 min of wash, the bursting has returned with the washout of the glutamate antagonists.

Fig. 6

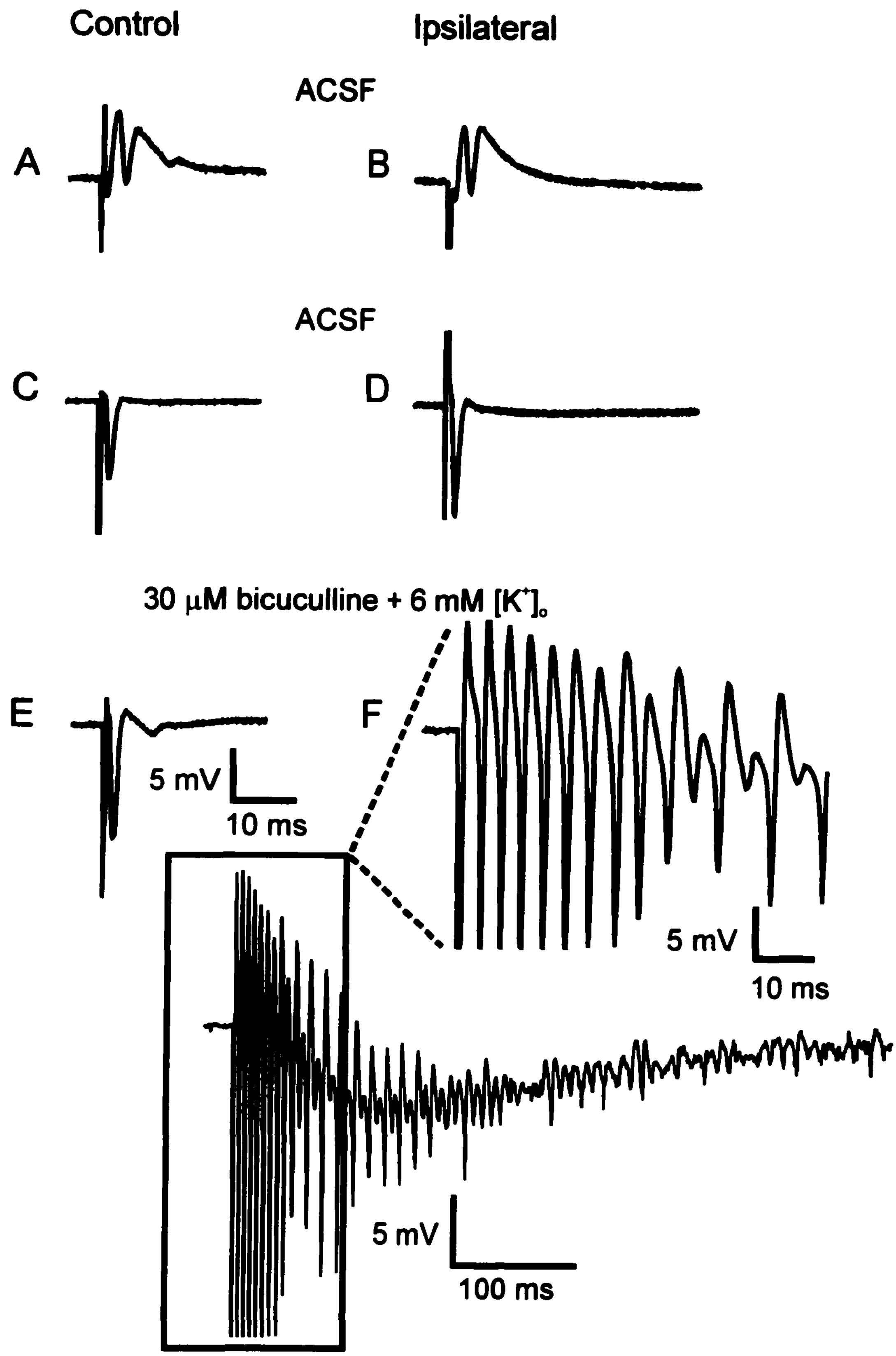

Control
Ipsilateral
ACSF
A
B
ACSF
C
D
30 μM bicuculline + 6 mM [K+]o
E
F
5 mV
10 ms
5 mV
10 ms
5 mV
100 ms

Fig. 7

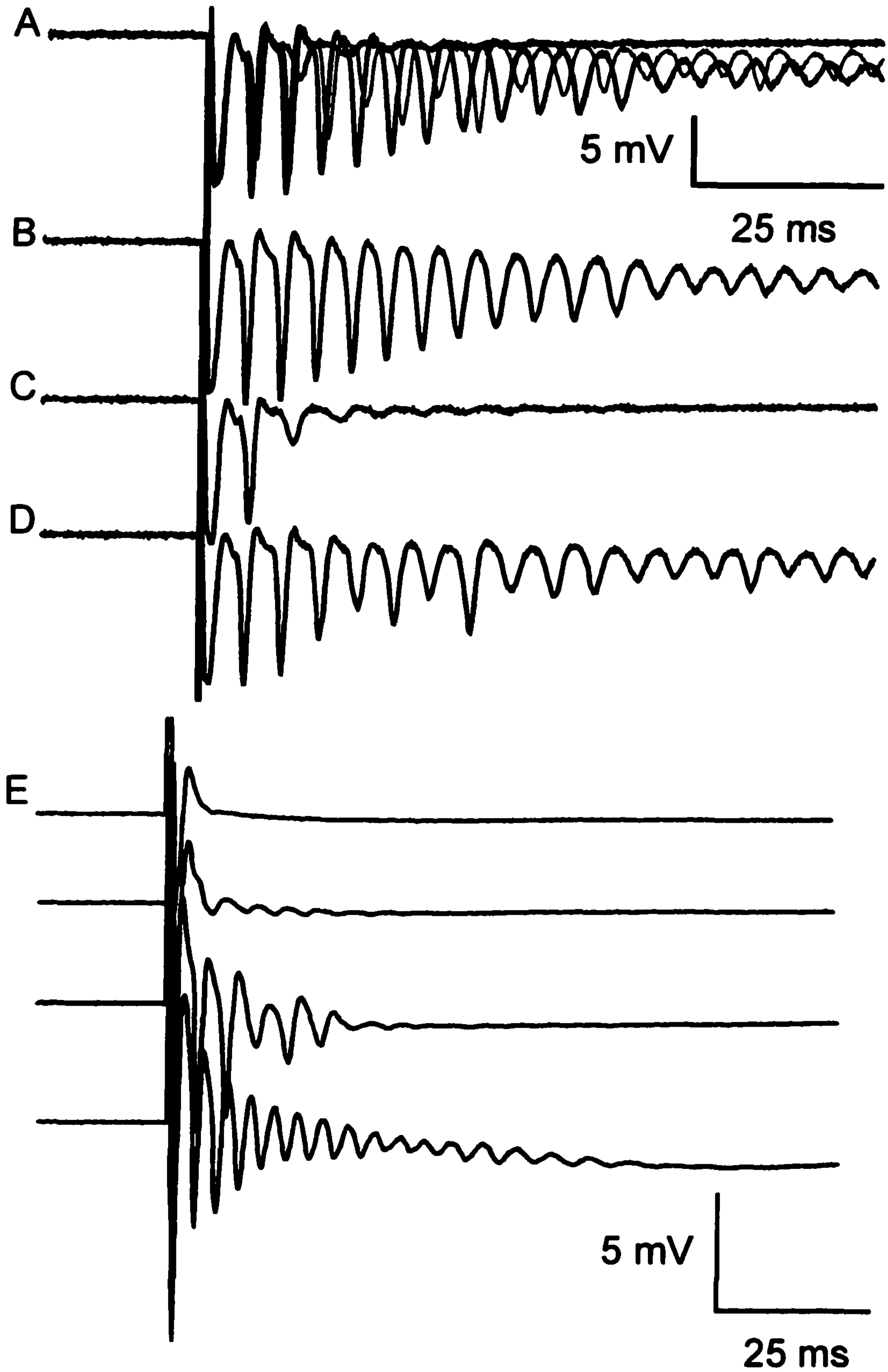
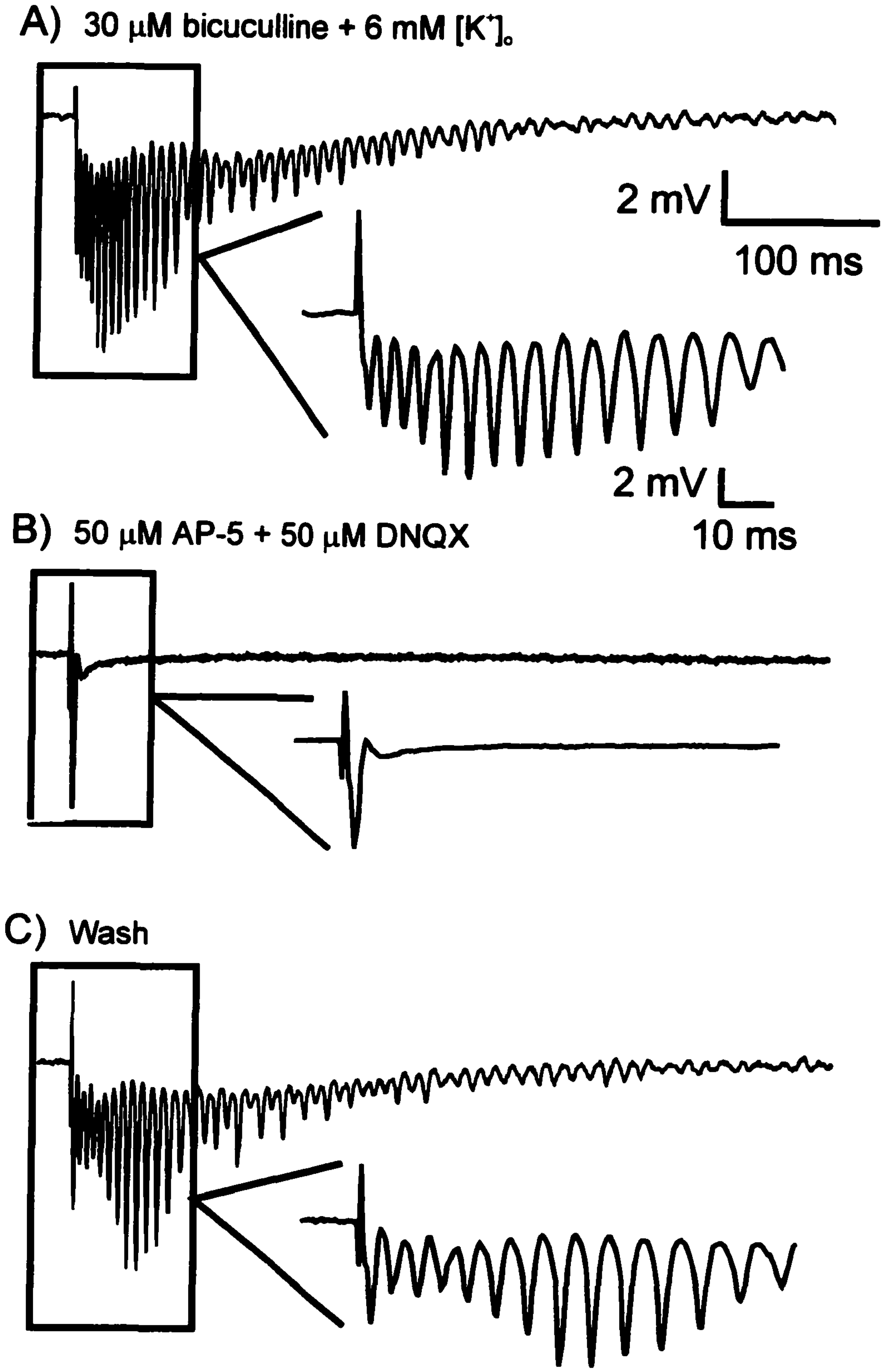


Fig. 8


A) 30 μM bicuculline + 6 mM [K+]o
2 mV
100 ms
2 mV
10 ms
B) 50 μM AP-5 + 50 μM DNQX
C) Wash

Spontaneous motor seizures and Timm stain

Rats were observed in the vivarium for 6-18 months following the H-I injury to detect any subsequent epileptic seizures. Sixteen percent of the H-I injured rats (3 out of 19 of all lesioned animals) developed multiple spontaneous motor seizures after a prolonged and variable latent period (fig. 9A). The H-I injured rats had a low seizure rate (0.0043 seizures/h) when compared to the seizure rate (0.027 seizures/h) of pilocarpine treated rats (a model of temporal lobe epilepsy) from a previous study (Williams et al., submitted*)*. The relative latent period (i.e., the number of days to the first observed seizure based upon 6 h of weekly monitoring) for the H-I rats was very long and variable (285±91 days) when compared to the pilocarpine treated rats from the previous study (49± 8 days). This data indicates that this model of brain injury can induce epilepsy in a small percentage of animals after a long and variable latent period, which is similar to what is seen in humans following a stroke. A further analysis of the Timm stain results consisted of placing the lesioned hippocampi into separate groups depending on whether or not the animals were seen to have spontaneous motor seizures (fig. 9B). The rats that did demonstrate spontaneous motor seizures had significantly more Timm stain in the IML of both the ipsilateral and contralateral hippocampi as compared to the H-I rats with no seizures ($p<0.001$, ANOVA, SNK). The ipsilateral hippocampus from the rats with seizures also had significantly elevated amounts of Timm stain in the IML as compared to the contralateral hippocampi from the animals with seizures ($p<0.05$, ANOVA, SNK).

Fig. 9

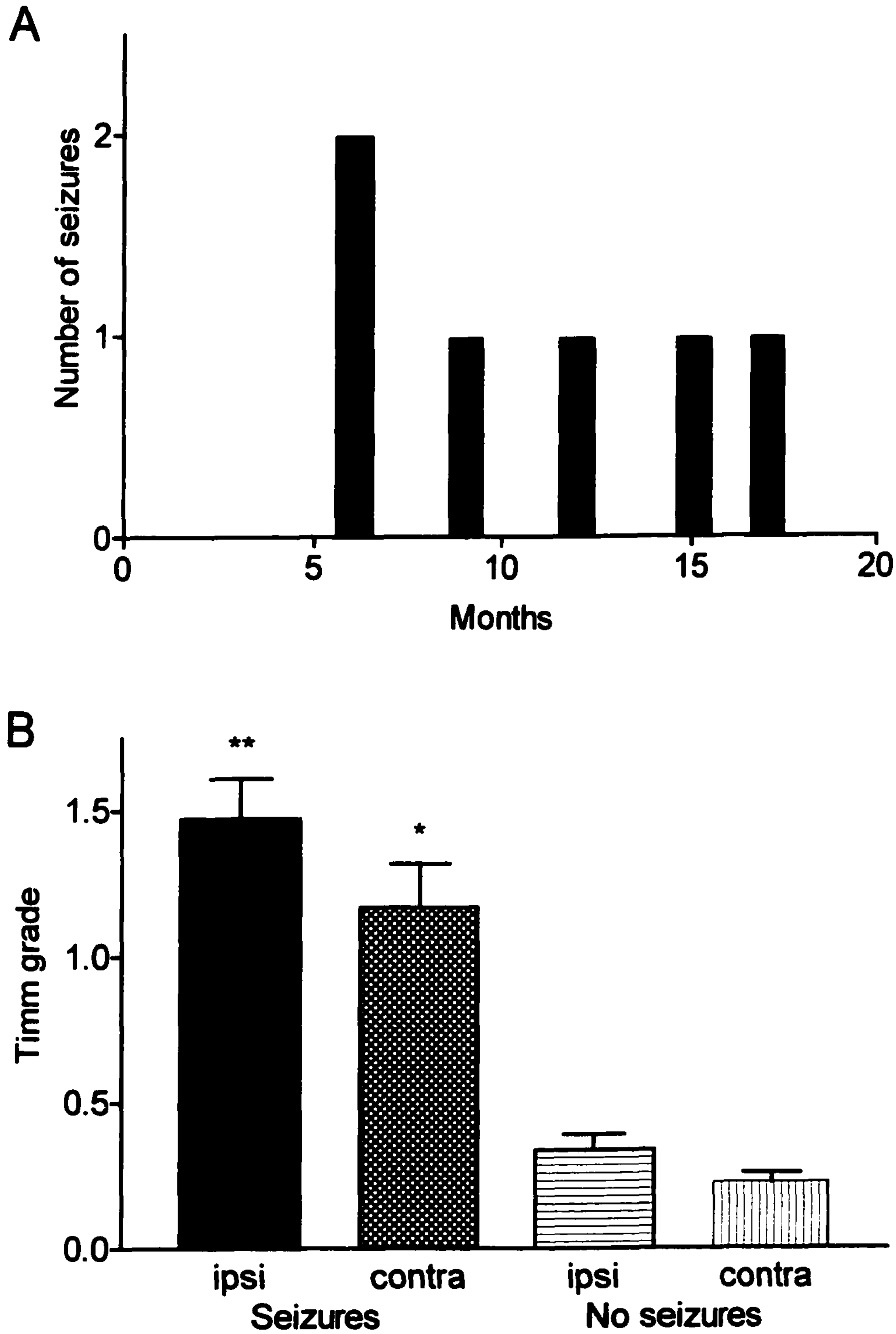

A
Number of seizures
0
1
2
0
5
10
15
20
Months
B
Timm grade
0.0
0.5
1.0
1.5
**
*
ipsi
contra
ipsi
contra
Seizures
No seizures

Figure 9 A) The distribution of seizures by month after the injury for the 3 rats that were seen to have seizures. B) Those animals with motor seizures had significantly more mean Timm stain in the IML of both the ipsilateral and contralateral hippocampi compared to the lesioned rats with no seizures ($p<0.001$, ANOVA, SNK). The ipsilateral hippocampi from the animals with seizures also had a significantly higher amount of mean Timm stain in the IML when compared to the contralateral hippocampi from the rats with seizures ($p<0.05$, ANOVA, SNK).

DISCUSSION

The present study provides evidence that neuron loss in the hippocampus following a unilateral H-I lesion appears to be limited to the ipsilateral hemisphere. This study also shows that a H-I injury involving the hippocampus can cause a small but significant amount of mossy fiber sprouting in the IML of both the ipsilateral and contralateral hemispheres. This study demonstrates that the electrophysiologic properties of the dentate gyrus can be chronically altered and are abnormal under conditions of reduced inhibition and increased extracellular potassium. We also found that a H-I injury to young adult rats can induce epilepsy in a proportion of the population that is similar to what is seen in the human population following a stroke.

The most common cause of late onset epilepsy (i.e., epilepsy that is seen in the elderly) is a stroke. The incidence of epilepsy following a stroke is 2-17% (Pohlman-Eden, 1997; Olsen, 2001). This incidence of seizures following a stroke closely matches what is seen in this model of H-I.

The seizure rate and relative latent period from the H-I rats have been compared to those seen in pilocarpine treated rats (a model of temporal lobe epilepsy) from another study (Williams et al. Submitted) in order to contrast the low seizure rate and long relative latent period in the H-I model to an established model of spontaneous motor seizures. There are some interesting similarities and differences between the H-I and pilocarpine models which may potentially help to explain the differences in seizure rate and relative latent period. The gross lesion in the H-I model appears to be limited to the ipsilateral hemisphere and involves the frontoparietal cortex and hippocampus (Towfighi et al., 1997,1998), while systemic pilocarpine has global effects, with the limbic structures like the hippocampus and amygdala being very sensitive to status epilepticus induced injury (Turski et al., 1984). The injury to the hippocampus is very similar in these two models with CA1 neurons being severely effected. The hilar inter-neurons are also injured, but to a lesser degree than is seen in CA1. The CA3 region appears to be the least susceptible area to both H-I and pilocarpine induced injury (Towfighi et al., 1997; Turski et al., 1984). The hilus contains a heterogenous population of neurons (Amaral, 1978), which are sensitive to ischemia and pilocarpine induced injury. It appears that the mossy cells are injured and lost in global ischemia and in pilocarpine induced status epilepticus (Hsu and Buzsaki, 1993; Scharfman et al., 2001). Somatostatin-like immunoreactive hilar neurons have been shown to be decreased in both global ischemia and in the kainate model of temporal lobe epilepsy (Johansen et al, 1987; Buckmaster and Dudek, 1997). It is unknown how hilar neurons may differ in their susceptibility to either type of injury, which could be a critical factor influencing seizure generation. It is also unknown what effect H-I may have on inhibitory interneurons in other areas of the hippocampus. It has been reported that inhibitory input to the granule cells after global ischemia is normal 3-4 months after the injury (Mody et al., 1995). Thus the hippocampal injury in both models is relatively similar, and it is unknown what differences in specific neuron loss exists between the H-I and pilocarpine models that could potentially account for the differences in seizure rate and relative latent period.

In both the H-I model and in the pilocarpine model, mossy fiber sprouting is abnormally present in the IML of the granule cells (Mello et al., 1993). However, the degree of sprouting is typically much greater in the pilocarpine model than what is seen here in the H-I rats. It is also interesting to note that the H-I rats that did have motor seizures in this study had significantly more sprouting than in the rats that were not seen to have spontaneous motor seizures. It is possible that the presence of mossy fiber sprouting is associated with an increased probability that an animal might experience a spontaneous motor seizure. The mossy fiber sprouting may be a marker for hippocampal injury and altered function, such that as the amount of sprouting increases so does the associated probability of spontaneous motor seizures. No studies to date, including this study (n=3 rats with seizures), have shown a strong correlation between the amount of mossy fiber sprouting in the IML and seizure rate. Though, as in this study, a correlation between hilar neuron loss and mossy fiber sprouting has been demonstrated in the kainate model of temporal lobe epilepsy (Buckmaster and Dudek, 1997). Thus, other factors besides mossy fiber sprouting are likely involved in seizure generation. Examples of other factors might include the formation of new recurrent excitatory circuits in other areas of the brain, and changes in either glutamate or GABA receptors on excitatory and/or inhibitory neurons (Kraus et al., 1994; Shumate et al., 1998; Esclapez et al., 1999; Smith and Dudek, 2001).

In this study both the H-I rats and the pilocarpine-treated animals were observed for 6 h per week until used for slice experiments. This rate of observation covers 4% of all possible time points that a rat may display a seizure. This amount of monitoring probably tends to overestimate the true latent period (i.e. the true latent period is probably shorter than presented here) and underestimates the true seizure rate (i.e., the true seizure rate is probably higher for both groups of rats). This problem may also be compounded by a low true seizure rate in the H-I rats. These data also imply that the percentage of rats seen to have spontaneous motor seizures may be higher than what is represented by our data, nor did we utilize chronic *in vivo* recording techniques in these animals to record electrographic seizures which may also be occurring at a relatively high frequency in a large number of hypoxic-ischemic rats.

A mirror focus occurs when a primary seizure focus generates a secondary focus in the homotopic contralateral cortex. Experimentally this has been induced by creating a focal lesion in one cortical hemisphere, with electrographic recordings from both the lesioned site and its homotopic contralateral site. The observation that seizures initially arise from the focal lesioned site is typically followed over time by seizure activity in the homotopic contralateral site. This seizure activity in the secondary site will continue even after the removal of the primary lesioned focus (see Morrel et al. 1993 for review). It has been hypothesized by Dudek and Spitz (1998) that deafferentation and synaptic reorganization in the homotopic cortex may lead to epileptogenesis of this secondary site. Electrophysiological abnormalities have been demonstrated in the contralateral somatosensory cortex (i.e., diaschisis) of a rat seven days after a photothrombotic lesion to the ipsilateral somatosensory cortex (Buckrehmer-Ratzmann et al., 1996). Hilar neurons, especially the mossy cells, have extensive commissural input to the inner molecular layer of the contralateral hippocampus (Blackstad, 1970; Frotscher et al., 1981; Frotscher, 1983; Seress and Ribak, 1984). Mossy cells are damaged or killed during global ischemia (Hsu and Buzsaki, 1993). In this model of H-I the neuron loss appears to be confined to the ipsilateral hemisphere. However, slight mossy fiber sprouting was found in the contralateral hemisphere where no neuron loss was detected. Thus it is possible that the ipsilateral loss of hilar neurons has led to deafferentation of the contralateral granule cell layer and the subsequent formation of mossy fiber sprouting, which could contribute to the formation of a secondary site of epileptogenesis.

This study found that the dentate gyrus from the ipsilateral hippocampus had all-or-none bursting of population spikes under conditions of decreased inhibition and increased extracellular potassium. However, we did not see large after-discharges of population spikes with a long and variable latency (see Cronin et al., 1992; Patrylo and Dudek, 1998). Another interesting finding associated with a small proportion of the ipsilateral slices was the finding that with the stimulus intensity held constant some of the slices in 30 μM bicuculline and 6 mM $[K^+]_o$ would slowly increase the length of the bursts of population spikes until it would reach a point and discharge a long burst of population spikes (i.e., 1-2 seconds, this data is not shown). Thus, our findings here are similar to what has been shown in hippocampal slices from kainate treated rats under the same recording conditions (i.e., the slices have all-or-none bursting in each model). However, the characteristics of the bursts generated from the H-I rats are different.

These differences could arise from the fact that the hippocampal slices taken from the H-I animals are from the septal hippocampus and the slices recorded in the kainate studies are from the temporal hippocampus. Septal slices were used in the H-I study because the gross lesion produced by H-I injury appeared to be predominately located in the dorsal ipsilateral hemisphere. It should not be assumed that bursting in slices from the temporal hippocampus would be identical to bursting in slices from the septal hippocampus. It has been shown that the septal hippocampus needs significantly more stimuli than the temporal hippocampus to become fully kindled (Racine et al, 1977), and that the frequency of CA3 spontaneous bursting was markedly higher in temporal hippocampal slices as compared to slices from the septal hippocampus (Bragdon et al., 1986). This data would suggest that the temporal end is more supportive of epileptiform activity.

Another potential reason that the bursting characteristics in the H-I rats are different from that seen in kainate-treated animals could be attributed to the weak sprouting seen in the IML of the H-I rats compared to the dense sprouting typically seen in the IML of the kainate treated rats (Patrylo and Dudek, 1998). If the amount of Timm staining in the IML is a reflection of the amount of new excitatory synapses (Wuarin and Dudek, 2001) then you would expect the slices from the kainate-treated rats to show more abnormal network bursting.

The H-I injury could also induce chronic alterations in glutamate receptor sub-unit composition in both NMDA and/or AMPA receptors. It has been shown in both models of epilepsy and global ischemia that the AMPA receptor may chronically change its sub-unit composition rendering the receptor more permeable to calcium (for review see Tanaka et al, 2000). The NMDA receptor sub-unit composition in CA3 may also be chronically altered following kindling (Kraus et al., 1994). Thus, any differential changes between the H-I-treated and kainate-treated rats in glutamate receptor composition, cellular and/or subcellular distribution of receptors could alter the characteristics of the epileptiform activity seen in the H-I ipsilateral slices.

CONCLUSION

This model of H-I brain injury in terms of inducing chronic spontaneous seizures is similar to what is seen in the human population following a stroke. This model also induces mossy fiber sprouting not only in the lesioned hemisphere but also in the unlesioned hemisphere, which could potentially lead to the formation of a secondary site of epileptogenesis. This study also demonstrates that mossy fiber sprouting is a phenomenon that may be common to multiple types of brain injury and may be an important component in the appearance of epilepsy following a stroke.

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Chapter 5

EPILEPSY AND SYNAPTIC REORGANIZATION IN A PERINATAL RAT MODEL OF HYPOXIA-ISCHEMIA

ABSTRACT

One of the potential chronic consequences of perinatal hypoxia-ischemia is the development of epilepsy. Synaptic reorganization in the hippocampus has been associated with epilepsy after an injury. We tested the hypothesis that perinatal hypoxia-ischemia will induce spontaneous motor seizures, synaptic reorganization in the dentate gyrus, and that these new circuits will lead to electrophysiological abnormalities. The right common carotid artery of 7-day-old rats was permanently ligated. The rats were placed for 120 min into a chamber filled with 8% oxygen (37° C). Animals were directly observed for chronic seizure activity for 6-18 months after the lesion. Extracellular and whole-cell patch-clamp recordings were obtained from the granule cell layer of the ipsilateral and contralateral septal hippocampus. The recorded slices were processed for Timm staining. Eight out of twenty rats were seen to have spontaneous motor seizures after the injury. The ipsilateral lesioned hippocampi from both the rats with seizures and the rats not seen to have seizures had increased amounts of Timm stain in the inner molecular layer (IML) compared to controls. The contralateral hippocampi from the rats with seizures, but not the hippocampi from the rats not seen to have seizures, had significantly increased amounts of Timm stain in the IML compared to controls. The dentate gyrus from ipsilateral hippocampal slices displayed all-or-none bursts of population spikes with antidromic stimulation, when bathed in 30 μM bicuculline and 6 mM $[K^+]_o$. Caged-glutamate flash-photolysis with whole-cell patch-clamp reveled a significant increase in granule cell-to-granule-cell interactions in the ipsilateral hippocampus. These results

suggest that perinatal hypoxia-ischemia can induce epilepsy and synaptic reorganization in the lesioned and non-lesioned hippocampus, and these new circuits can predispose the hippocampus to abnormal electrophysiological activity.

INTRODUCTION

Perinatal hypoxia-ischemia is the most common cause of cerebral palsy, and an important chronic consequence that can affect the quality of life for patients with cerebral palsy is the development of epilepsy. Epilepsy occurs in 15 to 60% of children with cerebral palsy (Hadjipanayis et al., 1997; Kwong et al., 1998; Mieszczanek, 2000). Cerebral palsy is a known risk factor for causing complex partial seizures (Rocca et al., 1987), and can also cause focal seizures, myoclonic seizures and generalized tonic-clonic seizures (Aneja et al., 2001). A relatively high proportion of the epilepsies associated with cerebral palsy are resistant to anti-epileptic pharmacotherapy (Gaggero et al., 2001).

One of the central features of human temporal-lobe epilepsy (which commonly manifests as complex partial seizures) is hippocampal sclerosis, where neurons in the hilus and in the CA3 and/or CA1 areas have died (Babb and Brown, 1987; Franck and Roberts, 1990). In the rat four-vessel-occlusion model of forebrain ischemia, the young-adult rat hypoxic-ischemic model, and in the human hippocampus after an ischemic injury, a qualitatively similar pattern of hippocampal neuronal death occurs, the CA1 area is the most susceptible neuronal population, followed by the hilus and then CA3 (see Pulsinelli and Brierly, 1979; Pulsinelli et al., 1982; Petito et al., 1987; Onodera et al., 1990; Franck, 1993; Towfighi et al, 1997, 1998). The pattern of neuronal loss in the perinatal rat model of hypoxia-ischemia is substantially different than that seen in the four-vessel occlusion model in adult rats and in 30-day hypoxic-ischemic rats (Towfighi et al., 1997), where CA3 is the most susceptible population followed by the granule cell layer, then CA1 and the hilus. Thus, the pattern of neuronal loss is dependent on the stage of development at the time of injury.

Treatment of rats with kainate or pilocarpine induces status epilepticus and leads to a pattern of hippocampal neurodegeneration very similar to what is observed in human temporal-lobe epilepsy (Nadler, 1981; Turski et al., 1993,1994). In both the kainate and pilocarpine models (e.g., Nadler et al., 1980, Mello et al., 1993) and in human temporal-lobe epilepsy, the Timm stain and other anatomical techniques have shown that the mossy fibers of the dentate granule cells appear to aberrantly innervate the inner molecular layer (IML) of the dentate gyrus. These new synaptic connections appear to be recurrent excitatory synapses and may be a source of an abnormal positive feedback loop. It is generally thought from both in vivo and in vitro studies that the dentate granule cells serve as a "gate" that prevents propagation of seizure activity into the hippocampus (Lothman et al., 1992), and that under certain physiologic conditions this gating function may breakdown and these new recurrent excitatory circuits may be a major factor in allowing seizure propagation through the dentate gyrus. Onodera et al, (1990) showed that the four-vessel occlusion model of global forebrain ischemia can induce a small amount of Timm stain in the IML. The finding that an ischemic injury could cause hippocampal synaptic reorganization led us to hypothesize that hypoxia-ischemia in the perinatal rat may also cause similar changes.

Tauck and Nadler (1985) advanced the hypothesis that enhanced recurrent excitation could underlie epileptogenesis when they showed that mossy fiber sprouting in kainate-treated rats was associated with paired-pulse potentiation and multiple population spikes to hilar stimulation, which would be expected to activate exclusively the mossy fiber axons of the dentate granule cells. Subsequent experiments (Cronin et al., 1992) showed that the responses to antidromic and orthodromic stimulation were relatively normal in most hippocampal slice preparations from kainate-treated rats; however, when $GABA_A$-mediated inhibition was blocked with bicuculline, some preparations with mossy fiber sprouting showed bursts to hilar stimulation. Some of these bursts had long and variable latencies when evoked at low stimulus intensities, a characteristic expected of circuits with recurrent excitation (Traub and Wong, 1982; Miles and Wong, 1986,1987; Christian and Dudek; 1988a,b). Sloviter (1992) recorded from kainate-treated rats in *in vivo* preparations and found that the dentate granule cells were hyperexcitable to perforant path stimulation 2-7 days after kainate treatment, but that perforant path responses were relatively normal 2 months later after sprouting had occurred. On the basis of these electrophysiological results, and anatomical observations suggesting mossy fiber collaterals synapse onto basket cells, Sloviter proposed that mossy fiber sprouting leads to enhanced recurrent inhibition and is primarily restorative in nature. Mody et al. (1995) reported that inhibition was intact 3 months after 15 min of global ischemia, and that hyperexcitability was not present. If synaptic reorganization occurs in the perinatal model of hypoxia-ischemia, and it is similar to the kainate model, then it may be necessary to block or depress inhibition to reveal these new recurrent excitatory circuits.

A "mirror focus" occurs when a primary seizure focus generates a secondary focus in the homotopic contralateral cortex (see Morrel et al. 1993 for review). One hypothesis holds that neuron loss in the ipsilateral cortex may led to deafferentation and subsequent synaptic reorganization in the homotopic cortex, which may be a major factor in the epileptogenesis of this secondary site (Dudek and Spitz, 1997). Electrophysiological abnormalities have been demonstrated in the contralateral somatosensory cortex (i.e., diaschisis) of rats seven days after a photothrombotic lesion to the ipsilateral somatosensory cortex (Buckrehmer-Ratzmann et al., 1996). Hilar neurons have extensive commissural input to the inner molecular layer of the contralateral hippocampus (Blackstad, 1970; Frotscher et al., 1981; Frotscher, 1983; Seress and Ribak, 1984). Many of these hilar neurons are damaged or killed during an ischemic or hypoxic-ischemic episode in 30-day-old rats (Hsu and Buzsaki, 1993). Thus it was hypothesized that an ipsilateral lesion that damages the hilus would potentially lead to mossy fiber sprouting in the contralateral hippocampus, but this may not necessarily be the case in the 7-day old model of hypoxia-ischemia in which the hilus may be relatively preserved (Towfighi et al., 1997).

To date it has been difficult to create perinatal models of temporal lobe epilepsy that demonstrate most of the features associated with human temporal lobe epilepsy. The models that utilize perinatal rats (hypoxia, febrile seizures, tetanus toxin, and daily injections of flurothyl) do not appear to cause neuronal loss or persistent spontaneous motor seizures, and only minimal to mild changes to the Timm stain in the IML have been identified in these models (Jensen, 1995;

Lee et al., 1995; Holmes et al., 1998; Toth et al., 1998; Huang et al., 1999; Dube et al., 2000). To different degrees these models demonstrate reduced seizure threshold, electrographic seizures, and impaired spatial memory. Models that have utilized kainic acid in perinatal rats have been unsuccessful in creating a epileptic rats with spontaneous seizures and hippocampal sclerosis (Sperber et al., 1991). As the animals mature, however, they become more sensitive to the effects of status epilepticus (Sankar et al., 2000). In contrast to the studies cited above, Romijn et al. (1994) showed that a hypoxic-ischemic injury in 12-13 day old rats induced spontaneous electrographic abnormalities in animals with severe lesions, although the threshold for electrically induced seizures was actually increased in these rats.

The goal of this study was to test the hypothesis that a hypoxic-ischemic injury at 7 days of age would induce epilepsy and synaptic reorganization in the hippocampus, and that these new circuits would lead to abnormal epileptiform activity in the dentate gyrus. We used Timm stain analysis of the IML to address the first portion of this hypothesis. The second part of the hypothesis was examined with in vitro extracellular and whole-cell patch-clamp recordings in hippocampal slices. We found that a moderate percentage of these animals went on to develop spontaneous motor seizures, in animals with hypoxic-ischemic injury and neuronal loss, Timm stain in the IML of both the ipsilateral and contralateral hippocampus was significantly increased above sham control rats. Those animals that displayed spontaneous motor seizures had significantly increased Timm stain in the IML in both the ipsilateral and contralateral hippocampus compared to the lesioned rats that were not seen to have seizures. The ipsilateral hippocampus from the hypoxic-ischemic rats showed abnormal electrophysiologic responses. These results suggest that the injury induced by hypoxia-ischemia is associated with synaptic reorganization in the lesioned hippocampus, and these new circuits can predispose the hippocampus to abnormal electrophysiological activity. They also suggest that increased amounts of sprouting in the IML of the dentate gyrus is associated with and is a marker for an increase in the probability of spontaneous motor seizures, and that the contralateral hippocampus may have synaptic reorganization with out obvious neuronal loss. The results from this study and from previous studies on rat models of temporal lobe epilepsy suggest that the formation of new recurrent excitatory circuits is a potentially common phenomena in the dentate gyrus.

MATERIALS AND METHODS

Hypoxic-ischemic treatment

A modified version of the Levine preparation, as described by Rice and coworkers (1980), was used to create a hypoxic-ischemic injury in perinatal rats. Male and female Sprague-Dawley rats 7 days of age were anesthetized using a 2% isoflurane/oxygen mixture. The ventral midline of the neck was surgically prepared and infused with bupivicaine (0.5%, 0.5 ml). A 1-cm ventral midline incision was made, and the right common carotid artery exposed and double ligated with 4-0 dexon. The skin was closed with 4-0 dermalon, and the rats allowed to recover in a heated cage with the dam. Sham controls had the carotid artery exposed but not ligated. After 2 hr of recovery, the ligated rats were placed in an airtight chamber where the temperature and humidity were maintained at 37^0 C and 90% respectively. The chamber was then filled with 8% oxygen and 92% nitrogen. The oxygen content of the chamber was monitored with an oxygen sensitive electrode (Microelectrode, Inc). The rats were exposed to 8% oxygen for 120 min, and allowed to recover in room air. The rats were placed in the vivarium for chronic seizure observation.

Seizure observation

A trained observer, who was blind to the animals treatment, directly monitored rats for an average of 6 h per week until the rats were used for electrophysiological experiments. Only grade 3 seizures or higher in the Racine scale (1972) were recorded.

Slice preparation and in vitro electrophysiological studies

Rats were deeply anesthetized (sodium pentobarbital 100 mg/kg I.P. or 4% halothane) and euthanized by decapitation. The brain was rapidly removed and stored for 1 min in ice-cold physiological solution (composition in mM: 124 NaCl, 3 KCl, 1.3 $CaCl_2$, 26 $NaHCO_3$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 11 glucose equilibrated with 95% O_2, 5% CO_2, pH 7.2-7.4). The brain was bisected along the mid-line, and the occipital pole was blocked and glued to the chuck of a vibroslicer in order to cut 400 μm-thick coronal slices of the hippocampus. The slices were kept in a storage chamber for at least 1.5 h prior to recording. The opposite hippocampus was used for anatomical studies. Slices were studied electrophysiologically in recording chambers that allowed multiple stimulating and recording electrodes.

Histology

A modified Timm histological procedure was used to label the zinc-containing axons of the granule cells. The hippocampus that was not used for *in vitro* electrophysiological experiments was dissected from the hemisphere and prefixed in phosphate buffered 0.37% Na_2S to precipitate the zinc in the mossy fibers. The hippocampus was immersion fixed in phosphate buffered 4% paraformaldehyde (pH=7.2). This same procedure was done on the recorded slices. The tissue was then saturated with 30% sucrose and sectioned at 35 μm on a sliding microtome. The hippocampus was straightened prior to mounting on the microtome (Buckmaster and Dudek, 1997). Every sixth section was mounted for Timm stain and cresyl violet counter stain. All sections from the recorded slices were mounted and processed for Timm stain analysis. The sections were coded and the intensity of the Timm stain in the IML was graded with blind procedures according to the rating scale of Tauck and Nadler (1985). An average and median score was obtained for the whole hippocampus and the recorded slices and grouped according to treatment and/or ipsilateral versus contralateral. A Wilcoxan ranked sum test was used to test for differences from a theoretical mean of zero.

Extracellular Recordings

Extracellular electrodes were filled with 1 M NaCl (2-20 MΩ). The recording pipette was placed into the granule cell layer, and the bipolar stimulating electrode (platinum/iridium teflon coated; SIU-SC100, Winston Electronics, Millbrae, CA; Grass S88, Quincy, MA) was placed in the hilus to evoke population spikes. Recordings were initially obtained in control solution (ACSF) and amplified with a DC-coupled axoprobe 1A (Axon Instruments, Foster City, CA) at a gain of 100x. Three stimulus intensities based upon the minimum current needed to evoke a 0.5 mV population spike (minimum, 2X-minimum, 4X-minimum) were used to assess population spike responses. Slices that could not produce population spikes, of 5 mV or greater, with hilar stimulation were not included in the analysis. The hippocampal slices were then bathed in 30 μM bicuculline, and the stimulation protocol was repeated as was established in the control solution. While still in 30 μM bicuculline, the extracellular potassium was raised from 3 mM to 6 mM, and the stimulation protocol was again repeated. Hippocampal slices that produced a graded response to hilar stimulation were not included in the analysis. A graded response was defined as; the number of population spikes increased as the stimulus intensity was increased, and no threshold for the generation of multiple population spikes could be determined. The glutamate receptor antagonists 2-amino-5-phosphonovaleric acid (AP-5, Sigma) 50 μM and 6,7-dinitroquinoxaline-2,3,(1H,4H)-dione (DNQX, Sigma) 50 μM, were used to assess the role of glutamate in the generation of population spike bursts. After the completion of the recordings, the slices were individually fixed for Timm stain analysis.

Whole-cell patch clamp recordings

Two to four 300 μm-thick slices were kept for 1-2 h in oxygenated ACSF at 32 °C before being transferred to the recording chamber, recordings were obtained at room temperature (21-23° C). Data were not analyzed if the resting membrane potential was less than –70 mV when whole-cell access was obtained. Perfusion solution (10 ml) containing caged glutamate (L-glutamic acid, γ-(α-carboxy-2-nitrobenzyl) ester (250 μM), Molecular Probes, Eugene OR) was oxygenated and recirculated at 2 ml/min. Pipettes for whole-cell recordings were made from borosilicate glass capillaries (KG-33, Garner Glass, Claremont, CA) with a horizontal pipette puller (P-87 Flaming-Brown pipette puller, Sutter Intruments, Novato, CA) to an open resistance of 2-4 MΩ. Pipette solution contained (in mM) 140 K-gluconate, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 4 magnesium ATP, pH 7.2. Whole-cell currents were amplified with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA), filtered (2 kHz), digitized (44 kHz) and stored on video tapes (Neuro-Corder, Neurodata Instruments, New York, NY). Data analysis was done off line with sampling rates 5-10 kHz (pClamp 6, Axon Instruments).

Flash photolysis of caged glutamate

Uncaging of glutamate (Callaway and Katz, 1993) was obtained with a xenon flash lamp (Chadwick-Helmuth, El Monte, CA). The flash of U.V. light was transmitted through a high-numerical-aperture, oil-immersion objective (X40, Nikon) mounted underneath the bottom of the recording chamber (coverslip) and focused approximately 200 μm into the tissue. An HeNe laser aimed directly through the objective into the tissue was used to determine the location of the photostimulation. A monochrome CCD camera (Cohu, San Diego, CA) and video monitor were used to determine the location of, and the distance between, the recorded cells and the spot of laser light. Flashes with intensities 50-100 mJ combined to a concentration of caged glutamate of 250 μM, produced a spatial resolution of approximately 100 μm. Photostimulations were applied in the granule cell layer at sites 200 μm apart.

RESULTS

Spontaneous motor seizures

Rats were observed for 6-18 months following the hypoxic-ischemic injury to assess subsequent motor seizures. Almost half of the hypoxic-ischemic injured rats (n=8; 40% or 8 out of 20 of all hypoxic-ischemic animals, Fig. 1) developed multiple spontaneous motor seizures after a prolonged and variable latent period (fig. 2A and 2B). The observed motor seizures were typical P3, P4 or P5 as described by the Racine scale (1972). The relative latent period (i.e., the days to the first observed seizure based upon 6 h of weekly monitoring) for the hypoxic-ischemic rats was long and variable (194±43 days) when compared to pilocarpine treated rats (a model of temporal lobe epilepsy) from a previous study (49± 8 days, Williams et al. 2001, submitted). The hypoxic-ischemic injured rats had a moderately low seizure rate (0.012±0.0035 seizures/h) when compared to the seizure rate (0.027±0.0081 seizures/h) of the pilocarpine treated rats from the previous study. This data indicates that this model of brain injury can induce epilepsy in a number of animals after a long and variable latent period.

Fig. 1

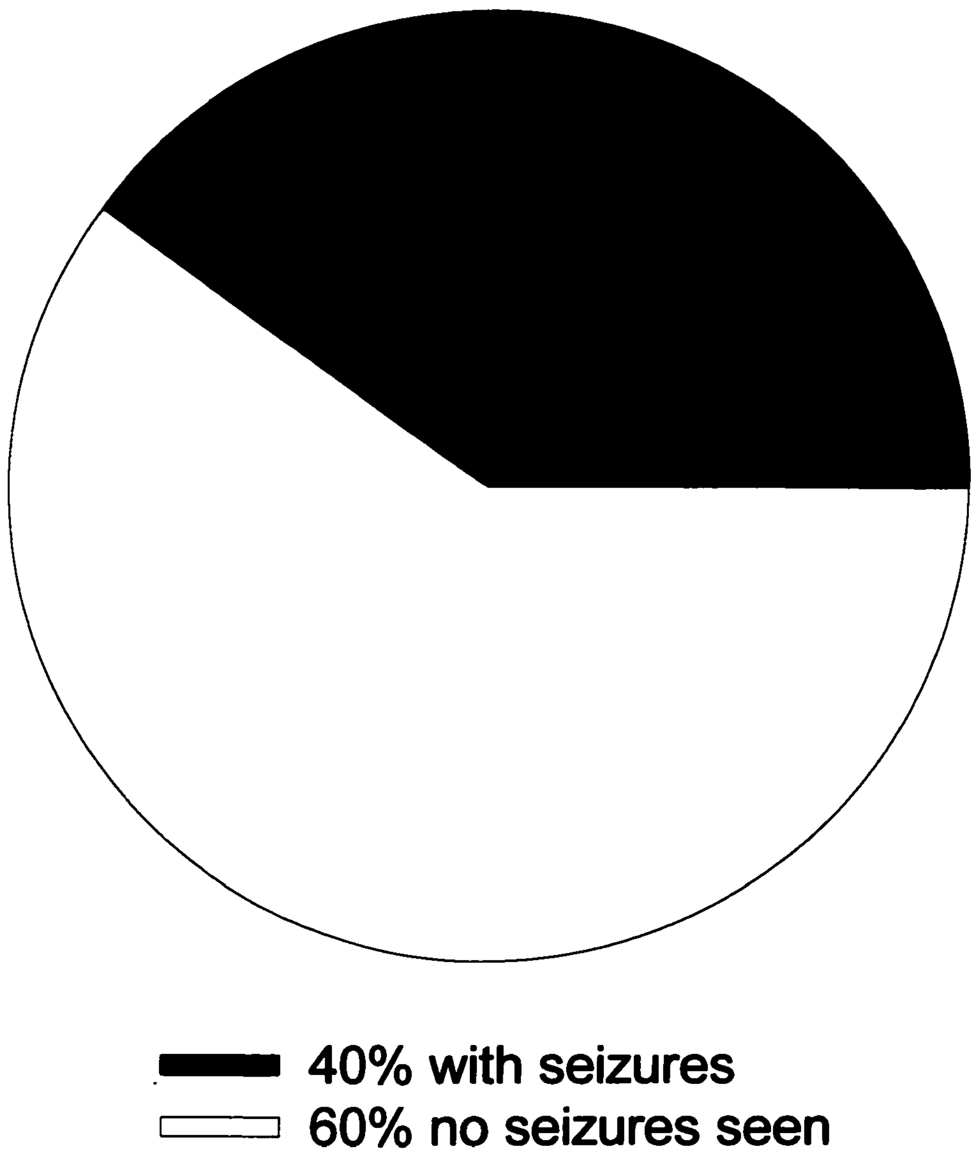
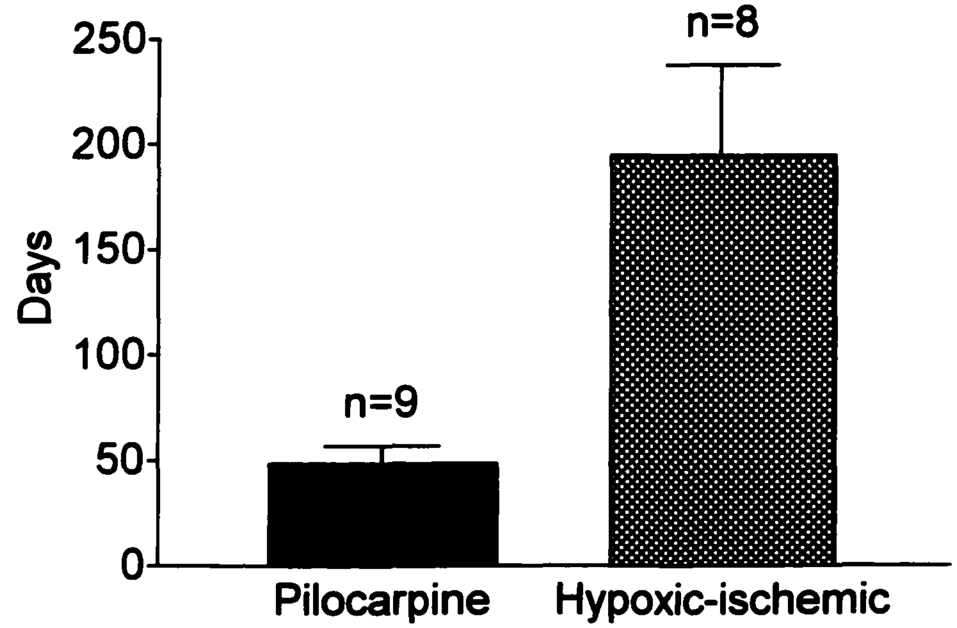


Fig. 2

A


n=8
n=9
250
200
150
100
50
0
Days
Pilocarpine
Hypoxic-ischemic

B

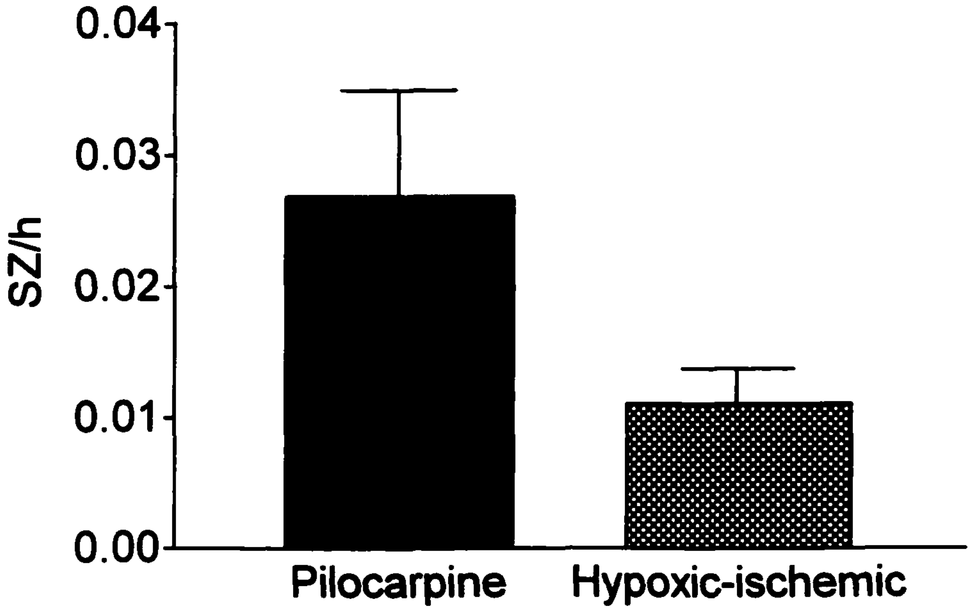

0.04
0.03
0.02
0.01
0.00
SZ/h
Pilocarpine
Hypoxic-ischemic

Figure 1 The percentage of rats that showed spontaneous motor seizures. In a population of twenty rats that were exposed to a hypoxic-ischemic injury 40% of the rats showed 1 or more spontaneous motor seizures (8 out of 20), and 5 out of the 8 rats had 3 or greater spontaneous motor seizures. Only seizures that were rated a 3 or greater as per Racine (1972) were recorded.

Figure 2 The relative latent period and seizure rate of the hypoxic-ischemic rats as compared to adult-pilocarpine-treated rats (from a previous study). Panel 1A shows the relative latent period (the number of days to the first observed seizure based upon 6 h of weekly observation). The average days to the first observed seizure was 195±43 for the hypoxic-ischemic animals and 49±8 for the pilocarpine treated rats. Thus the hypoxic-ischemic animals had a substantially longer relative latent period compared to the pilocarpine-treated animals. Panel 1B shows the seizure rate, which was also based upon 6 h of weekly observation. The seizure rate for the hypoxic-ischemic rats was 0.012±0.0035 (seizures per hour) and for the pilocarpine-treated rats the seizure rate was 0.027±0.008 (seizures per hour). The pilocarpine-treated rats seizure rates were two times higher than the hypoxic-ischemic rats.

Mossy Fiber Sprouting

Both recorded and whole hippocampi from the ipsilateral and contralateral hemispheres were examined for Timm stain in the IML of the dentate gyrus to assess for the presence of mossy fiber sprouting. A significant amount of Timm stain in the IML was found in both the ipsilateral hippocampi from rats that were seen to have seizures and from rats that were not seen to have seizures. The contralateral hippocampi from the hypoxic-ischemic injured rats that were seen to have seizures, also had a significant amount of Timm stain in the IML as compared to a theoretical median of 0 (fig. 3 and 4; $p<0.05$, Wilcoxan rank sum). The sham control animals and the contralateral hippocampi from rats that were not seen to have seizures were not significantly different from a theoretical median of 0. This data indicates that a hypoxic-ischemic injury at 7-days of age can induce abnormal amounts of Timm stain in the IML of both the ipsilateral lesioned and contralateral unlesioned hippocampus. This data also implies a strong association between the presence of spontaneous motor seizures and Timm stain in the IML of both the ipsilateral and contralateral hippocampi.

Figure 3 Timm stain in the IML of the dentate gyrus. Panels A and B show Timm stain from a control rat. Note the absence of Timm stain in the IML in this hippocampal section, which was scored a 0 on the Tauck and Nadler scale (1985). Panels C and D demonstrate Timm staining in the IML in an ipsilateral hippocampus from a hypoxic-ischemic rat that was seen to have spontaneous motor seizures. Note the dense band of Timm stain in the IML along the inner and outer blades, but not the apex (arrow) this hippocampal section was scored a 2 on the Tauck and Nadler scale. Panels E and F show Timm staining in the IML in the contralateral hippocampus from the same rat as shown in panels C and D. This hippocampal section was scored a 1 on the Tauck and Nadler scale (arrow is pointing to Timm stain in the IML).

Figure 4 The average Timm score from the hypoxic-ischemic rats. In both the control and contralateral hippocampi from rats that were not seen to have seizures the average and median Timm score was 0. The ipsilateral hippocampi from both the rats seen to have spontaneous motor seizures and not seen to have seizures had a significant amount of Timm stain the IML (the median score was 2 and 1 respectively, $p<0.05$, Wilcoxan rank sum test). The Timm score for the contralateral hippocampi of rats seen to have seizures was also significantly higher than the sham control rats (median score=1, $p<0.05$, Wilcoxan rank sum test).

Fig. 3

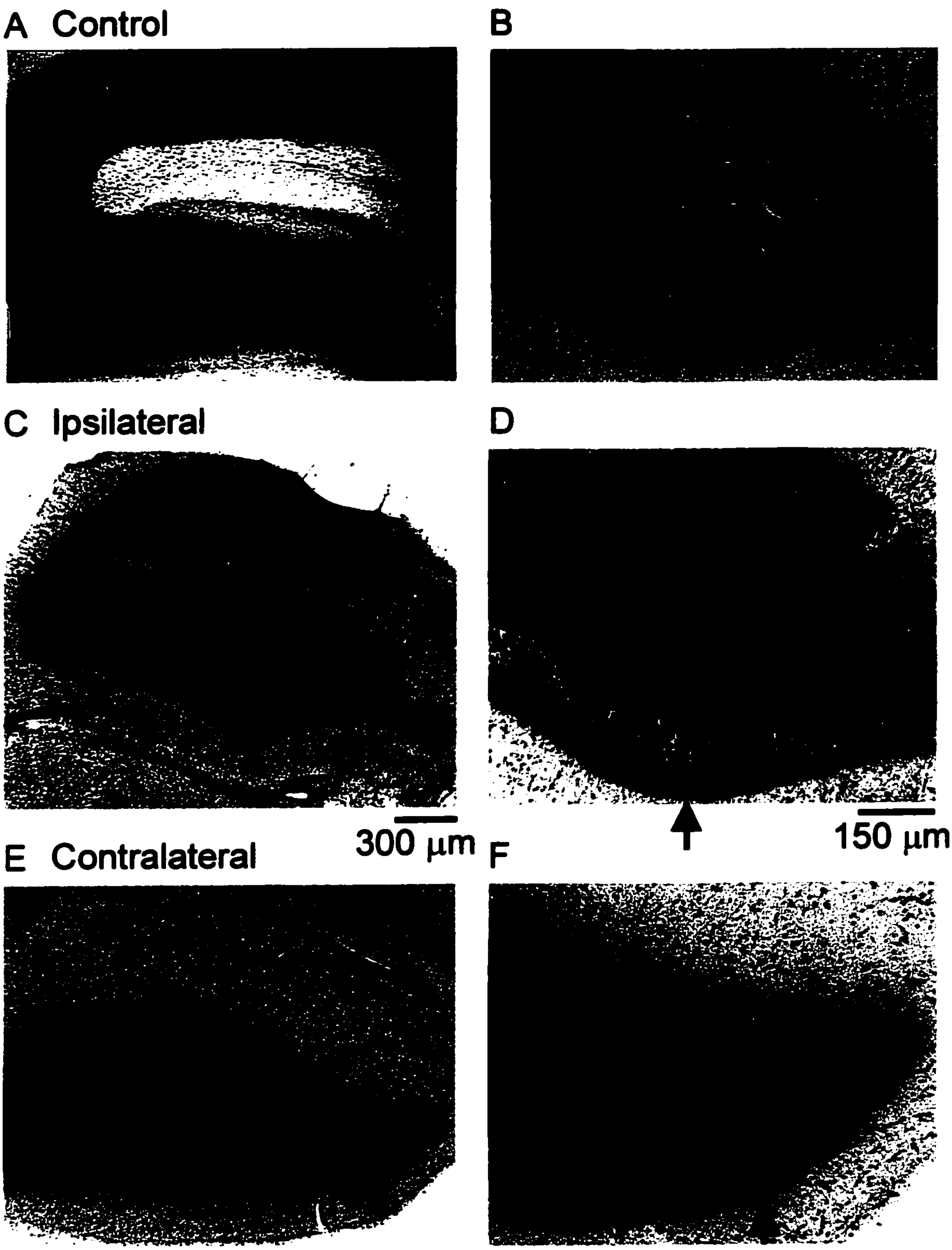
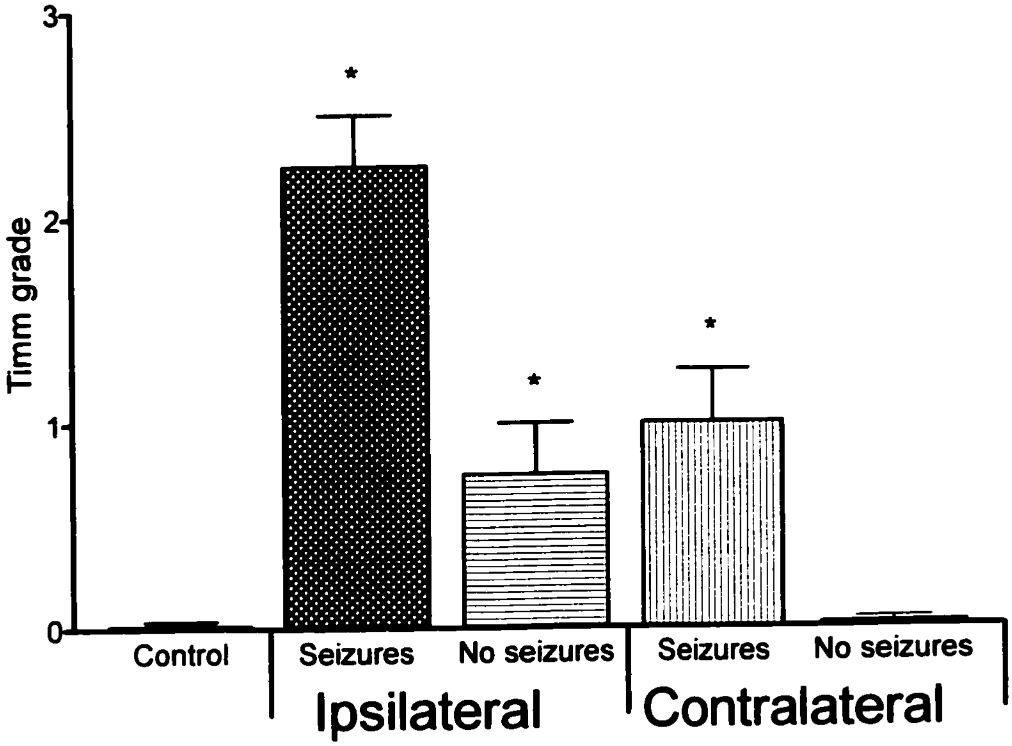


Fig. 4



Timm grade
3
2
1
0
*
*
*
Control
Seizures
No seizures
Seizures
No seizures
Ipsilateral
Contralateral

Extracellular electrophysiology

The ipsilateral hippocampi from hypoxic-ischemic injured rats were assessed for electrphysiological evidence of new recurrent excitatory synapses in the granule cell layer of the dentate gyrus. This was done in hippocampal slices by recording with extracellular electrodes placed into the granule cell layer, while a bipolar stimulating electrode was placed into the hilus (antidromic stimulation). We examined the hippocampal slices for evidence of all-or-none bursting with long and variable latencies. Slices that produced multiple population spikes, but had graded response (i.e., as stimulus intensity increased so did the number of population spikes) were not analyzed. All-or-none bursts of population spikes were never seen in the sham control animals (fig. 4B). Only when the ipsilateral hippocampal slices were bathed in 6 mM $[K^+]_o$ and 30 μM bicuculline did hippocampal slices produce all-or-none bursts of population spikes (Fig. 4A, 60% of the rats, 3 out of 5). In all the slices that demonstrated all-or-none bursting the bursts could be blocked by the application of 50 μM AP-5 and 50 μM DNQX (data not shown). This data implies that the hippocampus following a hypoxic-ischemic injury at seven days of age have abnormal electrophysiological responses that appear similar to what is seen in the granule cell layers from kainate-treated epileptic rats with mossy fiber sprouting. These abnormal responses are mediated by glutamate, and imply that new excitatory synapses have formed in the granule cell layer.

Fig. 5

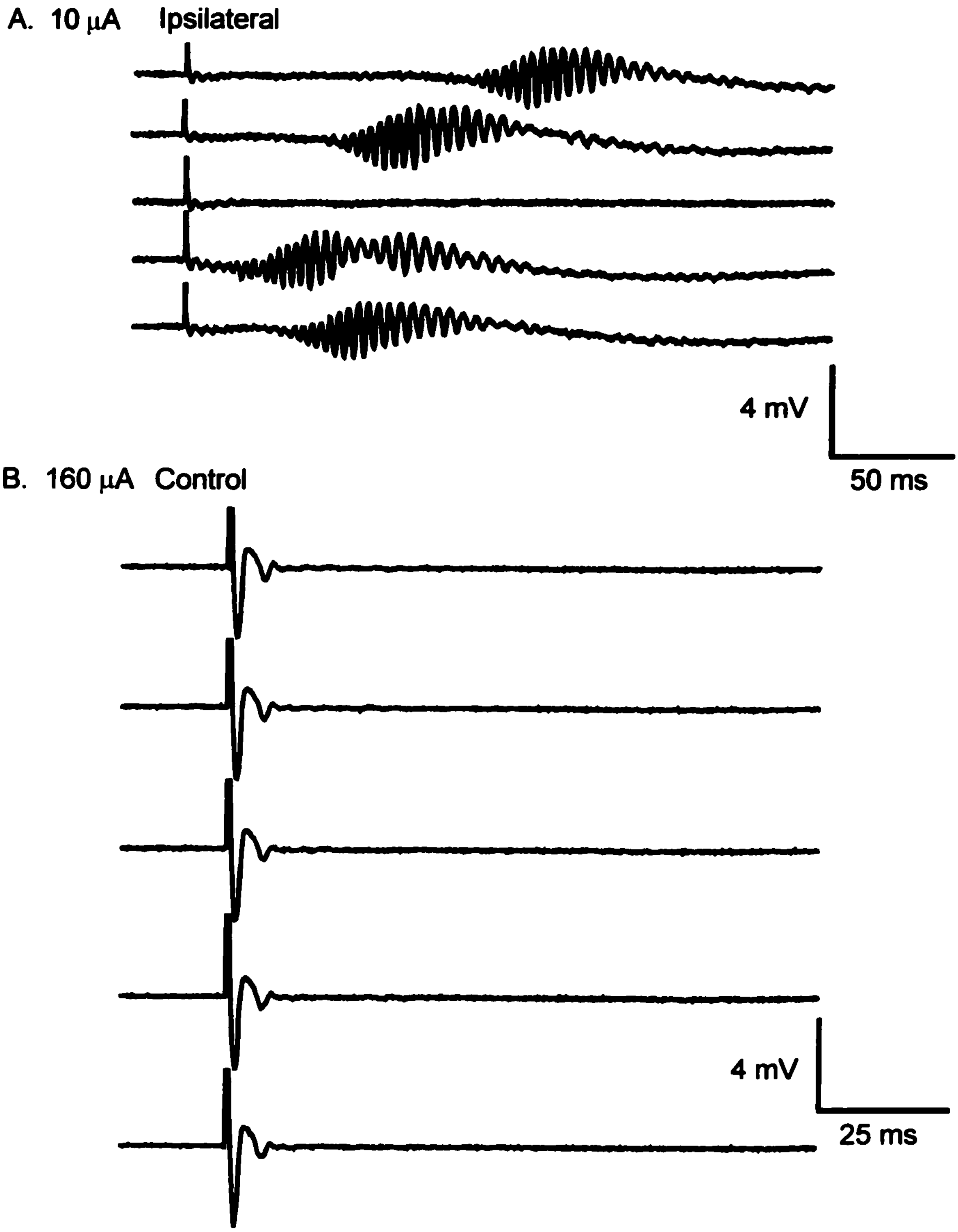


Figure 5 Panel A shows an all-or-none burst from the granule cell layer of an ipsilateral hippocampus evoked by a weak (10 μA) antidromic stimulus. Note the variable and long latency to bursting. The middle trace shows a burst failure. The slice was bathed in 30 μM bicuculline and 6 mM $[K^+]_o$. Panel B shows a recording from a control hippocampus with antidromic stimulus in 30 μM bicuculline and 6 mM $[K^+]_o$.

Flash photolysis

Focal stimulation with glutamate microdrops and photoactivation of caged glutamate has been shown to evoke repetitive EPSPs and EPSCs in hippocampal slices from rats with kainate-induced epilepsy (Wuarin and Dudek, 1996; Molnar and Nadler, 1999, Wuarin and Dudek, 2001). We used focal flash-photolysis of caged-glutamate to examine the granule cell layer for abnormal granule cell-to-granule cell interactions.

Direct stimulation of the recorded granule cell was done to assure that the flash of UV light was uncaging glutamate and producing EPSCs and action potentials. The flash was then moved away from the recorded cells in 200 μm steps around the entire granule cell layer. Each location was stimulated three times or greater and the presence or absence of repetitive EPSCs was assessed with whole-cell recordings at resting membrane potential. The response in the recorded neuron to photostimulation was consistent for a given location (i.e., photostimulation either evoked repetitive EPSCs or it failed to evoked repetitive EPSCs). In 1 out of 14 (8%) granule cells recorded from 4 control rats a positive response was found (Fig. 6A and 7). Significantly more granule cells recorded from ipsilateral hippocampi responded with positive responses (n=14 granule cells from 4 rats, p=0.033, Fishers's exact test, Fig. 7), and in one instance large bursts of unclamped inward spikes were noted while recording in control solution (fig. 6C). The contralateral hippocampus had 3 out of 11 (27%, from 4 rats) granule cells show a positive response to uncaging of glutamate (Fig. 6B and 7). This was not significantly different from the control response. These data show that after a perinatal hypoxic-ischemic injury the granule cell layer in the ipsilateral hippocampus has formed new recurrent excitatory connections.

Figure 6 Caged-glutamate flash-photolysis in the granule cell layer with whole-cell patch-clamp recordings in normal ACSF. The neurons were voltage-clamped at their resting membrane potentials ($\geq$ -70 mV upon whole-cell access). Panel A shows a response from a control hippocampal slice, no currents were evoked by flash-releasing glutamate 200 μm from the recorded neuron. Panel B shows a consistently evoked EPSC by flash-glutamate release 400 μm from a recorded neuron in a contralateral dentate gyrus. Panel C is a recording from an ipsilateral hippocampus with flash-release of glutamate 400 μm from the recorded neuron. This recording shows a burst of unclamped spikes. This was an atypical response, and most responses from the ipsilateral hippocampus were similar to what was shown in panel B. Both panels B and C were considered positive responses.

Figure 7 The percent of granule cell-to-granule cell interactions evoked by caged-glutamate flash-photolysis. In 14 recorded neurons from control hippocampi only 1 neuron (7%) was seen to produce a positive response. Ipsilateral slices had 50% of the granule cells show positive responses to caged-glutamate flash-photolysis, which was significantly different from controls ($p=0.033$, Fishers exact test). The contralateral hippocampus showed 27% of the granule cells with positive responses, which was not significantly different from controls.

Fig.6

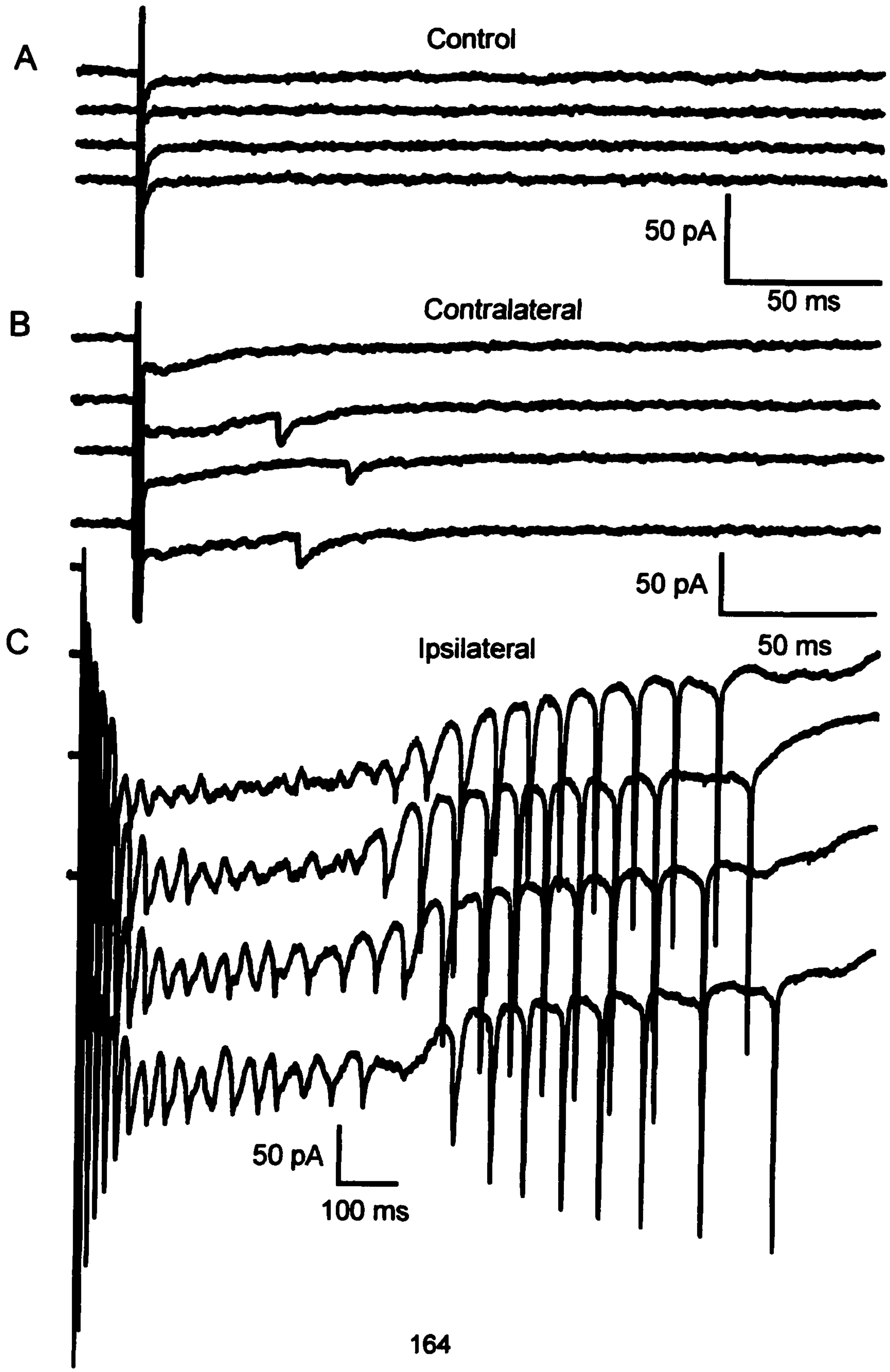
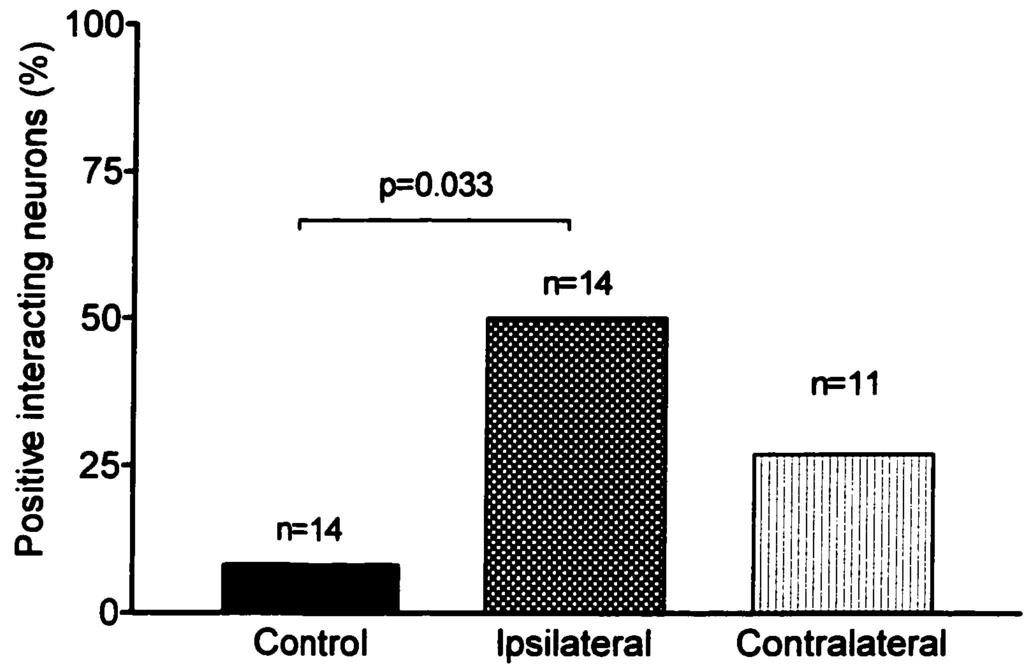

A
Control
50 pA
50 ms
B
Contralateral
50 pA
50 ms
C
Ipsilateral
50 pA
100 ms

Fig. 7



Positive interacting neurons (%)
100
75
50
25
0
p=0.033
n=14
n=14
n=11
Control
Ipsilateral
Contralateral

DISCUSSION

The present study provides evidence that a hypoxic-ischemic lesion in a perinatal rat induces: 1) spontaneous recurrent seizures, 2) Timm stain in the IML of both the injured and apparently non-injured hippocampus of the rats that display spontaneous motor seizures, 3) abnormal electrophysiologic responses from the granule cell layer of the injured hippocampus.

Spontaneous motor seizures

The rats in this study that displayed spontaneous motor seizures had seizures identical to the type of seizures (Racine scale, 1972) commonly displayed by kainate- and pilocarpine-treated rats. Although the seizures in the hypoxic-ischemic lesioned rats were similar to what was seen in rats that model temporal lobe epilepsy, we do not currently know where the seizures were generated. The current evidence suggests that the seizures involve the limbic system. The hypoxic-ischemic injury typically involves both the frontoparietal cortex as well as the dorsal to middle hippocampus, and the striatum is also commonly injured (Rice et al., 1980; Towfighi et al., 1997,1998). Thus there are many potential sites for seizure generation.

Most of the models that utilize perinatal rats involve the induction of seizures by either hypoxia (Jensen, 1995), hyperthermia (Toth et al., 1998), tetanus toxin (Lee et al., 1995), or daily injections of flurothyl (Holmes et al., 1998). We do not know if the seven-day-old rats are having seizures during the hypoxic period since we have not placed electrodes to record electrographic activity during the treatment. However, the rat pups did display abnormal movements during hypoxia that appeared to be seizure activity (swimming movements, lordotic posturing combined with tetanic movement of all four limbs).

In this study we have contrasted both the relative latent period and the seizure rate of the hypoxic-ischemic rats to pilocarpine-treated rats from a previous study. We did not do a comparative statistical analysis between these two groups because the studies were not run in conjunction nor were they designed with this comparison in mind, although both data sets are based upon six hours of direct observation per week. This amount of monitoring overestimated the true latent period (i.e. the true latent period is probably shorter than presented here) and underestimated the true seizure rate (i.e., the true seizure rate was presumably higher for both groups of rats). The rate of six hours per week of direct observation was only observing 4% of all the possible time points that an animal could have a seizure. This also implies that the percentage of rats seen to have spontaneous motor seizures may be higher than what is represented by our data. We did not utilize chronic *in vivo* recording techniques in these animals to record electrographic seizures, which may also be occurring at a relatively high frequency in a large number of hypoxic-ischemic rats. These observations relate to a study by Romijn et al. (1994) in which they utilized a hypoxic-ischemic lesion in12-13 day old rats, and found that a proportion of those rats with severe lesions displayed spontaneous electrographic abnormalities (i.e., 2 sec high frequency large amplitude spike activity) approximately 2.5 months following the lesion, but they never saw spontaneous motor seizures. Since this study did not appear to last much longer than 90-100 days it is possible that these animals would have started to have spontaneous motor seizures if allowed to survive longer. Thus, more work needs to be done in order to fully characterize the spontaneous motor seizures seen after perinatal hypoxia-ischemia.

Timm stain in the IML

Timm stain in the IML is a common finding in humans with temporal lobe epilepsy and in rat models of temporal lobe epilepsy and it is generally believed to be new mossy fiber collaterals (Nadler et al., 1980; Ben-Ari, 1985; Sutula et al., 1989; Houser et al., 1990; Babb et al., 1991; Buckmaster and Dudek, 1997a,b). Relatively weak amounts of Timm stain in the IML have been found in models of global ischemia (Onodera et al., 1990) and in 30-day-old hypoxic-ischemic rats (Williams et al., 1999). It has been hypothesized that hilar neuron loss and deafferentation of the IML is one of the primary factors leading to mossy fiber sprouting. It has also been reported that the hilus is relatively preserved in the 7-day hypoxic-ischemic model (Towfighi et al 1997). Thus the finding in this study of robust Timm stain in the IML of the ipsilateral and contralateral hippocampus is relatively surprising. However, it is currently unknown to what extent the hilus is quantitatively damaged. Specific populations of hilar neurons could be lost, and this would not be known unless specific staining procedures and a quantitative analysis are done to analyze this question. It is also unknown to what extent granule cell loss and potentially replacement (i.e., neurogenesis) of granule cells in this model may contribute synaptic reorganization.

The finding of this study that the contralateral hippocampus contained robust Timm stain in the IML in only those rats that were seen to have spontaneous motor seizures, but not in the contralateral hippocampus of rats that were not seen to have motor seizures implies a strong relationship between the presence of spontaneous motor seizures and Timm stain in the IML. Relatively few studies have shown a correlation between the Timm stain grade and the seizure rate, and if a correlation is found it is typically not very strong (Cavazos, et al., 1991; Mathern et al., 1993; Buckmaster and Dudek, 1997; Gorter et al., 2001). We did not find a correlation between the seizure rate and Timm stain in this study (data not shown). This finding does not negate the importance of mossy fiber sprouting in spontaneous seizures, but it does imply other factors are involved in generating seizures.

Electrophysiology

Extracellular recordings from the granule cell layer in hippocampi with mossy fiber sprouting from kainate- and pilocarpine-treated rats was first shown to produce hyperexcitability by Tauck and Nadler (1985). It was later shown that these potential new recurrent excitatory circuits under normal recording conditions are silent, however, when inhibition was blocked and/or extracellular potassium was raised these circuits become "unmasked" and demonstrate epileptiform bursting (Cronin et al., 1992; Wuarin and Dudek, 1996; Patrylo and

Dudek, 1998). One characteristic expected of circuits with recurrent excitation are bursts of population spikes or action potential with long and variable latencies (Traub and Wong, 1982; Miles and Wong, 1986,1987; Christian and Dudek; 1988a,b). Thus the experiments shown in this study were designed to demonstrate the presence of new recurrent excitatory circuits by utilizing bicuculline to block $GABA_A$ receptors and concurrently raise the extracellular potassium to 6 mM. A majority of the ipsilateral hippocampal slices tested showed all-or-none bursting with long and variable latencies, and we did not find these bursting characteristics if no Timm stain was seen in the IML. The main flaw in these extracellular experiments is that this work is under powered due to a lack of a sufficient population of rats studied for both the lesioned and control rats (n=5, lesioned; n=1, control). Thus based upon the available evidence we would suggest that the Timm stain in the IML represents new recurrent excitatory synapses, but this finding is preliminary based upon the limited number of animals studied.

Caged-glutamate is a photo-reactive compound that when exposed to U.V. light the caging component of the compound is released from the glutamate freeing it to bind with the glutamate receptor. Thus discrete areas in the tissue were stimulated by flashing focused U.V light onto the area of interest, which for this study was the granule cell layer. In this fashion focal flash photolysis of caged-glutamate has been used to map local neuronal circuitry (Callaway and Katz, 1993; Katz and Dalva, 1994; Dalva and Katz, 1994). It has also been shown that glutamate microdrops and minimal lasar photostimulation of the granule cell layer could repeatedly evoke EPSCs in recorded granule cells in tissue from kainate- and PILO-treated rats (Wuarin and Dudek, 1996; Molnar and Nadler, 1999; Lynch and Sutula, 2000). Thereby demonstrating granule cell-to-granule cell interactions, which are indicative of new excitatory synapses. This study demonstrates that in the ipsilateral hippocampus there is a significant increase in the granule cell-to-granule cell interactions. The contralateral hippocampus did not show a significant increase in granule cell-to-granule cell interactions. However, this included both the rats seen to have spontaneous motor seizures and those that were not seen to have seizures. Positive interacting granule cells were only found in contralateral hippocampi from rats seen to have spontaneous seizures. This aspect of the study was also limited by the number of rats studied, though there are an adequate number of neurons recorded for each group they derive from a limited population of rats (n=4 for each group).

SUMMARY

This study showed that a hypoxic-ischemic injury at seven days of age caused epilepsy in a proportion of the population that is similar to what is seen in humans with cerebral palsy. The animals with epilepsy demonstrated mossy fiber sprouting in both the lesioned and non-lesioned hippocampus, and the animals that were not seen to have spontaneous motor seizures did not have mossy fiber sprouting in the contralateral hippocampus. This data also showed that the mossy fiber sprouting represents new recurrent excitatory synapses in the granule cell layer. We conclude from this study that presence of mossy fiber sprouting is associated with an increased probability that animals with hippocampal injury will demonstrate chronic spontaneous motor seizures.

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Chapter 6

Experimental conclusions

In chapter 2 we found that cycloheximide was ineffective at blocking mossy fiber sprouting from pilocarpine-induced status epilepticus. However, rats could still have spontaneous motor seizures without detectable hippocampal sclerosis or mossy fiber sprouting. Chapter 4 showed that a hypoxic-ischemic injury at 30-days of age induces a small but significant amount of Timm stain in the IML of both the lesioned and unlesioned hippocampi. Electrophysiologic recordings from the lesioned hippocampi demonstrated large bursts of population spikes under conditions of reduced inhibition and increased extracellular potassium that were consistent with the formation of new recurrent excitatory synapses. However, the burst duration was much larger than would have been predicted by the amount of Timm stain that was present in the IML (see chapter 2, figure 10). Chapter 3 showed the temporal hippocampus to have a significantly longer burst duration and more Timm stain in the IML than the septal hippocampus from kainate-treated epileptic rats. If we compare the burst duration and amount of mossy fiber sprouting between the septal hippocampi from the epileptic rats and the lesioned hippocampi from the 30-day hypoxic-ischemic rats, we found that the kainate-treated epileptic rats had more mossy fiber sprouting, but a shorter burst duration. This implies that the mossy fiber sprouting found in the 30-day hypoxic-ischemic rats may not be the sole mechanism underlying the epileptiform bursts from the dentate gyrus. In chapter 5 we found that a large percentage of rats that received a hypoxic-ischemic injury at 7-days of age demonstrated spontaneous motor seizures, and these animals had significant amounts of mossy fiber sprouting in both the lesioned and unlesioned hippocampi. This is the first time spontaneous motor seizures have been demonstrated in any perinatal model of brain injury.

Table 1 Summary of results

Model	Neuron loss			Timm stain in the IML	Extracellular bursting	Flash photolysis positive	Seizures
	CA1	CA3	Hilus	heavy, medium, light	in dentate gyrus	in granule cells	
Kainate	yes	yes	yes	heavy	all-or-none	significant	~100%
pilocarpine	yes	yes	yes	heavy	all-or-none	significant	~100%
Pilo+CHX	yes	yes	yes	heavy	all-or-none	significant	~100%
Few Sz at Tx	no	no	no	not significant	slight bursting	no	33%-1 Sz
30-day H-I	yes	?	yes	light	all-or-none in ipsilateral	not yet significant in ipsialteral	16%
7-day H-I	?	yes	?	medium	all-or-none in ipsilateral	significant in ipsilateral	40%

Future directions

In order to resolve the contrasting results on cycloheximide described in chapter 2 with what has been previously published, this work should to be reproduced by other labs. The experiments should also utilize kainate-treated animals in order avoid the complication of protein synthesis inhibitors and IP3 signaling. These experiments should also use more than one protein synthesis inhibitor (anisomycin) in the study design.

Both models of hypoxia-ischemia need to be further characterized by chronic *in vivo* recordings from the hippocampus and cortex in order to determine to what degree these animals may be displaying electrographic seizures, where the seizures are likely being generated, and what structures are involved in the seizures. In the 30-day hypoxic-ischemic model, more work needs to be done to characterize changes to the granule cell layer. Preliminary studies have been done using glutamate flash-photolysis to map granule cell-to-granule cell interactions, and approximately one-third of the granule cells from the ipsilateral hippocampus showed a positive response.

Synaptic reorganization probably occurs in neuronal regions that have lost synaptic input. The hypoxic-ischemic models have severe neuronal loss in CA1 (30-day) and in CA3 (7-day). Thus, we would predict that in the case of the 30-day hypoxic-ischemic model, that the subiculum would undergo reorganization which could serve as an epileptic focus or could alter function. In the 7-day hypoxic-ischemic rats, CA1 would be the area that would hypothetically undergo synaptic reorganization. In humans with temporal lobe epilepsy it has been found that granule cells abnormally synapse onto CA2 pyramidal neurons (Williamson and Spencer, 1994). One coincidental finding in the perinatal rats was abnormal Timm staining in the stratum lacunae moleculare of CA1, implying that the granule cells have now made direct contact to CA1. How this potential new circuit might affect the hippocampus and seizure susceptibility is still unknown.