Excitatory Synaptic Input to Granule Cells Increases With Time After Kainate Treatment

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Wuarin, Jean-Pierre and F. Edward Dudek. Excitatory synaptic input to granule cells increases with time after kainate treatment. J Neurophysiol 85: 1067–1077, 2001. Temporal lobe epilepsy is usually associated with a latent period and an increased seizure frequency following a precipitating insult. After kainate treatment, the mossy fibers of the dentate gyrus are hypothesized to form recurrent excitatory circuits between granule cells, thus leading to a progressive increase in the excitatory input to granule cells. Three groups of animals were studied as a function of time after kainate treatment: 1–2 wk, 2–4 wk, and 10–51 wk. All the animals studied 10–51 wk after kainate treatment were observed to have repetitive spontaneous seizures. Whole cell patch-clamp recordings in hippocampal slices showed that the amplitude and frequency of spontaneous excitatory postsynaptic currents (EPSCs) in granule cells increased with time after kainate treatment. This increased excitatory synaptic input was correlated with the intensity of the Timm stain in the inner molecular layer (IML). Flash photolysis of caged glutamate applied in the granule cell layer evoked repetitive EPSCs in 10, 32, and 66% of the granule cells at the different times after kainate treatment. When inhibition was reduced with bicuculline, photostimulation of the granule cell layer evoked epileptiform bursts of action potentials only in granule cells from rats 10–51 wk after kainate treatment. These data support the hypothesis that kainate-induced mossy fiber sprouting in the IML results in the progressive formation of aberrant excitatory connections between granule cells. They also suggest that the probability of occurrence of electrographic seizures in the dentate gyrus increases with time after kainate treatment.

INTRODUCTION

Two important characteristics of temporal lobe epilepsy are (1) a latent period between the insult and the appearance of chronic seizures and 2) a progressive increase in the probability of seizure occurrence with time after the insult. Many types of injuries seem capable of producing a permanently increased susceptibility of the brain to generate seizures, and several hypothetical mechanisms have been proposed to contribute to or be responsible for the epileptogenic process. Although the nature of this hypothetical process is still unclear, synaptic reorganization following axonal sprouting has been proposed to play a central role in the generation of epileptic activity (Dudek and Spitz 1997; McNamara 1999). The progressive formation of recurrent excitatory circuits is an attractive hypothesis for temporal lobe epilepsy and post-traumatic epilepsy, because this hypothetical mechanism would seem likely to be associated with a latent period and an increase in chronic seizure frequency.

Kainate treatment can result in chronic epilepsy (Franck 1993; Sperk 1994). The seizure frequency usually increases with time after treatment (Hellier et al. 1998). Rats treated with kainate typically display neuron loss in the hippocampus (particularly in CA1, CA3, and the hilus) and sprouting of the mossy fibers into the inner molecular layer (IML) of the dentate gyrus (Babb et al. 1991; Ben-Ari 1985; Buckmaster and Dudek 1997a,b; Houser 1992). Several lines of evidence suggest that in the kainate model, as well as in other models of temporal lobe epilepsy (e.g., pilocarpine and kindling models), axonal sprouting in the IML forms recurrent excitatory connections between granule cells. Antidromic stimulation of the granule cells in slices from kainate-treated rats showing Timm stain in the IML could produce bursts of action potentials (Tauck and Nadler 1985), particularly when the inhibition was reduced with bicuculline (Cronin et al. 1992). Epileptiform bursts, both spontaneous and in response to electrical stimulation of the hilar region, were associated with Timm stain in the IML in slices from kainate-treated rats recorded after chronic epilepsy was established (Patrylo and Dudek 1998; Wuarin and Dudek 1996). More direct evidence in support of the hypothesis that sprouting of mossy fibers produces excitatory synapses between granule cells was provided by the demonstration that electrical stimulation of the mossy fibers at the level of the CA3 area could evoke excitatory postsynaptic currents (EPSCs) in granule cells from pilocarpine-treated rats (Okazaki et al. 1999). Also, electrical stimulation of the outer blade in slices from kainate-treated rats evoked excitatory postsynaptic potentials (EPSPs) in granule cells recorded in the inner blade (Lynch and Sutula 2000), corroborating the anatomical observation that sprouted axons from granule cells in the outer blade can cross the hilus and project into the IML of the inner blade in kainate-treated rats (Sutula et al. 1998). Studies using microstimulation with glutamate also strongly support the hypothesis that excitatory connections are formed between granule cells after kainate and pilocarpine treatment. Microdrops of glutamate applied in the granule cell layer evoked repetitive EPSPs in granule cells from rats with kainate-induced epilepsy (Lynch and Sutula 2000; Wuarin and Dudek 1996), and laser photostimulation of the granule cell layer could evoke single EPSCs in granule cells of pilocarpine-treated rats (Molnar and Nadler 1999). Thus several lines of in vitro experimentation using electrophysiological techniques support the hypothesis that excitatory connections are formed between granule cells after kainate and pilocarpine treatment.

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that the epileptogenic process in different animal models of temporal lobe epilepsy is associated with the formation of new recurrent excitatory circuits between dentate granule cells.

Although there is a relatively large body of data suggesting that new excitatory synapses are formed between granule cells in models of temporal lobe epilepsy, little is known about the changes in excitatory synaptic input that occur in granule cells with time after the initial insult. The present study aimed to test the general hypothesis that kainate treatment produces a continuous formation of new mossy fiber axons in the IML, which creates new excitatory synapses between granule cells and results in a progressive increase in the excitatory input to granule cells and in the probability of epileptiform activity. To test this hypothesis, three groups of animals were defined as a function of time after treatment: 1) the first 2 wk, 2) 2–4 wk, and 3) 10–51 wk (i.e., animals observed to have spontaneous recurrent seizures). We found that with time after kainate treatment 1) the intensity of the Timm stain in the IML increased, 2) the frequency and amplitude of spontaneous EPSCs also increased, 3) photostimulation of the granule cell layer revealed more excitatory interactions between granule cells, and 4) in bicuculline (30 μM) and high [K+]o (6 mM), epileptiform activity was evoked only in slices from animals 10–51 wk after kainate treatment. These results support the hypothesis that kainate treatment triggers the progression of an excitatory network between granule cells, which results in an increased probability of epileptiform activity when inhibition is simultaneously compromised.

METHODS

Kainate treatment and chronic seizures

Adult male rats (Sprague-Dawley; 150–250 g; Harlan) were injected with kainate (5 mg/kg in 150 mM NaCl ip) or saline every hour. Motor seizures generally appeared after three to four injections. The treatment was continued until the animals had class IV/V seizures for ≥3 h (Ben-Ari 1985; Racine 1972). Thus the total dose of kainate per rat was 20–50 mg/kg. At the end of the treatment, all animals received lactated Ringer (1–4 ml sc). Both kainate- and saline-injected rats were subsequently observed for 6–8 h/wk until the time of the slice experiments. Only class III–V seizures were recorded. These seizures represent unequivocal departure from normal rat behavior, and thus the observer was not blinded to the treatment. A total of 48 kainate- and 25 saline-injected control rats were divided into 3 groups as a function of time after treatment. Fifteen animals injected with kainate and 7 animals injected with saline were used 1–2 wk after treatment. The same number of kainate- and saline-injected animals was used 2–4 wk after treatment. Eighteen kainate-injected animals were maintained until after chronic epilepsy was established (10–51 wk), and 11 rats were maintained for 17–72 wk days after saline injection. No seizures were detected in any of the control rats. The delay between kainate treatment and the appearance of behavioral seizures (class III–V) was 4–23 wk (average, 10.8 ± 1.5 wk, mean ± SEM, n = 14 animals), when examined 6–8 h/wk (Fig. 1). The total number of observed seizures per animal was 6–48 (average, 25.1 ± 3.9). All the animals in the group maintained 10–51 wk after kainate treatment had spontaneous recurrent seizures for ≥3 wk by the time they were used for the slice experiments. Although no seizure was observed during the first 3 wk after kainate treatment with 6–8 h/wk direct observation, a recent study using 24-h video monitoring showed that 5/26 kainate-treated rats had at least 1 motor seizure during the first week posttreatment (see Hellier et al. 1999). This result indicates that chronic spontaneous seizures (i.e., epilepsy) following kainate treat-

FIG. 1. Cumulative probability distribution of onset of seizure activity (weeks) relative to kainate treatment (time 0). Data points are the time of the 1st recorded behavioral seizure for a given animal (class III–V; n = 1 animal/point except where indicated). The 1st behavioral seizures (class III–V) were observed 4 wk after kainate treatment with 6–8 h/wk monitoring, and by 23 wk after treatment, all animals exhibited spontaneous recurrent seizures. An event may occur earlier than suggested by the data from 6–8 h/wk direct observation.

Slice preparation and recording methods

Kainate-treated and saline-injected rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and their brains were dissected and placed for 30–60 s in oxygenated (95% O2-5% CO2), ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 26 NaHCO3, 3 KCl, 1.3 MgSO4, 1.4 NaH2PO4, 1.3 CaCl2, and 11 glucose, pH 7.4. The brains were bisected, and each half was glued on the stage of a vibratome (Campden Instruments, Lafayette, IN). Four to six 200- to 300-μm-thick slices, mostly from the middle third of the hippocampus, were cut parallel to the base of the brain. Slices were trimmed to isolate the hippocampus and incubated for 1–2 h in oxygenated ACSF at room temperature (21–23°C) before being transferred into the recording chamber. Slices were continuously perfused (2 ml/min) with oxygenated ACSF (10 ml, recirculated) containing caged glutamate [L-glutamate, γ-(α-carboxy-2-nitrobenzyl) ester (250 μM); Molecular Probes, Eugene, OR). Whole cell recordings were obtained at 21–23°C. Pipettes were pulled (P-87 Flaming-Brown pipette puller, Sutter Instruments, Novato, CA) from borosilicate glass capillaries (KG-33, Garner Glass, Claremont, CA), and filled with a solution containing (in mM) 140 K-glucoract, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 1 NaCl, 1 CaCl2, 1 MgCl2, 5 ethylene glycol-bis-(β-aminoethy)l ether)-N,N,N’,N’-tetraacetic acid (EGTA), and 4 magnesium ATP, pH 7.2. Open resistance was 2–4 MΩ. Whole cell currents were amplified with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 2 kHz, and digitized at 44 kHz for storage on video-tapes (Neuro-Corder, Neurodata Instruments, New York, NY). Data were analyzed off-line with sampling rates 5–10 kHz (pClamp 6, Axon Instruments). The spontaneous EPSCs were detected by defining two levels with horizontal cursors. One cursor was positioned approximately in the middle of the baseline noise and the second cursor outside of the peak noise. Currents with amplitude larger than 50% of the difference between the two levels were detected (Fetchan, pClamp6, Axon Instruments). Every detected event was examined and only EPSCs, characterized by a typical fast raising phase and slow decay phase, were included in the analysis. The amplitude of each EPSC was measured by placing a cursor manually at the peak of the raising phase. Recording and analysis of the electrophysiological data were done without knowledge of the treatment. Cumulative amplitude distributions of EPSCs were compared using the Kolmogorov-Smir
The presence of recurrent excitatory connections between pyramidal cells were done with tetrodotoxin (TTX) in the CA3 area where the increase in synaptic input produced by flash photolysis of caged glutamate. With a flash intensity of 50–100 mJ and a concentration of glutamate. With a flash intensity of 50–100 mJ and a concentration of glutamate. This supports earlier work using glutamate microdrops (Christian and Dudek 1988a) that changes in EPSCs frequency produced by photolysis of caged glutamate (250 µM) applied approximately 600 µm from the recorded cell on the side of the CA2 area evoked repetitive EPSCs in control conditions (A1). In the presence of TTX (2 µM), photostimulation of the same area did not evoke any change in synaptic input (B1). In control conditions, direct photostimulation of the recorded cell produced a large depolarizing potential superimposed with a burst of action potentials (A2), and intracellular injection of depolarizing current (800 pA) produced repetitive firing of action potentials (A3). Tetrodotoxin blocked action potential firing evoked by direct photostimulation (B2) and current injection (1.5 nA, B3). Arrows show artifact produced by the flash of ultraviolet (UV) light. This artifact is truncated in this and subsequent figures.

Flash photolysis of caged glutamate

A xenon flash lamp (Chadwick-Helmuth, El Monte, CA) was used to uncage glutamate (Callaway and Katz 1993). The flash of ultraviolet (UV) light was transmitted through the epifluorescence attachment of an upright Optiphot microscope (Nikon), mounted upside-down. A high-numerical aperture, oil-immersion objective (×40, Nikon) focused the flash of UV light approximately 200 µm into the tissue. The optical components were mounted on an X-Y stage to move the objective underneath the transparent bottom (coverslip) of the recording chamber. Location of the objective under the slice (i.e., the location of the photostimulation) was determined with a HeNe laser, mounted on the epi-fluorescence attachment and aimed directly through the objective into the tissue. The light produced by the laser was aligned with the center of the field and focused to a small spot (25–50 µm). Slices were viewed with a monochrome charge-coupled device (CCD) camera (Cohu, San Diego, CA). A video monitor was used to determine the location of the spot of laser light and of each recorded cell in the slice (approximated as the point of entry in the tissue of the recording electrode). The distance between recorded cells and the spot of laser light was measured with a calibrated grid superimposed on the video monitor. Preliminary experiments showed that the spatial resolution of the photostimulation was dependent on the intensity of the flash and also on the concentration of the caged glutamate. With a flash intensity of 50–100 mJ and a concentration of caged glutamate of 250 µM, the spatial resolution was approximately 100 µm. Therefore to optimize the probability of finding local circuits, photostimulations were applied at sites 150 µm apart, throughout the entire extent of the granule cell layer. To determine whether the increase in synaptic input produced by flash photolysis of caged glutamate was mediated by action potential firing, control experiments were done with tetrodotoxin (TTX) in the CA3 area where the presence of recurrent excitatory connections between pyramidal cells has been established (Miles and Wong 1986). In all the pyramidal cells tested (n = 7, from 4 untreated rats), TTX (2 µM) blocked the increase in excitatory synaptic evoked by photostimulation (Fig. 2). This supports earlier work using glutamate microdrops (Christian and Dudek 1988a) that changes in EPSCs frequency produced by photolysis of caged glutamate are due to firing in presynaptic cells evoked by glutamate.

Staining of mossy fiber sprouting

A modified Timm histological procedure was used to label the zinc-containing axons of the granule cells (Babé et al. 1991; Patrylo and Dudek 1998). Slices were processed with the Timm stain for sulfide precipitation of zinc and counterstained with cresyl violet. Slices treated with the Timm procedure included slices that were placed in fixative directly after the dissection, and slices processed after completion of the electrophysiological experiments. 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) (Fig. 3). A small proportion (4–7%) of the sections from the control animals displayed a thin, sometimes discontinuous band of black reaction product in the IML (i.e., grade 1). During the first 2 wk after kainate treatment, most sections (71%) did not show any Timm stain in the IML. However, a substantial proportion of sections (28%) already showed some degree of Timm stain in the IML (grade 1 and grade 2), and surprisingly, a small fraction (1%) had robust staining of the IML (grade 3). Between 2 and 4 wk after kainate treatment, 14% of sections did not show any Timm stain in the IML. Most sections (75%) showed Timm stain in the IML of grade 1 and grade 2. The proportion of sections showing robust Timm stain in the IML (grade 3) increased substantially (11%), compared with the results obtained to test the following specific hypotheses: 1) the intensity of the Timm stain in the IML increases with time after kainate treatment, 2) the amplitude and frequency of spontaneous EPSCs increase with time after kainate treatment, 3) the number of granule cells responding to locally applied flash photolysis of caged glutamate increases with time after treatment, and 4) in conditions of reduced inhibition [i.e., bicuculline (30 µM) and KCl (6 mM)], the probability of epileptiform bursts of action potentials increases with time after treatment.

Progression of staining in the IML with time after treatment

To test the hypothesis of a progressive increase of the Timm stain in the IML, sections obtained from the three post-kainate-treatment and post-saline-injection groups were processed with the Timm’s method and counterstained with cresyl violet. A total of 3,648 sections was processed and scored on a 0–3 rating scale (Tauck and Nadler 1985) (Fig. 3). A small proportion (4–7%) of the sections from the control animals displayed a thin, sometimes discontinuous band of black reaction product in the IML (i.e., grade 1). During the first 2 wk after kainate treatment, most sections (71%) did not show any Timm stain in the IML. However, a substantial proportion of sections (28%) already showed some degree of Timm stain in the IML (grade 1 and grade 2), and surprisingly, a small fraction (1%) had robust staining of the IML (grade 3). Between 2 and 4 wk after kainate treatment, 14% of sections did not show any Timm stain in the IML. Most sections (75%) showed Timm stain in the IML of grade 1 and grade 2. The proportion of sections showing robust Timm stain in the IML (grade 3) increased substantially (11%), compared with the results obtained

RESULTS

The Timm stain and whole cell patch-clamp recordings, combined with flash photolysis of caged glutamate, were used for differences in the effects of the flash photolysis of caged glutamate between the different treatment groups. Student-Newman-Keuls ANOVA was used to test differences in EPSC frequency. Data are expressed as means ± SE.

FIG. 2. Tetrodotoxin blocks the increase in excitatory postsynaptic currents (EPSCs) produced by flash photolysis of caged glutamate. Comparison of the effect of photostimulation of the CA3 area in control conditions (A) and in the presence of TTX (B). Whole cell voltage-clamp (A1 and B1) and current-clamp (A2, A3, B2, and B3) recordings at resting membrane potential (~69 mV) of a CA3c pyramidal cell. Flash photolysis of caged glutamate (250 µM) applied approximately 600 µm from the recorded cell on the side of the CA2 area evoked repetitive EPSCs in control conditions (A1). In the presence of TTX (2 µM), photostimulation of the same area did not evoke any change in synaptic input (B1). In control conditions, direct photostimulation of the recorded cell produced a large depolarizing potential superimposed with a burst of action potentials (A2), and intracellular injection of depolarizing current (800 pA) produced repetitive firing of action potentials (A3). Tetrodotoxin blocked action potential firing evoked by direct photostimulation (B2) and current injection (1.5 nA, B3). Arrows show artifact produced by the flash of ultraviolet (UV) light. This artifact is truncated in this and subsequent figures.
during the first 2 wk. Of the sections obtained from the animals 10–51 wk after kainate treatment (average, 190 ± 14 days), only 1% did not show any detectable Timm stain in the IML. In this group, most sections (67%) showed robust Timm stain in the IML (grade 3). The overall Timm score (see METHODS) for all sections obtained from control animals was 0.05 ± 0.01. For the sections obtained from kainate-treated animals during the first 2 wk after treatment, the Timm score was 0.36 ± 0.13. This score increased to 1.33 ± 0.09 in sections obtained 2–4 wk after treatment and to 2.6 ± 0.1 in sections from rats 10–51 wk after treatment (average, 190 ± 14 days). These data suggest that Timm stain in the IML is already apparent 2 wk after kainate treatment, and that its intensity increases with time after the kainate-induced injury.

Spontaneous EPSCs

To test the hypothesis that the amplitude and frequency of spontaneous EPSCs increase with time after kainate treatment, whole cell recordings were obtained from granule cells at resting membrane potential (Fig. 4). The amplitude and frequency of EPSCs in granule cells from kainate-treated and age-matched control groups were measured during 120-s periods for each cell and compared at three different times after treatment. Recordings were obtained from 8 granule cells in 7 slices from 6 kainate-treated rats 1–2 wk after treatment, 9 granule cells in 6 slices from 6 kainate-treated rats 2–4 wk after treatment, and 10 granule cells in 7 slices from 7 rats 10–51 wk after kainate treatment (average, 162 ± 30 days). Recordings from control, saline-injected animals were obtained from five granule cells in five slices from five animals 1–2 wk after injection, five granule cells in five slices from five animals 2–4 wk after injection, and six granule cells in five slices from five animals 1–2 wk after treatment, five granule cells in five slices from five animals 2–4 wk after injection, and six granule cells in five slices from five animals 17–72 wk after injection (average, 233 ± 47 days).

Amplitude of spontaneous EPSCs

Cumulative amplitude distributions showed a shift toward larger EPSC amplitude in granule cells with time after kainate treatment (Fig. 5, A and B). Comparisons between cumulative
amplitude distributions for spontaneous EPSCs in granule cells from kainate-treated animals using the KS test (2 samples, 1-tailed) revealed a highly significant increase in the EPSC amplitude between all three groups of animals treated with kainate ($P < 0.001$ for all comparisons between kainate-treated groups, $\chi^2$ test). In Fig. 5, A and B, the EPSCs from the control groups were lumped in one plot. However, statistical comparisons with controls were made between kainate-treated animals and aged-matched controls. Comparison between experimental and control groups showed highly significant differences in all three groups ($P < 0.001$ for comparisons between all kainate-treated groups and aged-matched controls, KS test, 2 samples, 1-tailed). To test for a potential change in the EPSC amplitude with age in the control groups, cumulative probability plots were constructed for granule cells from each control group and compared with each other (Fig. 5C). The KS test (2 samples, 2-tailed) showed no significant difference in the EPSC amplitude between any of the control groups ($P > 0.08$ for all comparisons between control groups). This result suggests that EPSC amplitude is larger in the kainate-treated animals than in age-matched controls and that this increase is progressive and begins within the first 2 wk after the insult produced by kainate treatment.

**Frequency of spontaneous EPSCs**

The average frequency of spontaneous EPSCs in granule cells from control animals did not differ significantly with time after saline injection ($P > 0.05$; Fig. 6). The average frequency of spontaneous EPSCs 1–2 wk after kainate treatment was not significantly different from any of the control groups ($P > 0.05$). The difference in the average frequency of spontaneous EPSCs between the groups 2–4 wk and 10–51 wk after kainate treatment was not significant ($P > 0.05$), but the average frequency in both groups was significantly higher than the frequency in all the other groups ($P < 0.05$). These data suggest that the increased frequency of EPSCs observed in granule cells from kainate-treated rats was induced by kainate and was not related to the age of the animals since the frequency of EPSCs in granule cells from control animals did not change significantly with time after saline injection. This result is consistent with the hypothesis that granule cells from kainate-treated rats receive input from more excitatory synapses, and/or from more active excitatory synapses than granule cells from saline-injected control animals.

**Relationship between Timm stain in the IML and properties of spontaneous EPSCs**

To determine whether the average amplitude and frequency of EPSCs in granule cells from rats studied 10–51 wk after kainate treatment were correlated with the intensity of the Timm stain in the IML, a score for the Timm stain was obtained for each animal (see METHODS), and the average EPSC amplitude and average EPSC frequency were plotted as a function of the Timm score (Fig. 7). Animals with lower Timm score generally had lower amplitude and lower frequency EPSCs, whereas animals with higher Timm score showed a wide range of results for both frequency and amplitude. Several granule cells from animals with high Timm score showed EPSC amplitude and frequency similar to those of granule cells from animals with low Timm score. However, EPSCs of high frequency and large amplitude were seen only in granule cells from animals with high Timm score. Linear regressions showed a significant correlation between Timm score and average EPSC amplitude (Pearson $R = 0.69$, $P < 0.05$) and between Timm score and average EPSC frequency (Pearson $R = 0.66$, $P < 0.05$). This result supports the hypothesis that increased intensities of Timm stain in the IML are associated with an overall increase of the excitatory synaptic input to granule cells.

**Photostimulation of the granule cell layer**

In hippocampal slices with Timm stain in the IML, local stimulation of the granule cell layer with glutamate microdrops
has been shown to evoke repetitive EPSPs in most granule cells (64%) (Wuarin and Dudek 1996). In the present study, we tested the hypothesis that the proportion of granule cells responding to glutamate microstimulation with repetitive EPSCs would increase with time after treatment. Specifically, we hypothesized that 1) glutamate microstimulation of the granule cell layer would produce repetitive EPSCs in relatively few granule cells during the first 2 wk after treatment, 2) this response should be observed in a larger proportion of granule cells 2–4 wk after treatment, and 3) glutamate microstimulation should evoke repetitive EPSCs in more granule cells from animals 10–51 wk after kainate treatment than in either of the other two groups.

Photostimulations were applied every 150 μm throughout the entire extent of the granule cell layer, and the postsynaptic response in granule cells was determined with whole cell recordings at resting membrane potential. Photostimulations produced either no change in the spontaneous EPSCs, or they evoked repetitive EPSCs (Fig. 8). This response varied from a few EPSCs (e.g., Fig. 8B) to periods of repetitive EPSCs lasting several hundreds of milliseconds (Fig. 9). Repeated stimulations of the same area in the granule layer produced consistent responses (Fig. 9). The ratio of the number of cells showing evoked excitatory synaptic responses to the total number of cells tested was determined for each group. A total of 53 granule cells from 23 saline-injected rats were tested with flash photolysis of caged glutamate (Fig. 10). Only one granule cell from an animal belonging to the group 2–4 wk after saline injection (n = 18 granule cells, 7 rats) showed repetitive EPSCs in response to photostimulation. No granule cell showed a change in EPSCs in response to photostimulation 1–2 wk (n = 18 granule cells, 7 rats) and 17–72 wk after saline injection (average, 337 ± 47 days, n = 17 granule cells, 9 rats). Control groups were not significantly different from each other (P = 0.31); therefore they were pooled for comparison. Ninety-two granule cells from 46 kainate-treated rats were tested with photostimulation (Fig. 10). The proportion of granule cells responding to photostimulation of the granule cell layer with repetitive EPSCs 1–2 wk after kainate treatment was not significantly different from controls (3/29 granule cells, 15 rats, P = 0.085). However, this proportion increased significantly to 32% during the period 2–4 wk after treatment (10/31 granule cells, 15 rats, P = 0.04). In the animals 10–51 wk after kainate treatment (average, 202 ± 19 days), photostimulation of the granule cell layer evoked repetitive EPSCs in 66% of the granule cells tested (21/32 granule cells, 16 rats, P = 0.008). This result supports the hypothesis that the number of functional excitatory connections between granule cells increases with time after kainate treatment.
Photostimulation-evoked epileptiform bursts

In conditions of reduced inhibition and increased excitability, hippocampal slices from rats 4–13 mo after kainate treatment have been shown to generate epileptiform bursts of action potentials in response to electrical stimulation of the hilar region (Patrylo and Dudek 1998; Wuarin and Dudek 1996). In the present study we tested the hypothesis that, in conditions of reduced inhibition, the probability of evoking epileptiform bursts of action potentials in the dentate gyrus would be relatively low during the first few weeks after kainate treatment compared with several months after treatment (i.e., when the animals had become epileptic). To evoke epileptiform bursting in the dentate gyrus, we used photostimulation of the granule cell layer in the presence of bicuculline (30 μM) to reduce inhibition, and high [K+]o (6 mM) to increase the probability of multisynaptic interactions. Current-clamp recordings of the electrical activity of granule cells were obtained in the whole cell configuration. In these conditions, photostimulation did not evoke bursts of action potentials in any of the granule cells recorded from saline-injected animals (0/8 granule cells, 7 rats). Four granule cells from 3 kainate-injected animals 1–2 wk after treatment and 13 granule cells from 7 kainate-injected rats 2–4 wk after treatment were tested with photostimulation applied throughout the granule cell layer. Photostimulation of the granule cell layer did not evoke bursts of action potentials in any of the granule cells tested during the first 4 wk after kainate treatment. In contrast, photostimulation of the granule cell layer evoked epileptiform bursts of action potentials in all the granule cells tested from rats 10–51 wk after kainate treatment (average, 209 ± 11 days, 11/11 granule cells, 7 rats; Fig. 11). Bursts of action potentials of similar duration were recorded regardless of the distance between the photostimulations and the recorded cell (e.g., recorded granule cell in the inner blade and photostimulation applied in the outer blade). Hyperpolarization of the recorded cells decreased the number of action potentials evoked by photostimulation.

FIG. 8. Comparison of the effect of flash photolysis of caged glutamate applied in the granule cell layer between a saline-injected (A) and a kainate-injected animal (B). Both experiments were done 4 wk after treatment. Diagrams of the outline of the granule cell layer show the position of the recording electrodes and the locations of the flashes of UV light (filled circles, 1–6). The effects of photoactivation of caged glutamate applied in locations 1–6 (A and B) are shown in the corresponding traces below. Distance between photostimulations was approximately 150 μm. Note repetitive EPSCs in trace 4 (B). Direct photostimulation of the recorded cells [trace 3 (A), trace 2 (B)], produced a large (clipped) inward current. This effect is shown in current clamp in the bottom two traces. Both granule cells were recorded at resting membrane potential (~76 mV), (~73 mV). Arrows show stimulus artifact produced by the flash. Outer blade, OB; inner blade, IB.

FIG. 9. Repeated photostimulations of the same area evoked repetitive EPSCs of similar duration in a granule cell from a rat 39 wk after kainate treatment. Whole cell patch-clamp recording at resting membrane potential (~72 mV) of a granule cell located in the outer blade. Fifteen consecutive photostimulations (0.05 Hz) were applied at the same site in the granule cell layer, approximately 600 μm from the recorded cell toward the tip of the outer blade. The numbers indicate stimulation number. Traces are continuous in A–C; the top traces in each panel show baseline activity. Arrows show the artifact produced by the flash. Bottom trace in C is an enlargement of the area outlined by the dashed lines.

FIG. 10. Plot of the percentage of granule cells from saline- and kainate-injected animals responding to photostimulation with an increase in EPSCs as a function of time after treatment. The number of cells tested is indicated for each age group.
tals, but did not block the bursts (not shown). These observations suggest that photostimulation-evoked firing of a relatively small fraction of the granule cell population in hippocampal slices from rats 10–51 wk after kainate treatment can induce the entire population of granule cells to fire epileptiform bursts when inhibition is depressed. This experimental approach revealed bursting activity only in slices from animals 10–51 wk after kainate treatment and not in slices from kainate-treated rats tested within the first 4 wk after treatment, suggesting that the dentate gyrus becomes more susceptible of generating epileptiform activity with time after treatment.

**DISCUSSION**

The present study provides several lines of evidence supporting the hypothesis that kainate treatment induces the progressive formation of new excitatory synapses between granule cells leading to an increased probability of epileptiform activity in the granule cell layer. The intensity of the Timm stain in the IML increased with time after treatment; this change in mossy fiber sprouting was correlated with an increase of both amplitude and frequency of spontaneous EPSCs in granule cells. The number of granule cells showing repetitive EPSCs in response to photostimulation of the granule cell layer increased dramatically with time after kainate treatment, and epileptiform bursts were observed only in slices with robust Timm stain in the IML from animals tested 10–51 wk after treatment.

**Increased excitatory input**

The first main finding of this study is the demonstration of a progressive increase in amplitude and frequency of spontaneous EPSCs with time after kainate treatment. A significant shift toward larger amplitude was already observed within 2 wk after kainate treatment. This trend continued during the 2- to 4-wk period after kainate treatment. In the animals studied 10–51 wk after kainate treatment, most EPSCs were larger than 10 pA. The frequency of spontaneous EPSCs in granule cells from both controls and kainate-treated animals was relatively low; therefore summation was unlikely to influence significantly the amplitude measurement of the EPSCs. No significant change in the frequency of EPSCs was observed during the first 2 wk after kainate treatment, but the frequency of spontaneous EPSCs more than doubled 2–4 wk after kainate treatment and was even higher in the animals 10–51 wk after treatment. No significant change in EPSC amplitude or frequency was observed with time after saline injection, indicating that the increase in excitatory input to granule cells from kainate-treated animals was not related to animal age but was caused by the kainate treatment.

Since kainate treatment has been shown to produce extensive cell loss in the hilus (e.g., Buckmaster and Dudek 1997b) and also probably in the entorhinal cortex (Eid et al. 1999), part of the excitatory input to granule cells is expected to be lost early after treatment, and thus one might expect a decrease in the frequency of EPSCs during the first few days after the treatment. The absence of a significant difference in the frequency of EPSCs between controls and rats tested during the first 2 wk after kainate treatment may result from a sampling problem (i.e., too few EPSCs sampled). Alternatively, as synaptic terminals from dying hilar neurons disappear, some of them may be replaced relatively rapidly by new synapses from sprouted mossy fibers. The presence of large-amplitude EPSCs during the first 2 wk after kainate treatment supports the idea that new synapses may be formed early after the kainate-induced injury.

**Mossy fiber sprouting**

Timm stain in the IML was detectable as early as 2 wk after treatment and was robust in most sections from rats 10–51 wk after kainate treatment. Recent studies have shown that mossy fiber sprouting can occur within the first 2 wk after kainate
treatment, and its expression seems more pronounced in the temporal pole of the hippocampus (Buckmaster and Dudek 1997b; Cantallops and Routtenberg 1996; Hellier et al. 1999; Simpson et al. 1997). Data derived from the pilocarpine model showed EPSCs of higher frequency and amplitude in granule cells from slices with more intense Timm stain in the IML (Simmons et al. 1997). Sutula et al. (1998) showed that in longitudinal slices from kainate-treated rats, sprouted axons in the inner molecular layer can extend 600–700 μm in the septotemporal axis. Corroborating this in vitro study, reconstruction of the axonal arbor of granule cells from kainate-treated rats recorded in vivo and labeled with biocytin revealed that the septotemporal span of sprouted axons in the IML was 600 μm (Buckmaster and Dudek 1999). We therefore hypothesized that the spontaneous EPSCs detected in granule cells from kainate-treated rats originated not only in neurons located in the slice but also from sprouted axons originating from granule cells outside of the slice, which were cut during the preparation of the slice. In consequence, we used a method to evaluate the intensity of the Timm in the IML stain throughout the hippocampus. We found that granule cells with spontaneous EPSCs of small amplitude and low frequency were from animals with low Timm score and that higher EPSC frequency and amplitude were generally associated with higher Timm score. Synaptic projections cut during the slice preparation may explain the few granule cells with low-frequency and small-amplitude EPSCs recorded in slices from animals with high Timm score. It is also likely that synaptic reorganization induced by kainate treatment did not result in increased excitatory input to all the granule cells. Our observation that changes in the EPSCs were correlated with increased mossy fiber sprouting in the IML supports the notion that this progressive increase in excitatory input to granule cells is due, at least in part, to mossy fiber sprouting.

Focal stimulation

The second result of this study is that the number of granule cells responding to photostimulation of the granule cell layer with repetitive EPSCs increased with time after kainate treatment. This approach was based on research by Katz and co-workers (Callaway and Katz 1993; Dalva and Katz 1994; Katz and Dalva 1994) demonstrating that photostimulation of caged glutamate can be used to map local neuronal circuitry in mammalian brain slices. Glutamate is known to activate selectively the somatodendritic area, but does not generate action potential firing when applied to axons (Christian and Dudek 1988a). Previous work has shown that glutamate microdrops applied in the granule layer of hippocampal slices from kainate-treated rats can evoke repetitive EPSPs in granule cells (Lynch and Sutula 2000; Wuarin and Dudek 1996), and minimal laser photostimulation of the granule cell layer from pilocarpine-treated rats could evoke single EPSCs in granule cells (Molnar and Nadler 1999). Taken together, these studies strongly support the hypothesis that both kainate and pilocarpine treatments result in the formation of an extensive excitatory network. In the present study, we showed that the mossy fiber sprouting in the IML, as well as the amplitude and frequency of spontaneous EPSCs, increased with time after kainate treatment. We next tested whether there was a parallel increase in the density of the putative excitatory network between granule cells. With flash photostimulation combined with whole cell patch-clamp recording, we found that <2% (1/53) of the granule cells tested from saline-injected animals showed an increase in EPSCs in response to flash photolysis of the granule cell layer. In contrast, within 2 wk after kainate treatment, the proportion of granule cells showing multiple EPSCs in response to photostimulation was 10%. This proportion increased to 32% by 4 wk and reached 66% in the animals 10–51 wk after kainate treatment. These data suggest that functional excitatory synapses between granule cells are formed relatively early after a kainate-induced injury and that the number of these connections (i.e., the number of granule cells connected together through excitatory synapses) increases progressively over time.

Temporal change in seizure frequency after injury

A central concept in temporal lobe epilepsy is that 1) a latent period (i.e., a period without chronic seizures) occurs after an injury before the onset of recurrent spontaneous seizures and 2) the frequency of seizures tends to increase with time after the precipitating insult. These characteristics of temporal lobe epilepsy and post-traumatic epilepsy suggest that the injury triggers a time-dependent process that increases the probability that epileptic seizures will occur. We hypothesized that after the kainate-induced injury, the granule cell layer becomes progressively more capable of generating epileptiform bursts as the number of newly formed excitatory connections between granule cells increases. Since inhibition has been shown to mask multisynaptic excitatory circuits (Christian and Dudek 1988a,b; Dichter and Spencer 1969a,b; Miles and Wong 1983, 1986, 1987; Miles et al. 1984; Patrylo and Dudek 1998), we tested this hypothesis in conditions of reduced inhibition. We used photostimulation of the granule cells to evoke activity in a few granule cells, and we then determined whether this localized firing could propagate synthaptically through the granule cell layer and generate epileptiform bursts. Photostimulation in conditions of reduced inhibition did not evoke bursts of action potentials in granule cells from control rats or in granule cells from rats tested within the first 4 wk after kainate treatment. In contrast, photostimulation of the granule cell layer evoked epileptiform bursts in all the granule cells tested from rats 10–51 wk after kainate treatment. Although Timm stain in the IML was already present in slices from the animals 1–4 wk after kainate treatment, it was generally more robust in slices from the animals 10–51 wk after treatment. This result is consistent with the hypothesis that with increased mossy fiber sprouting and synaptic connectivity, the granule cell layer becomes more susceptible to ictal-like events when inhibition is depressed.

Evidence for new recurrent excitatory circuits versus alternative mechanisms

When considered separately, part of the data in this study can be interpreted as resulting from a number of changes other than formation of excitatory circuits between granule cells. For example, increased amplitude and frequency of EPSCs support only indirectly the notion of increased excitatory synaptic input. Postsynaptic mechanisms, including changes in cell membrane properties and/or changes in glutamate receptor
number and/or function, could explain both the increase in frequency and amplitude of spontaneous EPSCs. Based only on this set of data, the argument can be made that the apparent increase in excitatory input may result not only from new excitatory connections between granule cells, but also from changes in synaptic projections from other areas including entorhinal cortex, hilar neurons (3), and/or from granule cells to CA3 (2). The data from the photostimulation experiments support the hypothesis of new excitatory synapses between granule cells (4). Dentate granule cell layer (DGL).

Conclusions

These results suggest that the kainate-induced injury triggers a process leading to increased seizure susceptibility of the dentate gyrus. A number of factors, including formation of new gap junctions between granule cells, altered inhibitory input to granule cells, and changes in glutamate and GABA receptor properties may contribute to the increased seizure susceptibility. However, our data indicate that synaptic reorganization plays a central role in the time-dependent changes in seizure susceptibility that follow an injury, such as the one produced by kainate-induced status epilepticus. Recent work has shown that kainate and pilocarpine treatments evoke axonal sprouting in CA1 pyramidal neurons cells (Esclapez et al. 1999; Perez et al. 1996), an increase in the spontaneous glutamatergic input to pyramidal cells (Esclapez et al. 1999), and an increased propensity to generate all-or-none, network bursts when inhibition is depressed (Meier and Dudek 1996; Smith and Dudek 2001). This suggests that injury-induced synaptic reorganization can produce epileptiform bursting not only in the dentate gyrus but also in other brain areas. The general hypothesis can be proposed that injury-induced synaptic reorganization accounts for the progressive formation of recurrent excitatory synapses resulting in an increased seizure probability.

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