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Calcium permeable AMPA receptors are expressed in a rodent model of status epilepticus

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Abstract

Objective—To characterize the plasticity of AMPA receptor (AMPA)-mediated neurotransmission in the hippocampus during status epilepticus (SE).

Methods—SE was induced by pilocarpine and animals were studied after 10 min (refractory-SE) or 60 min (late-SE) of the first grade V seizures. AMPAR-mediated currents were recorded from CA1 pyramidal neurons and dentate granule cells (DGCs) by voltage-clamp technique. The surface expression of GluA2 subunit on hippocampal membranes was determined using a biotinylation assay. GluA2 internalization and changes in intracellular calcium ($[Ca]_i$) levels were studied in hippocampal cultures using immunocytochemical and live-imaging techniques. AMPAR antagonist treatment of SE was evaluated by video and EEG.

Results—AMPA-mediated currents recorded from CA1 neurons from refractory and late SE animals were inwardly-rectifying and philanthotoxin-sensitive; similar changes were observed in recordings obtained from DGCs from refractory SE animals. GluA2 subunit surface expression was reduced in the hippocampus during refractory and late SE. In cultured hippocampal pyramidal neurons, recurrent bursting diminished surface expression of the GluA2 subunit and enhanced its internalization rate. Recurrent bursting-induced increase in $[Ca]_i$ levels was reduced by selective inhibition of GluA2-lacking AMPARs. GYKI-52466 terminated diazepam-refractory SE.

Interpretation—During SE, there is rapid, ongoing plasticity of AMPARs with the expression of GluA2-lacking AMPARs. These receptors provide another source of Ca^{2+} entry into the principal neurons. Benzodiazepam-refractory SE can be terminated by AMPAR antagonism. The data identify AMPARs as potential therapeutic target for the treatment of SE.

Introduction

Status epilepticus (SE), a prolonged self-sustaining seizure, is associated with significant mortality and morbidity including neuronal death, cognitive dysfunction and other systemic complications^{1,2}. Ionotropic glutamatergic receptors contribute to seizure sustenance and SE-induced cell death^{3,4}; however, the plasticity of glutamatergic transmission during SE has not been studied. Evidence that AMPA receptor (AMPA) antagonists can terminate kainic acid (KA)-induced SE^{5,6} raised the possibility that AMPAR-mediated neurotransmission is modified during SE. The strengthening of AMPAR-mediated synaptic transmission in response to physiological and pathophysiological stimuli is frequently associated with the synaptic incorporation of GluA2-lacking receptors⁷⁻⁹. There is an increased expression of GluA2-lacking AMPARs following hypoxia-induced seizures in neonatal animals¹⁰⁻¹²; however it is unclear if similar changes occur during SE in adult animals.

In this study, we tested the hypothesis that GluA2-lacking AMPARs are expressed on hippocampal principal neurons during SE. Further, we tested whether AMPAR antagonist GYKI-52466 can terminate diazepam-refractory SE.

Methods

All studies were performed in accordance with protocols approved by the Animal Care and Use Committee (ACUC) of the University of Virginia.

Pilocarpine-induced SE

For electrophysiological and biochemical studies adult male rats (170-220 g) were injected with pilocarpine (275-320 mg/kg IP) and monitored continuously for behavioral seizures. After 10 or 60 min of the first observed grade 5 seizure¹³, the animals were anesthetized with isoflurane and decapitated for preparation of hippocampal slices. The efficacy of GYKI-52466 on pilocarpine induced SE was tested using adult mice (25 - 30 g). Bipolar insulated stainless steel electrodes were implanted stereotactically over the hippocampus and cortex of the animals and they were allowed to recover for 5 – 7 days. After the recovery period, SE was induced by administration of pilocarpine and seizures were monitored for 5 hours by video-EEG as previously described¹⁴. SE onset was characterized by continuous electrographic spike wave and polyspike discharges with a frequency greater than 3 Hz. SE was considered terminated when the EEG returned back to baseline and spiking activity was arrhythmic and below 2 Hz during a 3 hr monitoring period. Diazepam or GYKI-52466 was injected intraperitoneally within the first 5 min of the onset of continuous seizures.

Hippocampal slice preparation

Horizontal hippocampal slices (300 μ m) were prepared with a vibratome (Leica VT1200S, Germany) using ice-cold oxygenated dissection buffer (4°C, 95% O₂, 5% CO₂) containing (in mM) 65.5 NaCl, 2 KCl, 5 MgSO₄, 1.1 KH₂PO₄, 1 CaCl₂, 10 dextrose, and 113 sucrose (300 mOsm). The slices were then placed in an interface chamber containing oxygenated artificial CSF (aCSF) at 30°C and allowed to equilibrate for 30 min. The aCSF contained (in mM) 124 NaCl, 4 KCl, 1 MgCl₂, 25.7 NaHCO₃, 1.1 KH₂PO₄, 10 dextrose, and 2.5 CaCl₂ (osmolality 300 mOsm). All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Hippocampal neuronal cultures

Hippocampal cultures were prepared as described previously^{15,16}. The experiments were performed on 16-18 days in vitro (DIV) high-density cultures consisting of 100,000 cells plated on 35 mm poly-L-lysine-coated coverslips.

Electrophysiology

The properties of AMPAR-mediated transmission on CA1 pyramidal neurons (CA1 neurons) and DGCs in hippocampal slices obtained from animals that experienced pilocarpine-induced SE and age-matched control animals were determined using whole-cell voltage clamp electrophysiology. The studies were performed on animals that had seizures for 10 or 60 min from their first stage 5 seizure¹³. Seizures become refractory to diazepam treatment when it is administered 10 or more minutes after the first stage 3 seizure¹⁷; the group of animals studied after 10 min of first stage 5 seizure will be referred to as “refractory SE” and the recordings obtained from the CA1 pyramidal neurons (CA1 neurons) and DGCs from this group as “refractory SE-CA1 neurons” and “refractory SE-DGC,” respectively. Another group of animals were studied 60 min after experiencing the

first stage 5 seizures, which will be referred to as “late SE.” The detailed procedure for electrophysiological studies is described in *Supplementary Materials and Methods*.

Biotinylation

Biotinylation and gel electrophoresis were performed as previously described¹⁸. The detailed procedure is described in *Supplementary Materials and Methods*.

Immunocytochemistry

To determine the changes in the surface expression and the rate of intracellular accumulation of GluA2-subunit containing AMPARs during 0-Mg²⁺-induced bursting, cultured pyramidal neurons (14-19 days *in vitro*) were studied. The trypan blue dye exclusion assay was performed to determine membrane integrity of neurons exposed to 0-Mg²⁺ aCSF. The detailed procedures are described in *Supplementary Materials and Methods*.

Calcium Imaging

The changes in [Ca]_i levels were studied in cultured pyramidal neurons using laser scanning confocal microscopy. Calcium dye, Fluo-4AM was used to detect changes in levels of [Ca]_i. The procedure is described in detail in *Supplementary Materials and Methods*.

Results

GluA2-lacking AMPARs are expressed on CA1 pyramidal neurons during refractory- and late SE

Control and SE-CA1 neurons were voltage clamped at various potentials ranging from -70 mV to +40 mV, and eEPSCs were recorded by the electrical stimulation of the Schaffer collateral pathway (Fig. 1A). In control CA1 neurons (n = 7 cells / 4 animals), the amplitude of eEPSCs recorded at -40 mV was equal to that evoked at +40 mV. In contrast, in recordings obtained from refractory SE-CA1 neurons (n = 11 cells / 5 animals), the amplitude of eEPSCs evoked at +40 mV was smaller than those recorded at -40 mV, suggesting an inward rectification of AMPAR currents. The current-voltage (I-V) relationship for mean eEPSCs recorded from control CA1 neurons was linear, but in recordings from refractory SE-CA1 neurons, the currents rectified at depolarized potentials (Fig 1B). The rectification index (RI), in refractory SE-CA1 neurons was significantly smaller (mean RI = 0.48 ± 0.08) than in control CA1 neurons (0.87 ± 0.04, p < 0.001, t-test).

Inward rectification of eEPSCs in SE-CA1 neurons suggested that AMPARs on Schaffer collateral-CA1 pyramidal neuron synapses lacked GluA2 subunits¹⁹. These receptors can be blocked by the polyamine, philanthotoxin-433 (PhTx)²⁰. PhTx (50 μM), did not alter the amplitudes of eEPSCs recorded from control CA1 neurons but diminished those recorded from refractory SE-CA1 neurons (Fig 1C). PhTx diminished eEPSC amplitude in refractory SE-CA1 neurons from -95.19 ± 17.6 pA to -81.5 ± 15.1 pA (n = 6 cells, 5 animals; p < 0.01, paired t test), whereas it did not alter the amplitude of eEPSCs recorded from control neurons. The mean eEPSC amplitude in control-CA1 neurons was -181.6 ± 34.5 pA and after application of PhTx it was -175.2 ± 30.42 pA (n = 6 cells, 5 animals; p > 0.05, paired t test). These observations further support the presence of GluA2-lacking AMPARs on refractory SE-CA1 neurons.

Activity-dependent plasticity of AMPARs on pyramidal neurons can be transient⁷. We therefore tested whether SE-induced AMPAR plasticity persisted into late SE. Similar to refractory SE-CA1 neurons, the eEPSC recordings obtained from late SE-CA1 neurons at +40 mV had smaller currents, compared with those recorded at -40 mV (Fig. 1B). The mean RI of eEPSCs recorded from late SE-CA1 neurons was 0.34 ± 0.06 (n = 7 cells / 6 animals,

$p < 0.05$, t-test). The eEPSCs recorded from these neurons were also inhibited by PhTx (baseline, 106 ± 13.2 pA vs. PhTx, -80.98 ± 16.16 pA, 7 cells, 5 animals; $p < 0.05$, paired t test; Fig. 1C).

DGCs expressed AMPARs with properties similar to those of GluA2-lacking receptors during refractory SE

To study whether the properties of AMPARs on DGCs were also modified during SE, eEPSCs were recorded from DGCs by electrical stimulation of the perforant pathway. The eEPSCs recorded from control DGCs ($n = 9$ cells / 5 animals) had similar evoked response amplitudes at both +40 and -40 mV. The I-V relationship for the mean eEPSCs was linear (mean RI = 0.85 ± 0.10 ; Fig. 2A). In contrast, the amplitude of eEPSCs recorded at +40 mV was smaller, compared with those recorded at -40 mV in 7 of 12 refractory SE-DGCs ($n = 7$ animals), and the I-V relationship for the mean eEPSCs revealed that the currents were inwardly rectifying (mean RI = 0.56 ± 0.09 , $p < 0.01$, t-test; Fig. 2B). The amplitude of eEPSCs recorded from refractory SE-DGCs was diminished by PhTx (baseline, -138.8 ± 20.6 pA vs. PhTx, -117.9 ± 18.6 pA, $n = 8$ cells, 5 animals; $p < 0.01$, paired t test; Fig 2C) compared to that in control-DGCs (baseline, -136.1 ± 20.4 pA vs. PhTx, -125.9 ± 19.3 pA, $n = 6$ cells, 3 animals; $p > 0.05$, paired t test; Fig 2C).

AMPA plasticity in DGCs did not persist into late SE. The amplitudes of eEPSCs recorded at +40 mV and -40 mV were similar, and the I-V relationship for the mean eEPSCs recorded from late SE-DGCs ($n = 11$ cells / 7 animals) was linear (mean RI = 0.88 ± 0.09 , $p > 0.05$, t-test; Fig. 2B). Further, similar to control DGCs, the application of PhTx did not diminish eEPSC amplitude in late SE-DGCs (baseline, -100.2 ± 8.6 pA vs. PhTx, -98.33 ± 8.3 pA, $n = 7$ cells, 5 animals; $p > 0.05$, paired t test). Thus, there was a transient expression of GluA2-lacking AMPARs on DGCs during SE, whereas it persisted on CA1 neurons. These findings were confirmed using a biochemical assay.

Surface expression of the GluA2 was reduced in hippocampi of animals that experience refractory or late SE

The surface expression of the GluA2 subunit in hippocampal slices of control, refractory and late SE animals were compared using a biotinylation pull-down assay. Representative Western blots (Fig. 3A) demonstrate reduced surface membrane expression of the GluA2 subunit in hippocampal slices from refractory SE animals compared to controls. The hippocampi of refractory SE animals and controls expressed similar amount of GluA2 protein ($p > 0.05$, t test). Surface GluA2 signal was normalized to the total expression, and the expression ratio in refractory SE hippocampi was less than that in control hippocampi (0.14 ± 0.05 vs. 0.23 ± 0.06 , $n = 8$; $p < 0.05$, t test). β -actin, a cytoplasmic protein, was not detected in the surface membrane fraction and its expression was similar in the total protein fraction of both control and refractory SE ($p > 0.05$, t test) group. This confirmed the purity of the surface membrane proteins and equal protein loading.

The changes in the surface expression of the GluA2 subunit during late SE were determined. Similar to refractory SE animals, there was less surface membrane-bound GluA2 protein in late SE hippocampi compared to control hippocampi (Fig. 3B). The ratio of surface to total expression in the hippocampi of late SE animals was 0.08 ± 0.01 , and 0.18 ± 0.02 in control animals ($n = 6$, $p < 0.05$, t test). Total GluA2 subunit expression was similar in hippocampi obtained from late SE and control ($p > 0.05$, t test) animals. β -actin expression was also similar in both control and late SE ($p > 0.05$, t test) groups.

SE induced cell injury could potentially cause in a general reduction in the surface expression of AMPARs; therefore changes in the surface expression of the GluA1 subunit

during SE were also determined. The fraction of surface expressed GluA1 subunit was greater in hippocampi of refractory SE (control vs. SE, 0.15 ± 0.06 vs. 0.45 ± 0.12 , $n = 6$; $p < 0.05$, t test) and late SE (control vs. SE, 0.23 ± 0.10 vs. 0.56 ± 0.15 , $n = 3$; $p < 0.05$, t test) animals. Together, these data demonstrate that distinct alterations, not cell injury, accounts for changes in the trafficking of AMPAR subunits during SE.

0-Mg²⁺-induced bursting in cultured pyramidal neurons decreased the surface expression of the GluA2 subunit, in part by increased rate of internalization

To further understand the mechanistic basis of AMPAR plasticity during SE, we used an *in vitro* model of prolonged recurrent bursting of cultured hippocampal pyramidal neurons¹⁶. Membrane potential was recorded from cultured neurons (14-19 DIV) in aCSF and then after perfusion with 0-Mg²⁺ aCSF. The mean resting membrane potential in aCSF was -68.48 ± 0.87 mV ($n = 6$ cells). The cells were then perfused with 0-Mg²⁺ aCSF, which depolarized the membrane to -51.75 ± 1.7 mV. We observed superimposed bursts of action potentials (data not shown). After 10 min, the membrane potential returned to baseline levels (-67.5 ± 2.1 mV), but the cells continued to fire bursts of action potentials, as we demonstrated previously¹⁶.

The surface expression of GluA2 subunits during 0-Mg²⁺-induced recurrent bursting was examined by treating cultures in parallel with aCSF or 0-Mg²⁺ aCSF for durations of 5, 10, 15, 20 or 30 min at 37°C before fixation. The non-permeabilized cultures were then incubated with an antibody directed against an epitope in the extracellular domain. Data were obtained from 3 replicates of 15-20 randomly selected neurons in each condition and GluA2 subunit surface expression was quantified by calculating the ratio of total area of surface immunoreactivity to that of the total cell area. In control neurons, the area of surface immunoreactivity of the GluA2 subunit was similar at each time point (Fig. 4A, B), but surface immunoreactivity was markedly reduced after 30 min of exposure to 0-Mg²⁺ aCSF (Fig. 4C, D). A study of the time course revealed a rapid decline in surface immunoreactivity of the GluA2 subunit following 0-Mg²⁺-induced recurrent bursting (Fig. 4E). After 30 min of exposure of 0-Mg²⁺ aCSF, the surface GluA2 immunoreactivity in treated neurons was ~70% less (0.13 ± 0.02) than that at 0 min (0.44 ± 0.06 , $p < 0.0001$, ANOVA followed by Dunnet's multiple comparison test). In contrast, surface GluA2-IR was similar after both 0 (0.54 ± 0.08) and 30 (0.41 ± 0.08 , $p > 0.05$, ANOVA) min incubation in aCSF. Thus, the reduction in surface expression of GluA2 protein observed during SE induced *in vivo* was replicated in an *in vitro* model.

To test whether 0-Mg²⁺ - induced bursting caused non-specific receptor internalization due to cell injury, the membrane integrity was monitored at 0, 10, 20 and 30 min of 0-Mg²⁺ treatment using a trypan blue dye exclusion assay. Cells treated with regular aCSF or Triton X-100 was used as negative and positive controls, respectively. All neurons treated with Triton X-100 showed intake of trypan blue, and this was significantly greater than in cells treated with aCSF or 0-Mg²⁺-aCSF ($p < 0.0001$, Chi square test). Cells treated with aCSF or 0-Mg²⁺-aCSF showed no difference ($p > 0.05$, Chi square test) in the proportion of trypan blue uptake at 10, 20 and 30 min. Thus, reduced surface expression of GluA2 subunit containing AMPARs during 0-Mg²⁺-induced bursting is unlikely due to cell injury.

Increased internalization may likely account for reduced surface expression of GluA2 subunit during 0-Mg²⁺-induced bursting. The rate of internalization was studied using an antibody feeding assay²¹. In control neurons, GluA2 immunoreactivity on the cell surface diminished with time and then began to stabilize (Fig. 5A, E). In contrast, GluA2 immunoreactivity in neurons maintained in 0-Mg²⁺ aCSF rapidly diminished and remained low (Fig. 5B, E). The decrease in surface expression also correlated with an increase in internalization (Fig. 5D). The data for the disappearance of the GluA2 subunit

immunoreactivity were fit to an equation for a single-phase association, and the best fit revealed that the rate of internalization in control neurons was $0.051 \pm 0.01 \text{ min}^{-1}$ with a time constant (τ) of 19.52 min (Fig. 5B, E). In contrast, the rate constant for disappearance of GluA2 immunoreactivity from cell surface in neurons maintained in 0-Mg^{2+} aCSF was $0.175 \pm 0.07 \text{ min}^{-1}$ with $\tau = 5.74 \text{ min}$ (Fig. 5F). These findings suggest that the reduced surface expression of GluA2 subunit during 0-Mg^{2+} -induced bursting is mediated, at least in part, by its accelerated internalization.

Increase in $[\text{Ca}]_i$ accumulation during 0-Mg^{2+} -induced SE in pyramidal neurons is attenuated by blockade of GluA2-lacking AMPARs

During 0-Mg^{2+} -induced bursting, intracellular calcium accumulates in the neurons²². If GluA2-lacking (and therefore calcium permeable) AMPARs are expressed during 0-Mg^{2+} -induced bursting, then blockade of these receptors should reduce $[\text{Ca}]_i$ accumulation. Exposure of neurons to 0-Mg^{2+} aCSF caused an exponential increase in Ca^{2+} fluorescence for 30 min with minimal associated blebbing (Fig. 6A). Within 10 min of 0-Mg^{2+} aCSF exposure, Ca^{2+} fluorescence increased to $53.7 \pm 3.9\%$ of its maximum ($n = 9$ cells). Application of IEM-1460 ($100\mu\text{M}$, a concentration that selectively blocks GluA2-lacking AMPARs) after 10 min of exposure to 0-Mg^{2+} aCSF resulted in either a stabilization or reduction in fluorescence signals (upto 20% of its maximum) in some regions of the dendritic processes ($n = 10$; Fig. 6A). After 20 min of IEM-1460 application, there was a significant reduction ($p < 0.05$, ANOVA) in fluorescence compared to that in bursting neurons. Thus, $[\text{Ca}]_i$ accumulation was slower in IEM-1460-treated neurons. Application of IEM-1460 to neurons maintained in aCSF did not show any change in fluorescence (data not shown).

To determine the rate of increase in $[\text{Ca}]_i$, the data were fitted to a single-exponential growth equation: intracellular accumulation (time) = $Y_{\text{max}} + [Y_{\text{max}} - Y_{\text{min}}][1 - e^{(K \times \text{time})}]$, where Y_{min} is the fluorescence at time 0, Y_{max} the maximum derived fluorescence, and K the rate constant of $[\text{Ca}]_i$. The rate constant $[\text{Ca}]_i$ accumulation during 0-Mg^{2+} treatment was $0.06 \pm 0.08 \text{ min}^{-1}$ and in IEM-1460-treated neurons it was $0.21 \pm 0.17 \text{ min}^{-1}$ (Fig. 6B). IEM-1460-induced reduction in $[\text{Ca}]_i$ levels was not due to reduction or termination of bursting activity because perfusion with IEM-1460 after 10 min of 0-Mg^{2+} -induced bursting did not terminate or reduce bursting over the next 30 min observed (data not shown).

AMPA-specific antagonist, GYKI-52466, successfully terminated diazepam-refractory pilocarpine-induced SE

AMPA antagonists have been previously shown to terminate KA-induced SE^{5,6}; however, the severity and pathology of pilocarpine-induced SE is significantly greater than that induced by KA²³. We tested whether AMPAR antagonist could terminate diazepam-refractory SE induced by pilocarpine. Treatment was initiated after the onset of continuous electrographic seizures. The onset of SE was characterized by the development of high amplitude and rhythmic spike wave and polyspike discharges with frequency greater than 3 Hz. This is the stage of seizures that is refractory to benzodiazepines in humans and rats²⁴⁻²⁶. Figure 7 shows representative EEG traces recorded from hippocampus and cortex of animals following pilocarpine induced SE and after drug treatments. The time to the onset of continuous electrographic seizures was $31.2 \pm 2.3 \text{ min}$ ($n = 45$). To test whether animals were refractory to benzodiazepines, the animals were treated with either diazepam (30 mg/kg , i.p) within 5 min of the appearance of continuous electrographic discharges and seizure termination was monitored for the next 3 hr. Treatment with diazepam (30 mg/kg , i.p; Fig 7A) did not terminate SE in any animal ($n = 7$). The animals continued to have seizures over the next 3 hr and 3/7 animals died. These observations indicated that the animals experienced diazepam-refractory SE.

A dose-response study was then performed to test the ability of GYKI-52466 to terminate SE. GYKI-52466 dose-dependently terminated SE (Fig 7A, B). At 30 mg/kg, GYKI-52466 did not terminate SE in any animal ($n = 5$). A dose of 50 mg/kg terminated SE within 3 hr in 3/5 animals in 106.04 ± 47.2 min; in 20% of the animals, SE was terminated in 17.3 min. A higher dose of GYKI-52466 (75 mg/kg) terminated seizures in 6/6 animals (100%) and the mean duration to SE termination was 63.17 ± 25.2 min; in 66.7% animals, SE was terminated in 24.5 ± 5.9 min of treatment. A dose of 100 mg/kg terminated SE in all animals ($n = 7$) and the mean duration of SE termination was 59.2 ± 28.3 min. In 5/7 animals (71.4%) SE was terminated in 14.9 ± 2.5 min. The dose – response relationship for seizure termination by GYKI-52466 within 1 hr of treatment could be well fit by a sigmoidal function (Fig 7C). The dose required to terminate seizures in 50% of animals within 1 hr of drug administration (ED_{50}) was 56.15 mg/kg.

Discussion

The major findings of the present study are 1) AMPAR-mediated currents recorded from CA1 neurons and DGCs became inwardly rectifying and philanthotoxin sensitive during refractory SE, and those recorded from CA1 neurons during late SE also had these properties; 2) the surface expression of the AMPAR GluA2 subunit was reduced during SE on hippocampal membranes obtained from refractory and late SE animals; 3) recurrent bursting in cultured hippocampal pyramidal neurons increased the rate of intracellular accumulation of GluA2 subunits and reduced its surface expression; and 4) GluA2-lacking AMPARs contributed to the elevation of $[Ca]_i$ during recurrent bursting. Together, these findings provide evidence for a dynamic, neuron-specific plasticity in AMPARs and the expression of GluA2-lacking AMPARs during SE.

The current study demonstrates for the first time that SE is associated with dynamic alterations in the AMPAR trafficking resulting in the functional expression of GluA2-lacking AMPARs in both DGCs and CA1 neurons during refractory SE and in CA1 neurons during late SE. Prior studies have found a transcriptional downregulation of the GluA2 subunit after hours or days after kindling²⁷, repeated chemically-induced seizures²⁸, or SE^{29,30}. However, the mechanisms that sustain SE likely involve the rapid synaptic plasticity of neurotransmitter receptors involving altered receptor trafficking. GluA2-lacking AMPARs have distinct biophysical characteristics, including greater conductance compared to GluA2-containing receptors, calcium permeability, and inwardly rectifying currents³¹. An increased expression of GluA2-lacking AMPARs has also been reported in models of neonatal seizures^{10,12}; however these receptors are functionally expressed until the 3rd week of life in rodents³². In contrast, AMPARs on adult principal neurons are predominantly expressed as calcium-impermeable heteromers comprising the GluA2 subunit; and the expression of GluA2-lacking AMPARs can not only enhance synaptic strength, but can also affect neuronal survival³³. In this study, neurons were studied at two distinct stages. While the refractory SE stage is characterized by resistance to benzodiazepines and continuous seizures, late SE is characterized by seizures interspersed with periodic discharges which are considered a marker for the onset of neuronal damage^{26,34}. The physiological significance of the transient expression of GluA2 subunit lacking AMPARs on DGCs during refractory SE is presently unclear; however, persistent expression of GluA2-lacking AMPARs on CA1 pyramidal neurons during SE could result in the prolonged elevation of $[Ca]_i$ levels that can contribute to excitotoxicity and potentially the development of epilepsy^{29,35}. In addition, prolonged expression of these receptors can also contribute to cell death via their enhanced permeability to Zn^{2+} ³⁶⁻³⁸.

Prolonged 0-Mg²⁺ induced bursting *in vitro* mimics many features observed *in vivo*. 0-Mg²⁺ exposure results in bursts of action potential discharges riding on paroxysmal depolarizing

shifts and the development of synchronous, electrographic discharges with similar electrographic properties observed in humans³⁹⁻⁴¹. Further, we have demonstrated that 0-Mg²⁺-induced bursting causes changes in the properties of GABA_A receptor similar to that observed in hippocampal slices obtained from animals that experienced SE for 1 hour including diminished mIPSC amplitude and surface expression of the GABA_A receptor γ 2 subunit^{21,42}.

The reduction in surface expression of GluA2 subunit was specific and unlikely due to cell injury because reduction in GluA2 subunit surface expression was associated with an increased expression of GluA1 subunit in hippocampi of animals experiencing SE. Further, the trypan blue dye exclusion assay did not produce evidence for cell injury in 0-Mg²⁺ treated cultures consistent with previous studies performed with propidium iodide uptake⁴³.

The mechanisms underlying the accelerated intracellular accumulation of the GluA2 subunit-containing AMPARs during refractory and late SE were not explored in the current study. It is possible that increased [Ca]_i during SE or 0-Mg²⁺-induced recurrent bursting activated post translational mechanisms that reduce surface expression of the GluA2 subunit containing AMPARs⁴⁴. The GluA2 subunit is internalized when it is phosphorylated by PKC α at the serine residue 880⁴⁵. This process is facilitated by its interaction with the PICK1-PKC α protein complex^{46,47}. In a recent report, Rakhade et al. demonstrated that increased phosphorylation of the GluA2 subunit at the serine residue 880 following hypoxia-induced seizures in neonatal rat was associated with enhanced functional expression of GluA2-lacking AMPARs¹². It is possible that pilocarpine-induced SE in adult animals similarly alters the phosphorylation of the GluA2 subunit. Alternately, accelerated endocytosis of the GluA2 subunit may be mediated by seizure-induced expression of the immediate early gene, activity-regulated cytoskeleton-associated gene (*Arc*)^{48,49}. *Arc* expression is induced in both the DGCs and CA1 neurons as early as 30 min following pilocarpine-induced SE⁵⁰. In an earlier report, Teber et al.⁵¹ demonstrated that even sub convulsive doses of pilocarpine induced a rapid expression of *Arc* in rat forebrain. Further studies are necessary to clarify whether *Arc* activation-induced GluA2 subunit endocytosis occurs during pilocarpine-induced SE in this study.

Several studies have found a transcriptional downregulation of the GluA2 subunit following SE²⁷⁻³⁰. One mechanism that is suggested to contribute to the transcriptional repression of the GluA2 subunit is the up-regulation of NRSF following SE (eg.,^{30,52}). A recent study elegantly demonstrated that preventing the interaction of NRSF with their target genes, that include among other, HCN1 and GluA2 genes, modified epileptogenesis⁵². There is also growing evidence that activity dependent internalization can also regulate transcription⁵³, suggesting the unexplored possibility that SE-induced internalization of the GluA2 subunit provides the signal for its genomic downregulation.

Recent reports on the ability of AMPAR antagonists^{5,6} to terminate KA-induced SE suggested that AMPAR-mediated transmission can be targeted to treat SE. The present study extends and confirms these observations using a pilocarpine model of SE. While similar to KA model, the intensity of seizures and neuronal damage induced by pilocarpine model are more severe²³. Together, the results of this study reveal significant plasticity of AMPARs during SE that may serve to regionally drive seizure activity and contribute to excitotoxicity. AMPAR blockade may be a promising therapeutic alternative to treat SE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

AMPA-mediated postsynaptic currents recorded from CA1 neurons of animals that had experienced refractory or late SE were rectifying and philanthotoxin sensitive. **A**, Representative superimposed averaged traces of AMPAR-mediated EPSCs evoked by Schaffer collateral stimulation and recorded from CA1 neurons obtained from control (black), refractory (red), and late (blue) SE animals. **B**, Plots of current-voltage (I-V) relationships for the recordings in panel **A**. **C**, Representative averaged traces of eEPSCs recorded from CA1 neurons from control, refractory and late SE animals before (black) and after (red) perfusion of philanthotoxin-433 (50 μ M).

Figure 2.

AMPA-mediated postsynaptic currents recorded from DGCs from animals that had experienced refractory SE, but not late SE, were rectifying and philanthotoxin sensitive. **A**, Representative superimposed averaged traces of eEPSCs recorded from DGCs obtained from control (black), refractory (red), and late (blue) SE animals. **B**, Current-voltage (I-V) relationships for the recordings shown in panel **A** demonstrate inward rectification of currents in DGCs obtained from refractory SE but not late SE animals. **C**, Representative averaged traces of eEPSCs recorded from CA1 neurons from control, refractory SE animals before (black) and after (red) perfusion of philanthotoxin-433 (50 μ M).

Figure 3.

Surface expression of the GluA2 subunit-containing AMPAR was reduced in hippocampus during SE. **A**, Sample Western blots of the total and surface protein fractions of GluA2 subunits in hippocampal slices obtained from refractory SE animals and age-matched controls. Note the absence of β -actin bands in surface samples confirming the purity of surface samples. **B**, Sample Western blots of the total and surface protein fractions of GluA2 subunits in hippocampal slices obtained from late SE animals and age-matched controls.

Figure 4.

Surface expression of the GluA2 AMPAR subunit was diminished following incubation of cultured pyramidal neurons in 0-Mg²⁺ aCSF. **A-B**, Representative images of surface GluA2 in neurons in aCSF for 0 and 30 min. Immunoreactivity was unchanged during the 30 min incubation period. **C-D**, Representative images of surface labeled GluA2 in neurons incubated in 0-Mg²⁺ for 0 and 30 min. GluA2 immunoreactivity decreased following 30 min incubation. **E**, Quantification of the time course of changes in surface GluA2 immunoreactivity following incubation with 0-Mg²⁺ aCSF.

Figure 5. 0-Mg²⁺-induced bursting increased GluA2 subunit internalization rate in cultured hippocampal pyramidal neurons. **A-D**, Representative images of surface (green, **A, B**) and intracellular (red, **C, D**) GluA2 subunits after various durations of incubation in aCSF (control) or 0-Mg²⁺ aCSF (0-Mg²⁺). **E**, Time course of internalization of GluA2 in pyramidal neurons incubated in aCSF (black) or 0-Mg²⁺ aCSF (red). Note that the rate of disappearance of the cell surface GluA2 subunit was significantly faster in 0-Mg²⁺ aCSF compared to that in controls.

Figure 6.

Bursting-induced increase in intracellular calcium was attenuated by selective block of GluA2-lacking AMPARs. **A**, Representative images of Fluo-4 AM-labeled dendrites of cultured neurons incubated in aCSF (top panel), 0-Mg²⁺ aCSF (middle panel) and 0-Mg²⁺-aCSF + IEM-1460 (lower panel) for 0-30 min. The red bar beneath the image indicates the duration of IEM-1460 application. **B**, Time course change in [Ca]_i levels following perfusion with control (grey), 0-Mg²⁺ (black) and 0-Mg²⁺ + IEM-1460 (red) aCSF. The red bar indicates the duration of IEM-1460 perfusion * $p < 0.05$ compared with control, ** $p < 0.01$ compared with control, # $p < 0.05$ compared with 0-Mg²⁺.

Figure 7.

AMPA antagonist, GYKI-52466 terminated diazepam-refractory SE. **A**, EEG traces from hippocampus and cortex of animals during pilocarpine-induced SE, and following treatment with diazepam (30 mg/kg) or GYKI-52466 (30 and 100 mg/kg). Diazepam or GYKI-52466 was administered within 5 min of continuous electrographic seizures and EEG was monitored for the next 3 hr. **B**, The time course of GYKI-52466 control of electrographic seizure is displayed. The onset and persistence of seizure freedom following GYKI-52466 treatment at 30 – 100 mg/kg doses were studied by analyzing seizure freedom at various time points following drug administration during a 3 hr monitoring period. Note that at doses greater than 50 mg/kg, GYKI-52466 terminated seizures in a majority of animals within 1 hr of treatment. **C**, The relationship between various doses of GYKI-52466 and the percentage of animals seizure-free by 60 min of drug administration is displayed. This relationship could be well fitted with a sigmoidal function, and the ED₅₀ value of GYKI-52466 to terminate SE within 60 min was determined to be 56.15 mg/kg.