Glutamate Injury–Induced Epileptogenesis in Hippocampal Neurons
An In Vitro Model of Stroke-Induced “Epilepsy”

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Background and Purpose—Stroke is the major cause of acquired epilepsy. The mechanisms of ischemia-induced epileptogenesis are not understood, but glutamate is associated with both ischemia-induced injury and epileptogenesis in several models. The objective of this study was to develop an in vitro model of epileptogenesis induced by glutamate injury in hippocampal neurons as observed during stroke.

Methods—Primary hippocampal cultures were exposed to 5 μmol/L glutamate for various durations. Whole-cell current clamp electrophysiology was used to monitor the acute effects of glutamate on neurons and chronic alterations in neuronal excitability up to 8 days after glutamate exposure.

Results—A single, 30-minute, 5-μmol/L glutamate exposure produced a subset of neurons that died and a larger population of injured neurons that survived. Neuronal injury was characterized by prolonged reversible membrane depolarization, loss of synaptic activity, and neuronal swelling. Surviving neurons manifested spontaneous, recurrent, epileptiform discharges in neural networks characterized by paroxysmal depolarizing shifts and high-frequency spike firing that persisted for the life of the culture.

Conclusions—This study demonstrates that glutamate injury produced a permanent epileptiform phenotype expressed as spontaneous, recurrent epileptiform discharges for the life of the hippocampal neuronal culture. These results suggest a novel in vitro model of glutamate injury–induced epileptogenesis that may help elucidate some of the mechanisms that underlie stroke-induced epilepsy. (Stroke. 2001;32:2344-2350.)

Key Words: epilepsy ■ excitotoxicity ■ glutamates ■ stroke

Epilepsy is one of the most common neurological disorders, affecting an estimated 40 to 50 million people worldwide. Approximately 30% to 50% of all epilepsy cases have a known cause and are termed acquired epilepsy. Cerebral ischemia, or stroke, is the most common cause of acquired epilepsy, accounting for ≈40% of these cases. Despite the important role of stroke in the development of epilepsy, little is known concerning the mechanisms by which an ischemic insult produces epilepsy.

Advances in the study of stroke, however, have demonstrated pathological events that may help elucidate the mechanisms underlying ischemia induced epilepsy. It has been shown that ischemia and anoxia during a stroke lead to massive release of the excitatory amino acid neurotransmitter glutamate, causing excessive activation of postsynaptic glutamate receptors believed to be the major cause of neuronal injury in stroke. In the core of a stroke, the majority of neurons undergo an irreversible membrane depolarization, the anoxic depolarization, and die forming an infarct. In the peri-infarct penumbra, the injury is less severe and produces a mixed population of neurons that undergo either irreversible anoxic depolarization and die or transient, reversible depolarization, the peri-infarct ischemic depolarization, and survive. Neurons that survive in the penumbra are the underlying substrates for ischemia-induced epileptogenesis.

As with stroke, glutamate receptor activation has been strongly associated with epileptogenesis. Therefore, we hypothesized that a less severe glutamate insult that produces prolonged, reversible neuronal depolarization like the glutamate injury in the penumbra could induce epileptogenesis in surviving neurons. To develop an in vitro model of stroke-induced epilepsy, this study was initiated to determine if an excitotoxic glutamate injury that produces prolonged, reversible depolarization could cause spontaneous, recurrent, epileptiform discharges (SREDs) that last for the life of hippocampal neurons in culture.

Materials and Methods

Unless otherwise noted, reagents were purchased from Sigma Chemical Co. Sodium pyruvate, minimum essential media containing

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Earle’s salts, fetal bovine serum, and horse serum were obtained from Gibco-BRL.

**Hippocampal Cell Culture**

Primary hippocampal cultures were prepared as described and characterized previously by our laboratory and confirmed to institutional guidelines. Briefly, hippocampal cells were prepared from 2-day postnatal Sprague-Dawley rats (Harlan) and plated on glial support layers at a density of 1 × 10⁶ cells/mL. Cultures were maintained at 37°C in a 5% CO₂/95% air atmosphere and fed twice weekly with neuronal feed. Cultures were used for experiments from 13 days in vitro through the life of the cultures (21 days). Glutamate concentrations in neuronal feed before culture feeding and at time of washout were analyzed by enzymatic fluorometric assay with a CMA 600 Microdialysis Analyser (CMA/Microdialysis) and were <1 μmol/L.

**Glutamate Injury**

At 13 days in vitro, neuronal media was removed, retained at 37°C, and replaced by a physiological treatment solution (145 mmol/L NaCl, 2.5 mmol/L KCl, 10 mmol/L N-[2-Hydroethyl]pipperazine-N'-[2-ethanesulfonic acid] (HEPES), 10 mmol/L glucose, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 2 μmol/L glycine, pH 7.3, osmolality adjusted to 325 mOsm with sucrose) in sham control cultures. Glutamate treatment was performed by supplementing this recording solution with 5 μmol/L glutamate. All exposures were performed at 37°C in the culture incubator. Glutamate exposure was terminated by 3 washes with recording solution and return of the original culture media.

**Neuronal Death Assay**

Neuronal death was assessed 24 hours after glutamate exposure using fluorescein diacetate (FDA)–propidium iodide (PI) microfluorometry. Neurons labeled with FDA or PI were quantified by means of the Ultraview image analysis software package (Perkin Elmer Life Sciences), and percent neuronal death was calculated as the number of neurons labeled by PI divided by the sum of the number of neurons labeled by PI and those labeled by FDA. Fluorescent images were compared with phase bright images to confirm that only pyramid-shaped neurons were counted. Three randomly selected fields were counted and averaged per culture (approximately 18 to 25 neurons per field).

**Measurements of Neuronal Injury**

Neuronal swelling was assessed before, during, and after exposure to 5 μmol/L glutamate with FDA. Images of FDA-stained neurons were captured as described above. The area of the neuronal soma before, during, and after exposure to 5 μmol/L glutamate was measured.

**Electrophysiology**

Whole-cell current-clamp recordings were performed on neurons by methods previously described in our laboratory. Cell culture media was replaced with a physiological recording solution (145 mmol/L NaCl, 2.5 mmol/L KCl, 10 mmol/L HEPES, 10 mmol/L glucose, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 0.5 μmol/L glycine, pH 7.3, osmolality adjusted to 325 mOsm with sucrose). Patch microelectrodes of 3 to 7 MΩ were filled with an internal solution of 140 mmol/L K⁺ gluconate, 1 mmol/L MgCl₂, 10 mmol/L HEPES, 1.1 mmol/L Ethylene glycol-bis (β-aminomethyl ether)-N, N',N',N’-tetraacetic acid (EGTA), 4 mmol/L Na₂ ATP, 15 mmol/L Tris Phosphocreatine, pH 7.2, osmolality adjusted to 310 mOsm with sucrose. Recordings were performed with an Axopatch 200A amplifier or an Axoclamp-2A amplifier (Axon Instruments) in current clamp mode. All data were digitized and stored on videocassette with a Neuro-corder DR-890 (Neurodata instruments Corp) and Sony VCR. In some experiments, hyperpolarizing square pulses (~0.5 nA, 200 ms) were injected every 5 seconds in the presence of 1 μmol/L tetrodotoxin to calculate input resistance. Data were later played back for analysis to a DASH IV chart recorder (Astro-Med Inc) or to a computer via a Digidata 1200 (Axon Instruments) and Strathclyde Electrophysiology Software (John Dempster, University of Strathclyde). Strathclyde Electrophysiology Software in event detector mode was used to calculate histograms of instantaneous spike frequency as defined as the inverse of the interval between 2 successive events. SREDs or electrographic seizures were defined as bursts of spike firing at a frequency of ≥3 Hz for durations of ≥20 seconds, analogous to electrographic seizures observed with EEG recordings. Neurons were categorized as “epileptic” on manifestation of 2 or more SREDs.

**Statistical Analysis**

Data are reported as mean±SEM. Student’s t test, 1-way ANOVA, or repeated-measures 1-way ANOVA and post hoc Tukey test were applied when appropriate, with SigmaStat 2.0 (Jandel Corp). For categoric data (percent of “epileptic” neurons), χ² tests were performed. A value of P<0.05 was considered statistically significant for all data analysis.

**Results**

**Glutamate Exposure Produces Hippocampal Neuronal Injury**

To determine if neurons exposed to 5 μmol/L glutamate underwent irreversible or reversible membrane depolarization, we performed whole-cell current-clamp experiments on neurons before, during, and after exposure to 5 μmol/L glutamate for 30 minutes. During glutamate exposure, neurons began to depolarize and spike briefly before succumbing to a larger prolonged depolarization (Figure 1A). This glutamate treatment did not produce continuous spiking but induced a membrane depolarization analogous to the depolarizations produced during ischemic or anoxic brain injury. This depolarization was also associated with a loss of synaptic potentials (Figure 1A). Figure 1B provides a quantitative analysis of the reversible depolarization. A smaller group of neurons (16%, 3 of 19 neurons) did not return to baseline potentials and manifested extended neuronal depolarization. In the neurons undergoing reversible depolarization (Figure 1, A and B), glutamate induced a significant and reversible decrease in membrane input resistance quantified in Figure 1C.

In addition, we evaluated neuronal swelling (Figure 1, D through F) as a measure of reversible injury by direct examination of fluorescein diacetate–stained pyramidal-shaped neurons (see Materials and Methods). Neurons swelled significantly, as assessed by increased somatic area (37±8% compared with baseline area, n=6 neurons, P<0.05). On washout of the glutamate, neuronal swelling decreased to 14±8% of baseline (n=6 neurons) within hours, not significantly different from preexposure value.

Glutamate exposure produced a mixed population of injured and dead neurons. Using the FDA/PI technique, we assessed the excitotoxicity of a 5 μmol/L glutamate exposure of increasing duration in hippocampal neuronal cultures 24 hours after treatment (Figure 2A). Glutamate exposure of 5 minutes resulted in 27±4% (n=8 cultures) neuronal death, not significantly different from sham controls (18±2%, n=13 cultures). Percent neuronal death increased significantly for...
Glutamate Induced Alterations in Neuronal Excitability

Because of the association between stroke and epilepsy, we hypothesized that neurons surviving the 5-μmol/L glutamate injury would display long-term changes in excitability. To test this hypothesis, we obtained current-clamp recordings from sham control neurons and neurons 1 to 8 days after exposure to glutamate. In the 30-minute treatment group, SREDs were observed in 86±4% of neurons per culture (n=17 cultures). “Epileptic” neurons were not observed in the sham control group and were observed significantly more often in the 30-minute treatment group than in the 5-minute or 90-minute groups (Figure 2B). The percentage of “epileptic” neurons ranged from 77% to 100% over the course of 1 to 8 days after exposure and was not significantly different from day to day (Figure 2C). SREDs were not observed immediately after glutamate exposure (Figure 1A) and were fully developed after a latency period of 24 hours after the injury.

A typical recording from a sham control neuron is shown in Figure 3A. Control neurons demonstrated spontaneous action potentials (spikes), excitatory postsynaptic potentials (EPSPs), and inhibitory postsynaptic potentials (IPSPs) typical of normal synaptic activity. Quantitative analysis of spike discharges in control neurons (n=12 neurons, over 4 hours of...
The SREDs or electrographic seizures in glutamate-injured neurons occurred spontaneously, randomly, and recurrently. In the 2.5-hour recording shown in Figure 5, 9 independent SREDs occurred, ranging in duration from 1.08 to 4.83 minutes. The average duration of SREDs and the average time interval between SREDs was 2.1±0.3 minutes and 7.2±1.0 minutes, respectively (n=10, over 7 hours of recording).

Neuronal Networks Displayed Synchronized SREDs After Glutamate Exposure
To determine if neurons with glutamate injury–induced SREDs were bursting in synchronized neural networks, we performed whole-cell current-clamp recordings on pairs of neurons 1 to 8 days after glutamate injury. Pairs of neurons ranged in distance from immediately adjacent to as far as 800 μm apart. Epileptiform discharges occurred simultaneously in 90% of neuron pairs (n=29 pairs). Both the onset and recording) revealed that the great majority of spike firings (88%) occurred in a frequency range <3 Hz (Figure 4A).

Occasionally, control neurons manifested spike discharges of >3 Hz. These bursts, however, never exceeded 10 seconds in duration (Figure 4C). Therefore, control neurons never manifested SREDs (see Materials and Methods).

Neurons treated with glutamate manifested SREDs (Figure 3B). The developing depolarizations (Figure 3C) started abruptly and were typical of the paroxysmal depolarizing shifts (PDSs) characteristic of epileptiform discharges.22 As episodes began to terminate, discrete PDSs became apparent. D, Regions of high-frequency spike firing throughout SRED (bars) are displayed at faster time scale. Initial depolarization of SRED triggered high-frequency spike firing of ~7 Hz. As the prolonged depolarization maximized, spike firing reached a frequency of 12 Hz, sufficient to reduce spike amplitude, presumably as the result of sodium channel inactivation. As the SRED began to terminate, PDSs still maintained high-frequency spike firing of 7 Hz.

Figure 4. Quantitative analysis of instantaneous spike frequency and high-frequency burst discharge duration revealed the presence of SREDs in glutamate treated neurons not seen in controls. A, Average histogram of instantaneous spike frequency with bins of 0.25 Hz determined from individual histograms of 12 control neurons making up >4 hours of recording. Majority of spike firing (61%) occurred at spike frequencies <0.25 Hz. Furthermore, 88% of all spikes had instantaneous frequency <3 Hz. B, Average histogram of instantaneous spike frequency with bins of 0.25 Hz determined from individual histograms of 10 neurons manifesting epileptiform activity (>7 hours of recording); 61% of all spike firing had instantaneous frequency >3 Hz.

C, Histogram of duration of high-frequency (<3 Hz) discharges in 12 control neurons. Bursts of >3 Hz never occurred for >10 seconds in control neurons. Therefore, no SREDs occurred in control neurons. D, Histogram of duration of high-frequency (>3 Hz) discharges in 10 glutamate-treated neurons; 60% of these discharges occurred with durations >20 seconds. These SREDs most often occurred with durations between 1 and 3 minutes. Glutamate-treated neurons also manifested high-frequency discharges of <10 seconds (31% of high-frequency discharges) during the interval between SREDs.
termination of the epileptiform discharges were highly synchronized (Figure 6B). Individual paroxysmal depolarizing shifts were also synchronized between neurons (Figure 6C). In addition, high-frequency spikes associated with both the prolonged initial depolarization and discrete PDSs near the end of SREDs occurred simultaneously (Figure 6D).

**Phenobarbital But Not Ethosuximide Inhibited Glutamate-Induced SREDs**

Phenobarbital terminated an ongoing SRED and reversibly inhibited the generation of SREDs (Figure 7A). On the other hand, ethosuximide, a t-type, voltage-gated Ca\(^{2+}\) channel blocker effective in treating generalized absence seizures,\(^{23}\) had no effect on SREDs in our model (Figure 7B). Thus, the SREDs induced by glutamate injury in our model responded to therapeutically relevant concentrations of anticonvulsants analogous to the setting of generalized tonic clonic and partial complex seizures.\(^{24}\)

**Discussion**

The experiments in this study document a new model of epileptogenesis in hippocampal neurons mediated by glutamate injury. Like the excitotoxic glutamate injuries associated with both ischemic and anoxic events,\(^{6}\) the excitotoxic injury in this model produced a mixed population of neurons characterized by both cell survival and cell death (Figure 2A). Glutamate exposure produced neuronal injury characterized by prolonged reversible membrane depolarization, decreased membrane input resistance, loss of synaptic potentials, and neuronal swelling (Figure 1). As suggested by our initial hypothesis, neurons that survived the glutamate exposure developed SREDS throughout the life of the cultures (Figure 2, B and C). SREDS occurred in 86±4% of the surviving neurons per culture (n = 17 cultures) and thus represented a model of glutamate injury–induced epileptogenesis. SREDS expressed many characteristics of overt epileptic seizures. SREDS started and terminated spontaneously (Figures 3 and 5) and were synchronized in nature (Figure 6). The SREDS produced by excitotoxict glutamate injury manifested PDSs that were typical of epileptiform activity\(^{22}\) with bursts of high-frequency spike firing (Figure 3) >3 Hz for 20 seconds or longer (Figures 4, B and D). Sham control neurons never displayed SREDS (Figure 3 and Figure 4, B and D, and Figure 6). Finally, the SREDS produced by glutamate injury responded to the anticonvulsant drug phenobarbital but not to ethosuximide (Figure 7). These results demonstrated that hippocampal cultures subjected to injury by glutamate exposure could be transformed into neuronal networks manifesting SREDS for the life of the culture, producing an in vitro model of epilepsy.

The involvement of glutamate in epileptogenesis has been implicated in whole animal,\(^{12–14}\) slice,\(^{15,16}\) and tissue culture models\(^{17}\) of epilepsy. To induce epileptogenesis, these models all used continuous neuronal spiking produced by sei-
Glutamate injury–induced epileptogenesis is clearly distinct from the low magnesium model of epileptogenesis and represents a separate cause of acquired epilepsy. These models use distinctly different physiological inputs. Whereas the low magnesium model uses continuous spike firing analogous to the status epilepticus to produce SREDs, the glutamate injury model used a prolonged, reversible depolarization to induce epileptogenesis, analogous to the peri-infarct ischemic depolarizations in ischemic stroke. Thus, two physiologically distinct mechanisms are used to induce epileptogenesis in these two models of epilepsy. In addition, the duration of the epileptogenic stimulus is very different between the glutamate injury model and the low magnesium model. The low magnesium model requires 3 hours of spike activity to induce SREDs, whereas only 30 minutes of glutamate exposure produces “epileptic” neurons in the glutamate injury model. The element of excitotoxic neuronal death in the glutamate injury model is another distinction from the low magnesium model. The low magnesium model is associated with little neuronal death. Thus, the induction mechanisms in the glutamate injury model and low magnesium model are as different as stroke and status epilepticus, known causes of acquired epilepsy. However, the inducers in these models may share common underlying mechanisms.

The low magnesium model requires calcium entry through the NMDAR as a second messenger for epileptogenesis. Results from our laboratory suggest that calcium may also act as a second messenger system in the glutamate injury model. Thus, although the models use the distinctly different physiological inputs of glutamate-induced depolarization and spike activity, they may converge on related molecular mechanisms for inducing and maintaining the epileptic condition. The potential role of selective neuronal death in glutamate injury–induced epileptogenesis requires further investigation, especially in light of the fact that inhibitory neurons are typically less vulnerable to excitotoxicity than excitatory neurons. Potential roles of selective neuronal death in glutamate injury–induced epileptogenesis requires further investigation, especially in light of the fact that inhibitory neurons are typically less vulnerable to excitotoxicity than excitatory neurons. Although differential cell death may affect the balance between the number of inhibitory and excitatory neurons, resulting in a larger number of surviving inhibitory neurons, the glutamate-induced injury produced “epilepsy” in the neurons despite the potential alterations in neuronal subpopulations. Further studies are needed to determine the role of selective cell death in this model. In addition, the possible roles of gap junctions, ischemia-induced alterations in second-messenger systems, and gene changes in mediating epileptogenesis represent important future directions for research that can be conveniently studied in this system.

The association between stroke and epilepsy has been demonstrated clinically, and stroke is the most common cause of acquired epilepsy. However, the mechanisms by which cerebral ischemia initiates epileptogenesis are not understood. The glutamate injury produced in this model of epileptogenesis resembles some of the phenomena associated with stroke. Increases in extracellular glutamate, reversible depolarization with loss of synaptic activity, acute neuronal swelling,
and excitotoxic delayed neuronal death associated with the ischemic penumbra are all present in this model. To our knowledge, this study demonstrates, for the first time, spontaneous, recurrent, epileptiform activity in hippocampal neurons induced by glutamate injury. This model of glutamate injury–induced epileptogenesis may offer new insights into the development and maintenance of the epileptic condition after a neurological trauma such as stroke and therefore may provide therapeutic strategies to develop both novel antiepileptic and anticonvulsant agents to prevent stroke-induced epilepsy.

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