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## Progressive, potassium-sensitive epileptiform activity in hippocampal area CA3 of pilocarpine-treated rats with recurrent seizures

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### Abstract

Rat hippocampal area CA3 pyramidal cells synchronously discharge in rhythmic bursts of action potentials after acute disinhibition or convulsant treatment *in vitro*. These burst discharges resemble epileptiform activity, and are of interest because they may shed light on mechanisms underlying limbic seizures. However, few studies have examined CA3 burst discharges in an animal model of epilepsy, because a period of prolonged, severe seizures (status epilepticus) is often used to induce the epileptic state, which can lead to extensive neuronal loss in CA3. Therefore, the severity of pilocarpine-induced status epilepticus was decreased with anticonvulsant treatment to reduce damage. Rhythmic burst discharges were recorded in the majority of slices from these animals, between two weeks and nine months after status epilepticus. The incidence and amplitude of bursts progressively increased with time after status, even after spontaneous behavioral seizures had begun. The results suggest that modifying the pilocarpine models of temporal lobe epilepsy to reduce neuronal loss leads to robust network synchronization in area CA3. The finding that these bursts increase long after spontaneous behavioral seizures begin supports previous arguments that temporal lobe epilepsy exhibits progressive pathophysiology.

### Keywords

status epilepticus; hippocampal slice electrophysiology; paroxysmal depolarizing shift; temporal lobe epilepsy

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## Introduction

Spontaneous, rhythmic bursts of synchronized action potentials can be induced in CA3 pyramidal cells in response to a variety of experimental manipulations. These events have been referred to as epileptiform burst discharges and have been widely studied to gain insight into the mechanisms underlying epileptiform activity. Historically, CA3 epileptiform burst discharges were produced in tissue removed from normal rodents by electrical stimulation (Stasheff et al. 1985), chemoconvulsants such as cobalt, penicillin, kainic acid, tetanus toxin, pilocarpine, bicuculline, and picrotoxin (Dichter & Spencer, 1969; Schwartzkroin & Prince 1977; Wong & Traub, 1983; Fisher & Alger, 1984; Swann & Brady, 1984; Ault et al. 1986; Müller & Misgeld, 1991; Nagao et al. 1996; Rutecki & Yang, 1998; He et al. 2009), or perturbation of the neuronal environment by manipulating concentrations of extracellular ions (e.g.  $\text{Cl}^-$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$ ; Schwartzkroin & Prince, 1978; Walther et al. 1986; Traynelis & Dingledine, 1988; Perreault & Avoli, 1991; Jensen & Yaari, 1997). More recent studies have measured the high frequency component of these events, and have addressed the relationship of burst discharges to sharp-wave ripples (Dzhala and Staley, 2004; Menendez de la Prida et al. 2006; Jones et al. 2007; Ellender et al. 2010).

Several studies have described CA3 burst discharges that have been recorded from slices of animals that have been treated to induce epilepsy, as well as other pathological conditions. The results have demonstrated that spontaneous epileptiform burst discharges can be recorded in area CA3 without any electrical, pharmacological, or chemical manipulation, when slices have been made from animals that either sustained a period of ischemia (Wu et al. 2005; Epsztein et al. 2006), intrahippocampal injection of tetanus toxin or kainate (Jefferys, 1989; Lee et al. 1995; Duigou, Bouilleret & Miles, 2008) or pilocarpine-induced status epilepticus (Scharfman et al. 2000; 2001; Klitgaard et al. 2002; McCloskey et al. 2005). Spontaneous epileptiform activity has also been recorded in the hippocampal and parahippocampal areas in patients with temporal lobe epilepsy (Staba et al. 2002) and in vitro after surgery (Cohen et al. 2002, Huberfeld et al. 2007). Presumably there are differences between the acute bursts recorded from slices of normal rodents and the bursts recorded weeks or months after epilepsy induction, or in slices from patients with intractable epilepsy, because relatively long periods of time have passed in the latter case, i.e., time between epilepsy induction and recordings of burst discharges. This idea is supported by in vivo recordings in chronic epilepsy rodent models, which show that spontaneous activity does not develop until days or weeks after seizure induction (Nagao et al. 1996; Hellier & Dudek, 1999; Bragin, et al. 2004). The purpose of the present study was to evaluate epileptiform bursts in area CA3 from slices of pilocarpine-treated epileptic rats at different intervals after status epilepticus, to determine when and where bursts originate in this model, and whether this pathophysiology changes over time.

## Methods

### Seizure induction

Male Sprague Dawley rats  $42 \pm 1$  days of age ( $N = 62$ ) received a single s.c. injection of atropine methylbromide (1 mg/kg s.c.), followed after 30 minutes by an s.c. injection of 380 mg/kg pilocarpine hydrochloride, and were monitored for at least 2 hours following injection for the development of status epilepticus. Status was defined by a stage 4 or 5 seizure that was not followed by resumption of normal behavior, but a period of behavioral seizures. When this period lasted 5 min, it was defined as status, because when 5 min of continuous seizures occurred, it did not terminate subsequently, at least for several hours. All animals received an injection of diazepam (5 mg/kg i.p.) one hour after the start of status epilepticus and an injection of 5% dextrose in lactate-Ringers solution (2.5 ml s.c.)

approximately 5 hours thereafter. Animals received one apple, cut open and laid at the base of their cage, each day for one-week following status epilepticus, or until normal food and water intake resumed. Animals that received pilocarpine, but did not exhibit status epilepticus, or animals that received saline (3 ml/kg) instead of pilocarpine, were used as controls, and received identical post-injection treatment. Animals were housed in clear cages so that spontaneous behavioral seizures could be confirmed. Animals were observed by investigators trained to detect behavioral seizures (Racine scale, Stages 1-5) and spent 5-20 min in the room approximately 3 times per day, 5-7 days a week. Although irregular, this schedule of observation was sufficient for the purposes of the study, i.e., to confirm that all animals with a history of status epilepticus developed more than one spontaneous stage 4-5 seizure, and therefore confirmed that the animals were epileptic.

### Hippocampal slice electrophysiology

After CO<sub>2</sub> anesthesia and decapitation, the brain was rapidly removed and immersed in ~4°C sucrose-based artificial cerebrospinal fluid (sucrose-ACSF, in mM: 252 sucrose, 5.0 KCl, 2.0 CaCl<sub>2</sub>, 2.0 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10 d-glucose; pH = 7.4). Sections (400µm) were cut horizontally, through the ventral 2/3 of the hippocampus, using a Vibroslice (Campden instruments), and immediately transferred to a recording chamber (modified from Fine Science Tools; Scharfman et al. 2001), where they were maintained at 32°C, oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>), and immersed in ACSF except for the upper surface. After 30 min, the sucrose-ACSF, which was delivered to the chamber by a peristaltic pump at a flow rate of approximately 1 ml/minute (Minipuls 2; Gilson), was replaced by ACSF containing 126 mM NaCl substituted for sucrose (NaCl-ACSF). The osmolarity of this ACSF solution was 310 ± 5 mOSM/kg (MicroOsmometer 210, Fiske Associates). Recordings were initiated between 30 and 60 minutes after the perfusate was switched from sucrose-ACSF to NaCl-ACSF.

Extracellular electrodes made of borosilicate glass (0.75 mm inner diameter, 1.0 mm outer diameter; 10-15 MΩ, World Precision Instruments) were pulled using a horizontal pipette puller (P-97, Sutter Instruments). A recording electrode was placed in the CA3 pyramidal cell layer to detect spontaneous bursts (described further below). Data were amplified (Axoclamp 2B; Axon Instruments), and recorded with a digital oscilloscope (Pro 10, Nicolet Instruments) and DAT recorder (DT-800; Microdata Instruments) for digitization and storage of data. Sharp intracellular microelectrodes weremade in the same manner as extracellular electrodes, but had a higher resistance (60-100 MΩ), and were filled with 1M potassium acetate instead of ACSF.

Spontaneous epileptiform bursts (often abbreviated as “bursts” below) were defined as a cluster of population spikes that were variable in amplitude (up to 6 mV) and were superimposed on a large positivity that was also variable, in both amplitude (2-10 mV) and duration (See Figure 1a). To ensure bursts that were relatively rare would not be missed in our slices, continuous recordings (> 2 minutes duration) were analyzed. Two minutes was chosen because it was far longer than the longest inter-burst interval (>8 times longer; see Results). When slices did not exhibit spontaneous bursts in CA3, electrical stimulation of the mossy fibers (with a stimulating electrode placed in the subgranular zone of the dentate gyrus; Scharfman 1997) was used to confirm that CA3 discharge was robust; i.e., the reason why bursts were not detected was not related to poor preservation of area CA3 in vitro. Preservation of CA3 neurons in slices was considered adequate when mossy fiber stimulation evoked a population spike > 2 mV (Scharfman 1997).

## Data analysis

Digitized data were quantified using Origin software (v. 7.5; OriginLabs). To measure burst amplitude and duration, a minimum of 3 bursts were averaged for each recording site. Burst amplitude was measured as the voltage difference between the maximum negative peak and the maximum positive peak. Burst duration was the time between the onset of the burst and the return to baseline. Burst onset was measured as the time when the amplitude exceeded 4 standard deviations of the root mean square (RMS) value, which was calculated using a custom-written routine in Igor Pro (Version 6.03, Wavemetrics Inc). Burst frequency was defined as the number of bursts recorded per minute, averaged over a 3 minute period.

Oscillation frequencies of epileptiform bursts were measured by creating power spectral density plots, using the DSP plugin for Igor Pro, based on a 200 millisecond time window initiating with the onset of the event waveform. To detect sharp wave ripple complexes (SPW-Rs) in control animals, a custom routine was written in Igor Pro to screen spontaneous activity filtered in the ripple range (80-200 Hz, Hamming window band passed) for events that exceeded 4 times the baseline RMS value, based on the methods of Maier and colleagues (Maier et al., 2003; Maier et al., 2009). Averages were made from a minimum of 10 events taken from each of 16 slices from 14 pilocarpine-treated rats. Predominant oscillation frequencies between 100-300 Hz were identified using a peak finding function in Igor-Pro for the PSD function of each waveform. Oscillation frequencies were determined for each slice by averaging the predominant frequencies from each event.

Changes in measures as a function of time after status were quantified using linear regression analysis. Data reported in text, tables, and figures are reported as mean  $\pm$  standard error of the mean. SPSS software (v.15.0, SPSS Inc.) was used to conduct a chi-square analysis to identify the location of the largest burst and a probit regression analysis of extracellular potassium concentration  $[K^+]_o$ , to determine the median effective concentration ( $EC_{50}$ ), and this number, along with confidence limits, was compared across groups.

## Results

### Epileptiform burst discharges recorded after status epilepticus

Data were collected from 170 slices made from 58 animals between 7 and 225 days after status epilepticus. Spontaneous epileptiform bursts were present in area CA3 in 81% of animals and 62% of slices overall. Slices from control animals treated with saline ( $n = 11$  animals) or animals treated with pilocarpine that did not experience status epilepticus ( $n = 4$  animals) did not show any evoked or spontaneous burst discharges, in any of 52 slices, up to 224 days after injection. Figure 1a illustrates a representative example of bursts that were recorded 4 months after status epilepticus. The mean amplitude of CA3 bursts from pilocarpine-treated rats, based on all experiments, was  $2.52 \pm 0.25$  mV, the mean duration was  $95.43 \pm 3.40$  msec, and the mean burst frequency was  $14 \pm 2$  bursts/minute ( $0.23 \pm 0.03$  Hz). However, mean values for amplitude and frequency increased with time after status (further described below), so the pooled data should be considered with this in mind. The peak oscillation frequency of the burst waveform was  $158.53 \pm 8.3$  Hz. Figure 1b shows the average power spectral density based on 16 slices from 14 animals that were evaluated between 2 and 20 weeks after status epilepticus, and a representative burst waveform after 0-50 Hz or 100-300 Hz filters were applied.

When recordings of spontaneous activity in area CA3 were filtered to evaluate SPW-Rs, only 2 of 9 slices (from 2 of 6 age-matched saline control animals) showed evidence of periodic SPW-Rs (that were greater than four times the RMS amplitude) under our recording conditions. These SPW-Rs were very small in amplitude (less than 0.5 mV in amplitude in

all cases). The peak frequency of these events in the range of 100-300 Hz was  $168.99 \pm 13.8$  in one slice and  $182.55 \pm 7.7$  in the other slice ( $n=60$  events total).

The morphology of epileptiform bursts from epileptic rats could vary considerably from animal to animal. Figure 1c illustrates this variability by comparing bursts recorded from different animals, and also illustrates the morphology of spontaneous bursts induced in control slices by elevating  $[K^+]_o$  (10-12 mM), or adding the GABA<sub>A</sub> receptor antagonist bicuculline (10  $\mu$ M) to the ACSF.

Sharp microelectrode recordings of CA3 pyramidal cells were used to evaluate the intracellular correlates of the extracellularly-recorded epileptiform bursts from pilocarpine-treated rats. At a membrane potential of  $-70 \pm 3$  mV, 17 of 18 CA3 pyramidal cells, recorded in slices from 11 animals, produced a large depolarization with multiple action potentials that was simultaneous to the extracellularly-recorded burst. In the case of the 18<sup>th</sup> cell, which did not show this large depolarization, a very large, sub-threshold depolarization was recorded. To confirm that large EPSPs were present at the hyperpolarized potential, and paroxysmal depolarization shift (PDS)-like events at depolarized potentials, cells were hyperpolarized or depolarized with DC current. (Figure 1d) and intracellular electrodes were filled with the lidocaine analog QX314 (200 mM in 1M potassium acetate; Alomone Labs) to block action potentials in a subset of recordings ( $n = 3$  cells). The results confirmed that a "giant EPSP" occurred during epileptiform discharges of the area CA3 network (Figure 1d), similar to previous descriptions of bursts recorded from disinhibited slices of normal rats (Johnston & Brown, 1984).

### The Role of CA3a/b

Simultaneous dual extracellular recordings were used to determine the location within the slice where bursts began, and also to determine areas outside of CA3 that might generate burst discharges ( $n = 6$  slices from 6 animals). Figure 2a shows the results of simultaneous recordings of CA3a and other areas of CA3, CA2, CA1, and the dentate gyrus. The lateral region of the CA3 cell layer, nearest to the fimbria and the border with area CA2 (CA3a according to Lorente de No, 1934), showed the earliest onset and largest burst amplitude, relative to other areas of CA3 (CA3b,  $3.46 \pm 0.19$  msec delay, CA3c  $4.5 \pm 0.16$  msec delay) and area CA2 ( $1.3 \pm 0.94$  msec delay;  $n=6$  slices from 6 animals). Bursts propagated to area CA1 in all 6 slices, producing a large field EPSP in the apical dendritic region of area CA1 in all slices, and a small burst in the pyramidal cell layer in most slices (4 of 6 slices). In the two exceptional slices, there was a small positivity in the cell layer, but no evidence of population spikes. The average latency to the onset of the CA1 field potential was  $4.31 \pm 0.78$  msec after CA3a burst onset. CA3 bursts also influenced the dentate gyrus, because a large field EPSP in the molecular layer followed the CA3 burst ( $n = 3$  of 3 slices from 3 animals). However, population spikes were not detected in recordings from the granule cell layer in any of the 6 slices. These data are similar to recordings in bicuculline-treated slices from normal animals, where CA3 bursts lead to subthreshold potentials in granule cells via connections with hilar mossy cells and interneurons (Scharfman, 1994). However, the latency to onset of the event in the inner molecular layer following CA3a burst onset ( $1.67 \pm 0.62$  msec), was much shorter in slices made from epileptic rats compared to the disinhibited hippocampal slice (Scharfman, 1994).

In a subset of slices ( $n = 4$ ), sequential extracellular recordings were made from the alveus to stratum lacunosum-moleculare, along an axis tangential to the CA3b cell layer (i.e., a laminar profile). The results showed that the largest field EPSPs were recorded in the apical dendrites (Figure 2b) and therefore the most likely site of origin of the burst discharges. To determine whether isolated CA3 subregions could support burst discharges, 4 slices from 4 animals were used, and CA3a or CA3a/b was recorded initially, to confirm that large,

spontaneous rhythmic bursts occurred. Then the recording electrode was raised, and the slice was cut with dura scissors. The first cut removed the entorhinal cortex (Figure 2c). The second cut removed the dentate gyrus (Figure 2c). Next, CA1 and the subiculum were severed. Finally, a cut was made to remove the region of CA2/CA3 that is dorsal to the fimbria. After each cut, the electrode was lowered to record bursts in CA3a/b, using landmarks in the slice to reposition the recording electrode as close as possible to the original recording site. In these experiments, bursts were similar in amplitude, morphology and frequency after all transections (Figure 2c). Recordings that were made outside of the isolated CA3 region did not detect burst discharges. To ensure that bursts were not missed, even if they were rare, recordings were made for up to 2 hours after transections. These data suggest that the burst initiation site was within the area that had been isolated.

A complementary approach to the same question produced similar results. “Minislices” containing only the lateral CA3 (CA3a) region were made immediately after removal of the brain (n = 13 slices from 3 animals). These CA3a minislices exhibited robust epileptiform bursts, and similar minislices from control animals (n= 10 slices in 3 animals) did not produce any such activity (data not shown), suggesting that the observation of bursts were related to disconnection and not related to trauma after cutting slices. Taken together, the data suggest that a small subpopulation CA3a/b cells was sufficient to generate and maintain bursts *in vitro*.

The location of the largest burst recorded in the CA3 pyramidal cell layer was evaluated in 101 slices from 35 animals. For this analysis, the CA3 cell layer was subdivided into 5 regions (CA3a, CA3a/b, CA3b, CA3b/c, and CA3c), and hippocampal slices were divided into 7 dorso-ventral regions (the most ventral region corresponding to slices made ~2.5 mm dorsal to the interaural point, the most dorsal region corresponding to slices made ~6.2 mm dorsal to the interaural point; based on a rat stereotaxic atlas (Paxinos & Watson, 1982)). For each animal, all slices were placed in the recording chamber together, and all CA3 subregions were screened in all slices. The results of this analysis, shown in Figure 2d, indicate that the majority of bursts were largest in the center of the dorsal-ventral axis (58.1% of animals) and the subfield with the largest bursts was CA3a (38.7 % of animals; compared to CA3b and c (Figure 2d). Chi square analysis supported this interpretation, because it revealed that the distribution of the largest burst-generating region was significantly heterogeneous when the axes were combined ( $\chi^2$  (24) = 72.7,  $p < 0.05$ ), and when the dorsal-ventral axis ( $\chi^2$  (6) = 55.8,  $p < 0.05$ ), and CA3-CA2 axis ( $\chi^2$  (4) = 16.3,  $p < 0.05$ ) were analyzed separately.

### Progression of epileptiform burst discharges

Thirty-four animals were used to determine whether CA3 bursts change as a function of time after status epilepticus. Most animals were evaluated at least one week after status, because pilot studies showed that bursts were not evident in slices of the 4 animals that were evaluated between the first and second week after status (n=19 slices). Of the three animals recorded between the second and third week, bursts were detected only in one animal. Slices made from this animal (16 days after status) produced bursts in 2 of 4 slices (50%). Between the third and fourth week, 2 of 4 animals exhibited bursts in area CA3 in 6 of 21 slices (29%). This percentage increased with time after status (Table 1). Together, these data suggest that bursts develop during the second week after status but are not always present at that time. Moreover, burst discharges appear to increase with time after status epilepticus.

Table 1 summarizes the percentage of slices demonstrating bursts as a function of the number of months after status epilepticus. Overall, 17.6% of slices recorded in the first month after status exhibited bursts. This number increased to 57.6% in the second month, and began to plateau at approximately 90% of slices after the second month. Regression

analysis revealed that the relationship of burst incidence to the delay after status was significant ( $r^2 = 0.16$ ,  $p < 0.05$ ). Of the 34 animals that were used in this analysis, 3 were observed to have spontaneous seizures, but not exhibit bursts. They were evaluated 43, 44, and 56 days after status. The results suggest that spontaneous seizures could occur in animals without epileptiform bursts in CA3 in vitro.

Figures 3a, 3b and 3c show the changes in burst amplitude and frequency that occurred with time after status epilepticus, respectively. Burst amplitude ( $r^2 = 0.21$ ,  $p < 0.05$ ) and frequency ( $r^2 = 0.49$ ,  $p < 0.05$ ) increased as a function of the week after status when the slices were evaluated. Burst duration ( $r^2 = 0.05$ ,  $p > 0.05$ ) and the peak frequency ( $r^2 = 0.079$ ;  $p > 0.05$ ) did not appear to change as a function of time (See Figure 3c). Interestingly, burst incidence, frequency, and amplitude continued to increase after the first spontaneous behavioral seizure was observed, which was  $27 \pm 3$  days ( $n=54$ ) after status epilepticus. These data argue against the “step-wise” concept of epileptogenesis, that changes in excitability reach a plateau or cease altogether once spontaneous seizures begin, and argue that changes continue to occur (See Jung et al. 2007; Williams et al. 2009).

### Regulation of bursts by $[K^+]_o$

The relationship between  $[K^+]_o$  and epileptiform activity has been well documented in slices from normal rats (Rutecki et al. 1985; Stringer & Lothman, 1988; Traynelis & Dingledine, 1988; Jensen & Yaari, 1997). To evaluate this relationship in pilocarpine-treated rats,  $[K^+]_o$  was varied systematically in slices from 51 animals when slices were made 2-3 months after status epilepticus. For these experiments, slices were initially prepared and recorded in a solution containing 2.0 mM KCl, and the KCl concentration was raised in 1.5 mM increments, every 30 minutes, and all slices were monitored until spontaneous bursts began. The incidence of bursts in these slices showed a relationship to  $[K^+]_o$ , which is illustrated in Figure 3d. When the ACSF contained 2.0 mM KCl, 26% of animals demonstrated bursts in at least one slice, 39% of animals demonstrated bursts when KCl was raised to 3.5mM, 80% of animals at 5.0 mM KCl, 92% of the animals at 6.5mM KCl, 96 % at 8.0 mM KCl, and 98% at 9.5 mM KCl, the highest concentration tested. For comparison,  $[K^+]_o$  was raised in the same manner in slices from control animals ( $n = 5$ ) to determine the minimum  $[K^+]_o$  required to produce bursts (Figure 3c). In these experiments, bursts larger than 1 mV in amplitude did not begin until KCl was above 8.0 mM (20% of animals). A probit regression analysis was conducted with the proportion of animals producing bursts at each concentration of KCl. The concentration of KCl required to produce bursts in 50% of pilocarpine-treated animals ( $EC_{50}$ ) was 5.14 mM KCl (95% CI: 3.95 to 7.65), which was significantly lower than the  $EC_{50}$  in control rats (11.74 mM KCl; 95% CI: 8.45 to 18.47), demonstrated by the non-overlapping confidence intervals. Therefore, slices from pilocarpine-treated rats had a significantly increased sensitivity to  $[K^+]_o$ .

### Discussion

Our results extend previous observations that spontaneous rhythmic epileptiform bursts develop in area CA3 of rat hippocampus in animal models of temporal lobe epilepsy (Jefferys, 1989; Lee et al. 1995; Scharfman et al. 2000; 2001; Klitgaard et al. 2002; McCloskey et al. 2005; Duigou, Bouilleret & Miles, 2008). This study extends these observations by showing that bursts are robust, propagate throughout CA3 and neighboring regions, and are sensitive to  $[K^+]_o$ . The results also demonstrate the progressive nature of the burst discharges, because the percentage of slices exhibiting bursts, and burst amplitude and frequency, increased significantly as a function of time after status epilepticus. Notably, the increases continued to occur after the onset of spontaneous convulsive seizures, supporting the perspective that progressive changes continue to occur in animal models of epilepsy even after the time when many would suggest ‘epileptogenesis’ is over.

Simultaneous recordings suggested that the bursts were generated in area CA3a – CA3a/b. Interestingly, this is similar to the location of the pacemaker region that has been reported in studies of bursts produced by pharmacological manipulation in slices from guinea pigs (Menendez de la Prida et al. 2006; Knowles, Traub, & Stowbridge 1987, Wittner & Miles, 2007), but is distinct from the burst-generator region resulting from pharmacological manipulation in slices from mouse and rat, which is closer to CA3 b/c (Korn et al. 1987). The location of the CA3a-CA3b region as the most common site of burst origin in the chronic epileptic hippocampus is intriguing because this area has been shown to receive the greatest recurrent collateral input (Wittner & Miles, 2007). The CA3 recurrent collateral system is important for the synchronization among CA3 pyramidal cells (Miles & Wong, 1983). Perhaps this pathway becomes intensified as a result of increase CA3 axon collateralization, which has been shown following status (Siddiqui & Joseph, 2005), and the result is a hypersynchronous epileptic network.

Recent evidence suggests that emergence of fast ripple activity in area CA3 of the chronic epileptic rat is the result of CA3 pyramidal cell desynchronization, rather than hypersynchronization, and, consequently, the amplitude or ripple activity decreases after status (Foffani et al. 2007). The present results are not in conflict with this finding, because the population burst discharge evaluated here is not the same as the high frequency component. In this study, the predominant frequency measured on bursts was often in the ripple (80-200 Hz) band, although some fast ripple (250-500 Hz) activity could be detected. Extracellular and intracellular recordings additionally suggested that an all-or-none, PDS-like event was fundamental to burst discharges, similar to those that have been reported by investigators in slices of normal rats exposed to convulsants (Ayala et al. 1973; Schwartzkroin & Prince, 1978). The results are consistent with the recent finding by Ellender and colleagues (2010) that CA3 pyramidal cells are highly likely to participate in epileptiform bursts. Hyperpolarization of CA3 neurons did not prevent epileptiform bursts, suggesting that they were mediated by “giant” EPSPs, which is also similar to previous reports of area CA3 burst discharges in normal rats after disinhibition or convulsant treatment (Johnston & Brown, 1984).

Hippocampal slices were made from pilocarpine-treated animals as early as one week after status epilepticus, but epileptiform bursts were not detected until 16 days after status, and at that time, they were not consistent. As time increased after status, the incidence and amplitude of bursts increased. Therefore, even if some bursts develop soon after status, the results suggest that they become more robust as the time after status increases. The reason may be related to the time course of spontaneous convulsive seizures in each animal, because spontaneous convulsive seizures can occur within the first week, but are often clustered before developing a more regular pattern (Goffin et al. 2007). The reason may also be related to an intensified collateral network, in CA3 that develops with time, as mentioned above.

An interesting finding of the present study was that the bursts appeared to progress in frequency and amplitude, often months after the animals had exhibited their first spontaneous behavioral seizures. The fact that excitability appears to continue to increase long after animals begin to have spontaneous convulsive seizures is consistent with previous studies of others who have shown that structural or functional changes following status continue well after the first recurrent EEG or behavioral seizure (Dudek et al. 2002; Pitkänen et al. 2002; Pitkänen & Sutula, 2002). It also supports clinical observations of individuals with temporal lobe epilepsy who have increased temporal lobe damage with time after the first presentation of epilepsy (Bernasconi et al. 2002; Fuerst et al. 2001; Cendes, 2005), although this may not apply to all cases (Mathern et al. 2002).

### Bursts are sensitive to $[K^+]_o$

Previous work has demonstrated an increased sensitivity of the hippocampus to  $[K^+]_o$  in kindled rats (King et al. 1985; Stringer & Lothman, 1988). Therefore, it is important to determine whether slices made from rats after status in the pilocarpine model also had an altered sensitivity to  $[K^+]_o$ . Here, bursts were present regardless of the  $[K^+]_o$  used, and nearly half of the animals had bursts when  $[K^+]_o$  was 3.5 mM, while bursts occurred in nearly all of the animals when  $[K^+]_o$  was 5.0 mM. In comparison, control slices did not exhibit bursts in area CA3 until  $[K^+]_o$  was elevated to 8.0 mM. The increase in  $[K^+]_o$  sensitivity is intriguing because it suggests that area CA3 in the epileptic animal may have an impaired ability to buffer  $[K^+]_o$ , which could contribute to chronic hyperexcitability and, potentially, seizures.

Previous work suggests that the rat hippocampus may have an enhanced  $[K^+]_o$  buffering capacity if pilocarpine-induced status epilepticus is not treated with an anticonvulsant (Köhling et al. 1995). Our data would seem to be inconsistent with that conclusion. One explanation for this discrepancy is the difference in model, because animals that experience status epilepticus without anticonvulsant treatment develop more neuronal damage, which is associated with increased gliosis. Reactive astrogliosis following prolonged status epilepticus may enhance  $[K^+]_o$  buffering, but increase neuronal excitability through other mechanisms (Ortinski et al. 2010). Impaired buffering capacity may reflect altered function of cation transport channels such as the KCC2 channel, which could cause disrupted chloride homeostasis and lead to depolarization rather than hyperpolarization in response to GABA release. This mechanism appears to drive burst activity in the surgically-resected subiculum in humans with temporal lobe epilepsy (Huberfeld et al. 2007), and is supported by a recent study by Duigou, Bouilleret and Miles (2008) of spontaneous hippocampal epileptiform activity in the months following intrahippocampal kainate injection. When focal application of potassium chloride was combined with GABA, epileptiform bursts increased in duration and ictal discharges developed.

### Burst generation in area CA3 in other animal models of epilepsy

Reports of spontaneous area CA3 bursts with the large amplitudes observed in the present study have been rare. There are several potential explanations. In the current study, status was decreased in severity at 1 hr by diazepam, and this approach may influence the subsequent development of bursts in CA3. The degree of CA3 pyramidal cell loss appears to be proportional to the duration of status epilepticus (Sperk, 1994; Klitgaard et al. 2002), so the development of bursts may be sensitive to the severity and length of status. Intervening with anticonvulsant drug treatment less than 30 minutes after status appears to prevent the development of area CA3 bursts (Klitgaard et al. 2002). Indeed, areas that had sclerosis-like lesions were less likely to show spontaneous epileptiform activity in slices made ipsilateral to an intrahippocampal kainate injection, than the less damaged areas, or the contralateral hippocampus (Duigou, Bouilleret & Miles, 2008). Therefore, it may be that moderate CA3 cell death is required to cause reorganization among surviving neurons to produce network bursts, while too much damage will prevent a sufficient population to survive, and to ultimately synchronize. Support for this idea comes from the lateral entorhinal cortex kindling model, which causes minimal CA3 cell loss and does not result in spontaneous bursts in CA3 when hippocampal slices are made from the kindled animals. However, there was a chronic increase in sensitivity to elevated potassium in those slices (King et al. 1985).

It is important to note that it is unlikely that there was greater preservation of CA3a/b cells in our study, relative to other areas, and this contributed to the results. Instead, the finding that the number of slices producing burst discharges increases with time after status epilepticus argues for a role of network reorganization or other slow changes, which take

time to develop. The finding argues against a role of neuronal loss, because the majority of neuronal loss occurs in the days or week after status epilepticus (Pitkänen and Sutula, 2002). Moreover, very little evidence exists in our hands for differential preservation within area CA3a,b and c (Scharfman et al 2000; Scharfman et al 2002).

The site of initiation of epileptiform bursts recorded in the current study was most often the CA3a subregion, which is different than results using intrahippocampal tetanus toxin injection, which produces bursts with remarkably similar amplitude, duration, and morphology, but with initiation closer to CA3c (Jeffereys, 1989; Lee et al. 1995). This discrepancy may be due to location of the tetanus toxin injection site (near the CA3c region, at the tip of the hippocampal fissure). However, it is possible that many areas within CA3 (a, b or c) can become the site of burst origin, depending on the methods used to initiate epileptogenesis. When longitudinal slices were made following intrahippocampal kainate injection (Duigou, Bouilleret & Miles, 2008), the burst-initiation region could be either in area CA3 or CA1, suggesting the slice orientation may also play a role in the burst pattern.

### Implications

The reliability with which the CA3 region displays epileptiform activity in slices makes it a potential resource for anti-epileptic drug development, because it may be better to evaluate drugs in slices from epileptic rats rather than normal rats which are altered pharmacologically or electrically. The support for this view comes from previous studies, where it was shown that peptides which reduced epileptiform burst discharges in slices from epileptic rats were less effective in blocking bursts in control animals that were produced by disinhibition (McCloskey et al. 2005). Therefore, drug screening using slices from epileptic rats might produce a different pharmacological profile compared to control tissue. The model may also provide a tractable approach to study progressive worsening of excitability, which is germane to TLE (Dudek et al. 2002; Pitkänen et al. 2002; Pitkänen & Sutula, 2002; Williams et al. 2009).

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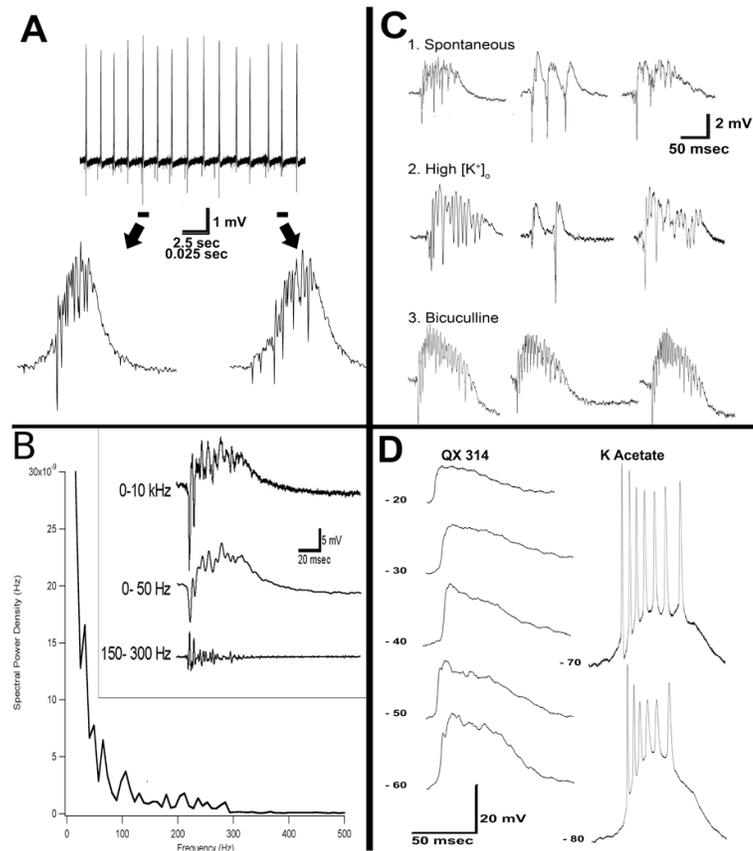
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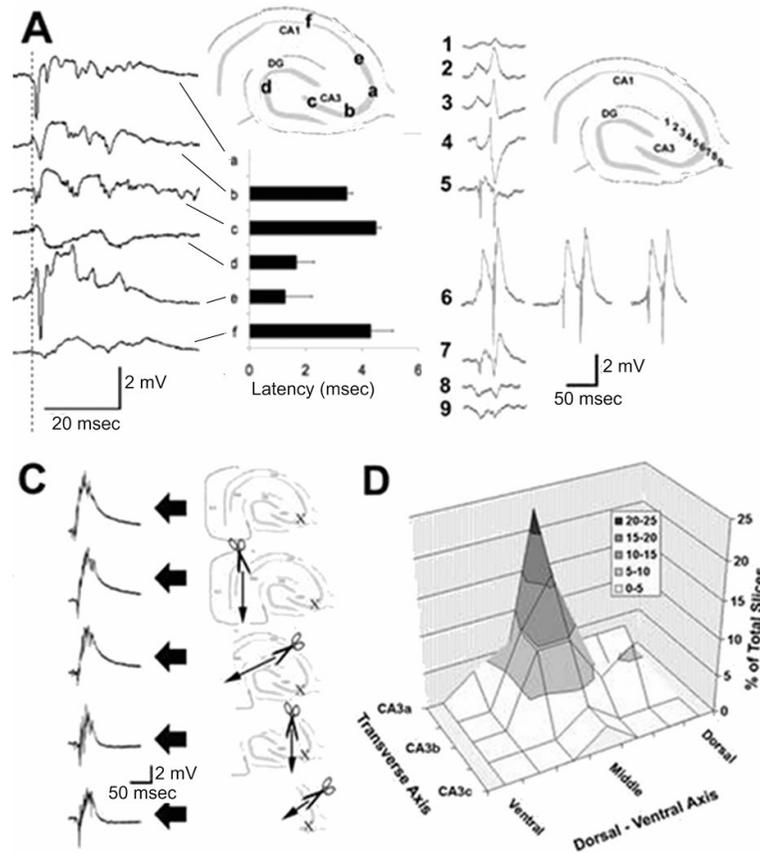
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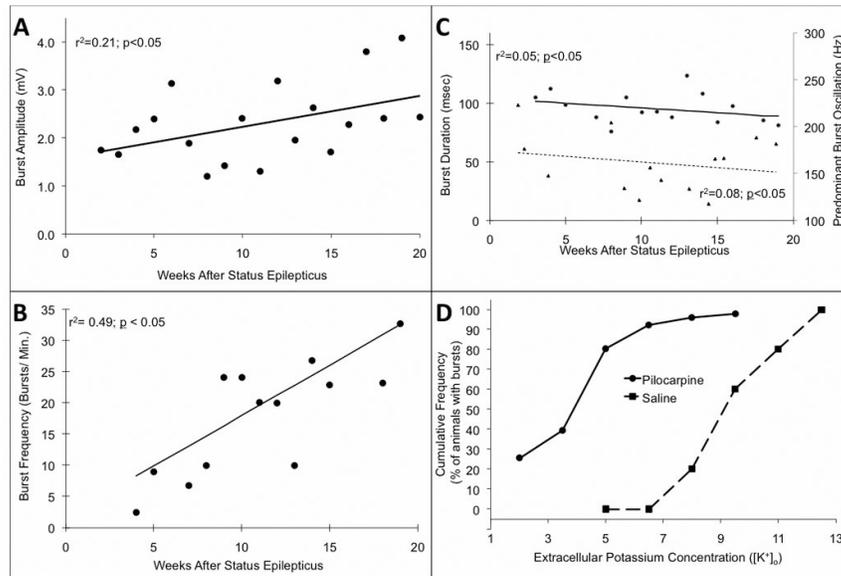
**Figure 1.**

**A.** Spontaneous bursts recorded in the pyramidal cell layer of area CA3 in a pilocarpine-treated animal 4 months after status epilepticus. **B.** Mean power spectral density is plotted for 16 slices from 14 pilocarpine-treated rats, based on a minimum of 10 bursts per slice. The peak frequency within the 100-300 Hz range was  $158.53 \pm 8.3$  Hz. **Inset:** Example of a spontaneous burst from a pilocarpine-treated rat after digital filters were applied using a Hamming filter in Igor Pro. Wideband events were filtered using a 0-50 Hz low pass filter, and a 100-300 Hz band pass filter to show the corresponding activity in these ranges. **C.** Comparison of burst morphology in slices from 3 different pilocarpine-treated rats (1) with bursts induced by 10-12 mM  $[K^+]_o$  in slices from 3 different naïve rats (2), and bursts induced by 10  $\mu$ M bicuculline in slices from 3 naïve rats (3). **C.** Spontaneous bursts recorded in an individual CA3 pyramidal cell at depolarized ( $-20$  to  $-60$  mV) and hyperpolarized ( $-70$  to  $-80$  mV) potentials. Recordings at depolarized potentials were made with QX314-filled microelectrodes ( $-20$  to  $-60$  mV from top to bottom). The voltage dependence and discharge at the peak of the depolarization are consistent with the suggestions that “giant” EPSPs underlie the bursts (Schwartzkoin and Prince, 1978; Brown and Johnston, 1981).



**Figure 2.**

**A.** Simultaneous extracellular recordings of spontaneous bursts in the pyramidal cell layer of area CA3a/b (region a in the diagram) and other subfields (area CA3, area CA1, and the dentate gyrus; regions b-f in the diagram). Once initiated, bursts propagated to other regions of area CA3 (b-c), area CA2 (e), area CA1 (f), and the dentate gyrus (d). The dotted line represents the point during the CA3a burst where the extracellular potential exceeded 4 standard deviations from the baseline root mean square value. Inset: Latency data are plotted for 6 slices (6 animals). They demonstrate the mean latency for bursts to propagate from CA3a to other areas. **B.** Laminar profile of spontaneous bursts in area CA3. Recording electrodes were placed sequentially through the layers of CA3a or b, along an axis perpendicular to the cell layer, starting at the fissure and ending at the alveus. Note that the largest field EPSP was recorded in the apical dendritic region (regions 4-5). Three consecutive bursts are displayed from the pyramidal cell layer (region 6) to demonstrate their slight variations in morphology. **C.** Extracellular recordings were made in area CA3a//b (recording site indicated by the “X” in the diagrams) and the slice was transected so that a smaller portion of CA3 remained. Recordings showed that even the isolated CA3a/b area was sufficient to generate bursts. **D.** The location of the largest burst within area CA3 (a,b or c) is plotted as function of the location of the slice along the dorso-ventral axis based on 101 slices from 31 animals. The largest burst was recorded in the CA3a- CA3a/b portion of the cell layer, and in the center of the dorsal -ventral axis.



**Figure 3.**

Burst amplitude (A) and burst frequency (B) showed a significant increase with time after status. C. Combined results of analyses of burst duration (left axis, circles, solid line) and peak oscillation frequency (right axis, triangles, dashed line) as a function of time after status. Neither of these measures changed significantly in a time-dependent manner (for statistical comparisons, see text). D. Cumulative frequency (% animals producing bursts in slices) as a function of  $[K^+]_o$  in slices from pilocarpine-treated (solid line), and saline treated (dashed line) animals. Pilocarpine treated-rats demonstrated an increased sensitivity to  $[K^+]_o$  with a significantly lower median effective concentration ( $EC_{50}$ ) than control rats.

**Table 1**  
**Incidence of CA3 Bursts Following Status Epilepticus**

Summary of the proportion of slices producing bursts in area CA3 between 30 minutes and 6 hours after slices were perfused with saline-ACSF. Slices from control animals did not produce epileptiform burst larger than 1 mV for up to 8 months after animals were treated with saline or pilocarpine. When slices were made from pilocarpine-treated animals with status, the percent of slices with bursts increased as a function of the number of months after status that the recording was made.

Months after treatment	n	# of recorded slices	% of slices with bursts
<b>Control</b>			
1-8 months	15	52	0
<b>Pilocarpine</b>			
0-1	7	34	17.6
1-2	7	33	57.6
2-3	7	31	90.3
3-4	7	37	91.9
4-6	6	26	92.3