

# Time course and mechanism of hippocampal neuronal death in an in vitro model of status epilepticus: Role of NMDA receptor activation and NMDA dependent calcium entry

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## Abstract

The hippocampus is especially vulnerable to seizure-induced damage and excitotoxic neuronal injury. This study examined the time course of neuronal death in relationship to seizure duration and the pharmacological mechanisms underlying seizure-induced cell death using low magnesium ( $Mg^{2+}$ ) induced continuous high frequency epileptiform discharges (in vitro status epilepticus) in hippocampal neuronal cultures. Neuronal death was assessed using cell morphology and fluorescein diacetate–propidium iodide staining. Effects of low  $Mg^{2+}$  and various receptor antagonists on spike frequency were assessed using patch clamp electrophysiology. We observed a linear and time-dependent increase in neuronal death with increasing durations of status epilepticus. This cell death was dependent upon extracellular calcium ( $Ca^{2+}$ ) that entered primarily through the *N*-methyl-D-aspartate (NMDA) glutamate receptor channel subtype. Neuronal death was significantly decreased by co-incubation with the NMDA receptor antagonists and was also inhibited by reduction of extracellular ( $Ca^{2+}$ ) during status epilepticus. In contrast, neuronal death from in vitro status epilepticus was not significantly prevented by inhibition of other glutamate receptor subtypes or voltage-gated  $Ca^{2+}$  channels. Interestingly this NMDA- $Ca^{2+}$  dependent neuronal death was much more gradual in onset compared to cell death from excitotoxic glutamate exposure. The results provide evidence that in vitro status epilepticus results in increased activation of the NMDA- $Ca^{2+}$  transduction pathway leading to neuronal death in a time-dependent fashion. The results also indicate that there is a significant window of opportunity during the initial time of continuous seizure activity to be able to intervene, protect neurons and decrease the high morbidity and mortality associated with status epilepticus.

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**Keywords:** Low  $Mg^{2+}$  model of status epilepticus; Neuronal death; NMDA- $Ca^{2+}$  pathway

## 1. Introduction

Status epilepticus is a prolonged seizure condition representing a major medical and neurological emergency (DeLorenzo, 2006; Lowenstein and Alldredge, 1998). The International

League Against Epilepsy defines status epilepticus as continuous seizure activity or intermittent seizures without regaining consciousness lasting 30 min or longer (DeLorenzo, 2006; Lowenstein and Alldredge, 1998) and recent evaluations have extended this definition to include seizure activity lasting even less than 30 min (DeLorenzo et al., 1999; Lowenstein et al., 1999; Treiman et al., 1998). The importance of status epilepticus is that it is associated with a significant morbidity and mortality despite aggressive treatment protocols and it can produce permanent brain damage, especially in the limbic system, resulting in cognitive dysfunction, epilepsy and other

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serious neurological conditions (Delorenzo, 2006; Delorenzo et al., 2005; Fountain, 2000; Lowenstein and Alldredge, 1998). The number of cases of status epilepticus in the United States alone has been estimated from epidemiological studies to be approximately 102,000–152,000 episodes per year and as many as 55,000 deaths per year have been attributed to status epilepticus (Delorenzo, 2006; Hauser, 1983). In addition, 12–30% of adult patients with a new diagnosis of epilepsy initially present with a first seizure that meets the definition of status epilepticus (Drislane, 2000; Fountain, 2000). It is important to investigate the basic mechanism and pharmacology of neuronal death produced by status epilepticus.

Significant progress has been made in recent years in the characterization of status epilepticus induced cell damage and neuronal tissue alteration after seizure activity (Fujikawa, 2005; Holmes, 2002; Sutula and Pitkänen, 2002). In vivo studies have correlated status epilepticus with caspase-induced apoptosis (Ekdahl et al., 2003), suppression of hippocampal neurogenesis in association with glucocorticoid levels and the frequency of seizure episodes (Liu et al., 2003), CA3 injury in the hippocampus due to GluR2B knockdown (Friedman et al., 2003), reorganization of alpha and beta integrin subunits (Fasen et al., 2003), and *N*-methyl-D-aspartate (NMDA) receptor activation (Brandt et al., 2003). Meldrum and co-workers have demonstrated that status epilepticus can cause neuronal injury in ventilated and monitored animals even in the absence of underlying systemic physiological complications (Meldrum et al., 1973). However, in vivo studies of status epilepticus evaluating the molecular mechanisms of neuronal injury have had difficulty in clearly delineating the basic mechanisms involved in cell death because of the inherent problems associated with controlling the neuronal environment. In intact animal models, cellular responses to status epilepticus are associated with complex effects in multiple body systems, making it difficult to evaluate basic mechanisms. For example, changes in glucose utilization, hormonal production, extracellular fluid composition, and the involvement of other physiological processes may vary under different conditions inducing status epilepticus and alter the environment of neurons under study, complicating mechanistic studies in vivo.

This study was initiated to directly evaluate the effect of electrographic epileptiform discharges over time on neuronal viability and characterize the pharmacological mechanisms underlying status epilepticus induced cell death in a controlled neuronal environment utilizing the well-established hippocampal neuronal culture model of in vitro status epilepticus. Exposure to a low magnesium ( $Mg^{2+}$ ) medium induces continuous high frequency epileptiform discharges in hippocampal neurons in culture. These high frequency discharges are produced because of the elimination of  $Mg^{2+}$  block of the ionotropic NMDA receptor channels, which is normally re-established after neuronal depolarization (Mayer and Westbrook, 1984). Lack of  $Mg^{2+}$  block of NMDA receptors produces repetitive depolarizations manifested as high frequency spiking characteristic of in vitro status epilepticus. In an elegant study, Mody et al. (1987) suggested that lowering extracellular  $Mg^{2+}$  may produce a reduction in the electric field across the neuronal membrane

by reducing surface charge screening causing a membrane depolarization. Further, Mangan and Kapur (2004) have shown that an increased probability of transmitter release presynaptically, enhanced NMDA receptor-mediated excitability postsynaptically, and extent of neuronal interconnectivity contributes to initiation and maintenance of elevated network excitability upon exposure to low  $Mg^{2+}$  medium. The spike frequency and epileptiform discharges manifested upon exposure to a low  $Mg^{2+}$  medium are identical to the electrographic features of status epilepticus observed with animal models and in humans (Delorenzo et al., 2005; Lothman et al., 1991; Mangan and Kapur, 2004; Sombati and DeLorenzo, 1995). While the neuronal cultures do not manifest anatomical structures or behavioral seizures, the hippocampal neuronal culture model has the unique advantage of providing excellent control of the neuronal environment and allowing the application of powerful in vitro techniques to study biochemical, electrophysiological and molecular mechanisms underlying in vitro status epilepticus (Delorenzo et al., 2005; Deshpande et al., 2007a; Goodkin et al., 2005; Mangan and Kapur, 2004; Sombati and DeLorenzo, 1995). Thus, this model is ideally suited to evaluate the effects of electrographic epileptiform activity with synaptic activation on neuronal viability.

The results from this study indicate that low  $Mg^{2+}$  induced in vitro status epilepticus can cause neuronal death, but that cell death occurs over a prolonged time period measured in hours. The degree of status epilepticus induced neuronal death was linear, increased with increasing durations of exposure to continuous high frequency epileptiform discharges and was primarily dependent on NMDA receptor activated  $Ca^{2+}$  entry. The results also indicate that status epilepticus induced neuronal death due to NMDA receptor activation takes much longer to initiate compared to stroke or excitotoxic glutamate induced cell death (Choi et al., 1987; Deshpande et al., 2007b). Understanding the basic mechanism and pharmacology of neuronal death induced by in vitro status epilepticus may provide important insights into obtaining a better understanding of the molecular mechanisms causing neuronal death in this common neurological condition. It may also lead to the development of novel therapeutic agents aimed at reducing the neuronal damage produced by prolonged seizure activity and ultimately decrease the high morbidity and mortality associated with status epilepticus.

## 2. Materials and methods

All the reagents including fluorescein diacetate and propidium iodide were purchased from Sigma Chemical Co (St. Louis, MO) unless otherwise noted. Sodium pyruvate, minimum essential media containing Earle's salts, fetal bovine serum and horse serum were obtained from Gibco-BRL (Invitrogen Corp., Carlsbad, CA).

### 2.1. Hippocampal neuronal culture

All animal use procedures were in strict accordance with the National Institute of Health Guide for the Care and Use of

Laboratory Animals and approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Studies were conducted on primary mixed hippocampal neuronal cultures prepared as described previously with slight modifications (Deshpande et al., 2007a; Sombati and DeLorenzo, 1995). In brief, hippocampal cells were obtained from 2-day postnatal Sprague–Dawley rats (Harlan, Frederick, MD) and plated at a density of  $1 \times 10^5$  cells/cm<sup>2</sup> onto 35-mm grid cell culture dishes (Nunc, Naperville, IL) previously coated with poly-L-lysine (0.05 mg/ml). Glial cultures were maintained at 37 °C in a 5% CO<sub>2</sub>/95% air atmosphere and fed thrice weekly with glial feed (minimal essential media with Earle's Salts, 25 mM HEPES, 2 mM L-Glutamine, 3 mM Glucose, and 10% fetal bovine serum). When confluent, glial beds were treated with 5-μM cytosine arabinoside for two days to curtail cell division. On the 13th day in vitro, the media was fully replaced with a 5% horse serum supplemented neuronal feed (composition given below) in preparation for neuronal plating on the following day. At this time, these cultures predominantly consisted of glial cells with few, if any, neurons. On the 14th day in vitro, hippocampal cell suspension was plated on these confluent glial beds at a density of  $2 \times 10^5$  cells/cm<sup>2</sup>. Twenty-four hours after plating, cultures were treated with 5-μM cytosine arabinoside to inhibit non-neuronal growth. Cultures were maintained at 37 °C in a 5% CO<sub>2</sub>/95% air atmosphere and fed twice weekly with neuronal feed (minimal essential media with Earle's Salts, 25 mM HEPES, 2 mM L-Glutamine, 3 mM Glucose, 100 μg/ml transferrin, 5 μg/ml insulin, 100 μM putrescine, 3 nM sodium selenite, 200 nM progesterone, 1 mM sodium pyruvate, 0.1% ovalbumin, 0.2 ng/ml triiodothyroxine and 0.4 ng/ml corticosterone supplemented with 5% horse serum). These mixed cultures were used for experiments from 13 days in vitro following neuronal plating through the life of the cultures.

## 2.2. Hippocampal neuronal culture model of status epilepticus

After 2 weeks, cultures were utilized for experimentation and status epilepticus was induced as described previously (Deshpande et al., 2007a; Sombati and DeLorenzo, 1995). Maintenance medium was replaced with physiological recording solution with or without MgCl<sub>2</sub> containing (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 2 CaCl<sub>2</sub>, 10 glucose, and 0.002 glycine, pH 7.3, and osmolarity adjusted to  $290 \pm 10$  mOsm with sucrose. Continuous high frequency epileptiform bursts were induced by exposing neuronal cultures to physiological recording solution without added MgCl<sub>2</sub> (low Mg<sup>2+</sup>). The status epilepticus continued until physiological recording solution containing 1 mM MgCl<sub>2</sub> was added back to the cultures. Unless designated as low Mg<sup>2+</sup> treatment, experimental protocols utilized physiological recording solution containing 1 mM MgCl<sub>2</sub>. For the perfusion studies, fresh low Mg<sup>2+</sup> solution was constantly perfused over the culture plate using the gravity feed perfusion system (Warner Instrument Corp., Hamden, CT) at the rate of 2-ml/min. This procedure does not disrupt the neurons and provides a complete exchange of the experimental media every 1 min.

## 2.3. Whole-cell current clamp recordings

Whole-cell current clamp recordings were performed using previously established procedures (Deshpande et al., 2007a; Sombati and DeLorenzo, 1995). Briefly, cell culture dishes were mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). Patch electrodes with a resistance of 2 to 4 MΩ were pulled on a Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA), fire-polished and filled with a solution containing (in mM): 140 K<sup>+</sup> gluconate, 1.1 EGTA, 1 MgCl<sub>2</sub>, and 10 Na-HEPES, pH 7.2, osmolarity adjusted to  $290 \pm 10$  mOsm with sucrose. Whole-cell recordings were carried out using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in a current clamp mode. Data were digitized via Digidata 1322A (Axon Instruments, Foster City, CA) and transferred to VHS tape using a PCM device (Neurocorder, New York, NY) and then played back on a DC-500 Hz chart recorder (Astro-Med Dash II, Warwick, RI). Electrophysiological data was obtained under both perfusion and non-perfusion conditions. The spike frequency did not alter between these two experimental protocols. The electrophysiological data represent recordings from the non-perfusion condition, wherein drug-containing solution was exchanged with maintenance media before start of the experiment.

## 2.4. Neuronal death assay

Neuronal death was assessed at various time points after low-Mg<sup>2+</sup> exposure using fluorescein diacetate (FDA)–propidium iodide (PI) microfluorometry (Deshpande et al., 2007c; Didier et al., 1990). Morphological assessment of the cultures was also carried out to determine neuronal viability (Deshpande et al., 2007c; Limbrick et al., 1995). Three randomly selected fields were marked and photographed. Both fluorescent and phase bright images were captured. These same fields were examined at various time points during continual exposure to low Mg<sup>2+</sup> solution. 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. To quantify neuronal cell death with this technique, cells in three randomly selected fields were counted manually and averaged per culture (approximately 18 to 25 neurons per field).

## 2.5. Data analyses

Data are reported as mean  $\pm$  S.E.M. Whole-cell patch clamp experiments and cell death analyses were carried out in parallel under similar experimental conditions in sister neuronal cultures. Neuronal culture plates were incubated in either low Mg<sup>2+</sup> solution or low Mg<sup>2+</sup> + drug-containing solution for specified durations and then subjected to various experimental protocols at the end of the treatment. Thus, the drugs were



applied during the low  $Mg^{2+}$  treatment for the duration of status epilepticus and chronically for both patch clamp and cell death experiments. Status epilepticus frequency was determined by counting individual epileptiform bursts and spikes over a recording duration of 5 min for each neuron analyzed before and after application of the drug. Neuronal cell death data were examined using one-way analysis of variance (ANOVA) followed by post-hoc Tukey test where appropriate. Paired *t*-tests were utilized to compare differences in neuronal death data between the fluorescent techniques and phase contrast microscopy. Each neuronal culture plate was treated as  $n=1$ . All experiments had their own sham controls that were handled identically to the treatment condition. Equal number of sham controls and drug conditions were kept for each experiment. Net neuronal death was calculated by subtracting neuronal death observed in respective sham control groups in order to adjust for cell death due to handling artifacts. Data were analyzed using SigmaStat 2.0 and plotted using SigmaPlot 8.02 (SPSS Inc., Chicago, IL).

### 3. Results

#### 3.1. Status epilepticus in hippocampal neuronal cultures

Treatment of hippocampal neurons in culture with low  $Mg^{2+}$  media induced continuous high frequency epileptiform activity characterized by repetitive burst discharges. Each burst was comprised of multiple spikes overlaying a depolarization shift identical to those observed in vivo during status epilepticus (Delorenzo et al., 2005; Lothman et al., 1991; Mangan and Kapur, 2004; Sombati and DeLorenzo, 1995). In marked contrast, recording from control neurons showed only occasional spontaneous action potentials (Fig. 1A). Fig. 1B and C illustrates continuous recordings from a neuron after 2-h and 8-h of exposure to low  $Mg^{2+}$ . The data demonstrate that the resultant high frequency continuous epileptiform discharges were

sustained for the entire duration of low  $Mg^{2+}$  exposure. Expansion of the time scale indicated that spike frequency was greater than 3 Hz and lasted longer than 30 min and thus satisfied the definition criteria of status epilepticus (Hauser, 1983).

#### 3.2. Duration of status epilepticus and neuronal cell death

Having established that status epilepticus occurs throughout the duration of low  $Mg^{2+}$  exposure, we then investigated the effects of increasing durations of electrographic status epilepticus in a controlled neuronal environment on hippocampal cell death. In vitro status epilepticus was induced as described in the Materials and methods by removing  $Mg^{2+}$  from the culture medium. Cell death was assessed using both FDA–PI staining and morphological measurements in the same marked field. Neuronal cultures were subjected to varying durations of status epilepticus which included 2, 4, 8 and 12-h exposures to low  $Mg^{2+}$ . As shown in Fig. 2A, increasing the duration of status epilepticus caused a corresponding increase in neuronal death. There was a linear and time-dependent increase in neuronal death with status epilepticus. Neuronal death increased from approximately 30% to 100% when the duration of low  $Mg^{2+}$  exposure was increased from 2 to 12 h. Thus, all the neurons die if status epilepticus continues for long enough. Neuronal death assessment using the FDA–PI intravital dye staining method or the morphological examination technique were consistent and produced essentially identical determinations of the degree of neuronal death and the results from these two techniques were not statistically divergent (paired *t*-test). Thus, we carefully compared the effects of continuous epileptiform discharges on cell death to control for the time and conditions of handling by using identical conditions except for the replacement of physiological  $Mg^{2+}$ .

Status epilepticus is known to cause elevations in extracellular levels of glutamate (Smolders et al., 1997; Ueda et al., 2002). However, in vivo evidence indicates this accumulation should be minimal (Bruhn et al., 1992; Millan et al., 1993). Prolonged exposure to elevated extracellular glutamate is known to kill the neurons via excitotoxic mechanisms (Choi et al., 1987; Deshpande et al., 2007a; Limbrick et al., 1995). In order to rule out involvement of neurotoxic effects of glutamate or other seizure-released metabolites, we performed experiments wherein fresh low  $Mg^{2+}$  solution was continuously perfused over the culture plate throughout the duration of status epilepticus. This technique prevents the build up of glutamate or metabolites released during the in vitro status epilepticus providing a more controlled environment for the neurons. Both control and status epilepticus neurons were treated identically. As shown in Fig. 2B, similar to non-perfusion experiments, we observed a linear and time-dependent increase in neuronal death despite continuous perfusion of low  $Mg^{2+}$  solution. Differences in neuronal cell death between sham control and low  $Mg^{2+}$  conditions (perfusion and non-perfusion) were significant for all the low  $Mg^{2+}$  exposure durations ( $p<0.001$ ,  $n=6–10$ ). Interestingly, cell death under perfusion conditions was lower than non-perfusion conditions, but it was not statistically significant

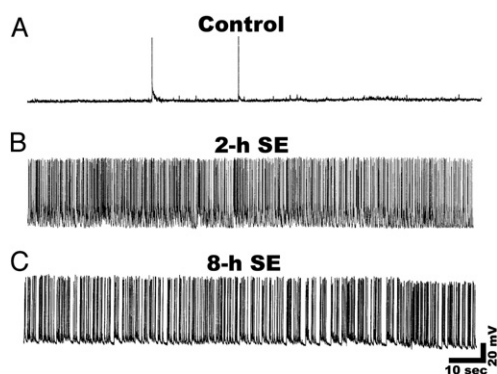


Fig. 1. Induction of continuous high frequency epileptiform discharges or in vitro status epilepticus like activity in cultured hippocampal neurons during exposure to low  $Mg^{2+}$  solution. A. Representative current clamp recording from a control neuron showing occasional spontaneous action potentials. B and C. Representative current clamp recordings showing induction of tonic high frequency epileptiform bursts (in vitro status epilepticus, SE) from neurons subjected to 2 and 8 h of low  $Mg^{2+}$  treatment respectively. Tonic high frequency spiking was observed at all the durations of status epilepticus. The results shown were representative of over 20 recordings.

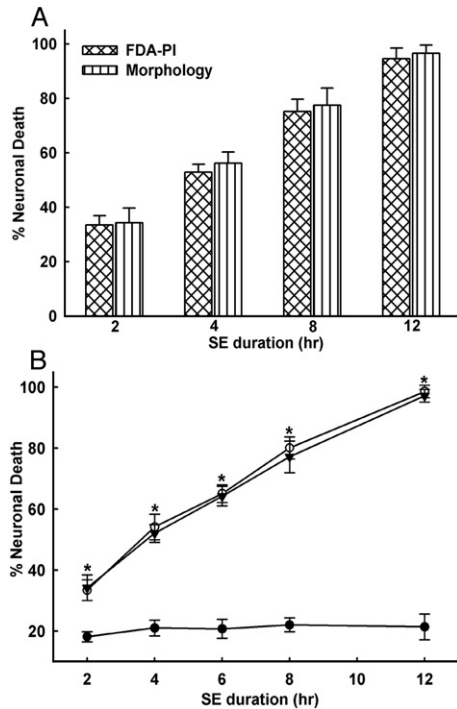


Fig. 2. Increase in neuronal cell death with increasing durations of in vitro status epilepticus. A. Cultures were exposed to various durations of low  $Mg^{2+}$  treatment ranging from 2 to 12 h and neuronal death was assessed using the FDA–PI intravital dye staining method and the morphological technique in the same marked field. Differences between the two techniques to evaluate cell death were not statistically significant. B. Accumulation of glutamate or other seizure-released metabolites into the media does not contribute to neuronal cell death. Continuous perfusion of culture plate with fresh low  $Mg^{2+}$  solution throughout the duration of status epilepticus using gravity feed perfusion system prevented possible accumulation of glutamate or other substances in the culture plate. Duration of status epilepticus ranged from 2 to 12 h and low  $Mg^{2+}$  solution was perfused throughout the experiment. Black circles (●) indicate sham controls. Open circles (○) represent percent cell death from non-perfusion status epilepticus cultures. Black triangles (▼) represent percent cell death from continuous perfusion status epilepticus cultures. Control and treated neurons were subjected to similar perfusion protocol. Significant differences were observed for all the time points of status epilepticus between sham and low  $Mg^{2+}$  treated neurons ( $n=6-10$ ,  $*P<0.001$ , one-way ANOVA, post-hoc Tukey test). However, there were no significant differences in cell death between perfusion and non-perfusion conditions ( $n=6-10$ ,  $P=0.7$ ). Data are represented as mean  $\pm$  S.E.M.

( $P=0.81$ ). These experiments indicate that status epilepticus alone, and not the build up of glutamate or other seizure-released metabolites were responsible for neuronal death. Cell death in non-status epilepticus control cultures ( $n=6-10$ ) was estimated around 10–15% and represented the cell death associated with handling procedures involved with washing neurons and exposure to the control recording solution (Fig. 2B). For the subsequent experiments, cell death from sham controls was subtracted from drug treatment groups to correctly represent net neuronal death due to low  $Mg^{2+}$  exposure alone.

### 3.3. Role of $Ca^{2+}$ in status epilepticus induced neuronal death

Calcium is a major signaling molecule and has been implicated to play an important role in epileptogenesis and neuronal death in various acute neurological diseases (Delorenzo et al., 2005). To

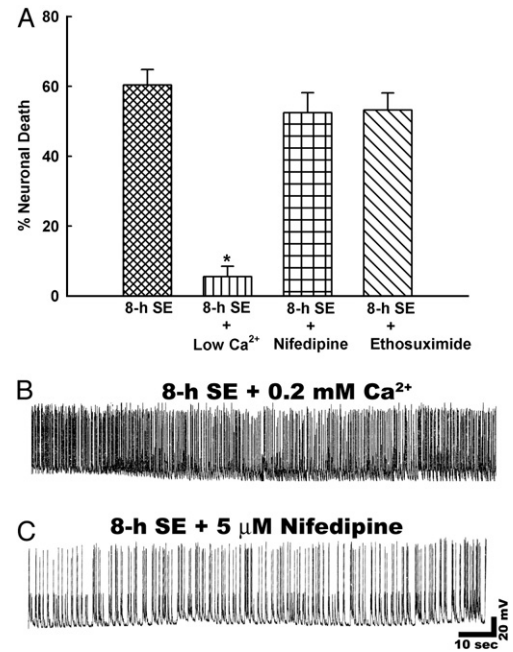


Fig. 3. In vitro status epilepticus induced neuronal death is dependent upon extracellular calcium. A. Neuronal death assessment revealed that lowering extracellular  $Ca^{2+}$  significantly attenuated net neuronal cell death compared to 8-h status epilepticus alone group ( $n=6$ ,  $*P<0.001$ , one-way ANOVA, post-hoc Tukey test). In contrast, percent neuronal death was not significantly different between 8-h status epilepticus and nifedipine or ethosuximide treated cultures ( $n=6$ ,  $P=0.8$ ). Data are represented as mean  $\pm$  S.E.M. B. Representative current clamp recording from a neuron subjected to low  $Mg^{2+}$  induced status epilepticus in the presence of low  $Ca^{2+}$  (0.2 mM). Lowering extracellular  $Ca^{2+}$  had no effect on low  $Mg^{2+}$  induced high frequency spiking. C. Representative recording from a neuron treated with nifedipine (10  $\mu$ M) during low  $Mg^{2+}$  induced status epilepticus. Similar to low  $Ca^{2+}$  condition, nifedipine treatment didn't affect low  $Mg^{2+}$  induced high frequency spiking.

determine if  $Ca^{2+}$  was a major component in status epilepticus induced neuronal death, the concentration of extracellular  $Ca^{2+}$  was manipulated. This method has been routinely used to investigate the possible role of extracellular  $Ca^{2+}$  in neuronal

Table 1

Spike frequency before and during low- $Mg^{2+}$  treatment in the presence or absence of various pharmacological manipulations

Treatment condition	Spike frequency
Control	0
Low $Mg^{2+}$	11 $\pm$ 3.4
+0 mM $Ca^{2+}$	12 $\pm$ 4.2
+Nifedipine (5 $\mu$ M)	9 $\pm$ 3.2
+Ethosuximide (1 mM)	11 $\pm$ 4.1
+CNQX (10 $\mu$ M)	10 $\pm$ 2.8
+NBQX (10 $\mu$ M)	8 $\pm$ 4.1
+mCPG (250 $\mu$ M)	10 $\pm$ 3.2
+D-APV (25 $\mu$ M)	11 $\pm$ 3.8
+MK-801 (10 $\mu$ M)	10 $\pm$ 4.4
+ $\omega$ -conotoxin (1 $\mu$ M)	0
+Tetrodotoxin (1 $\mu$ M)	0

Data are represented as the mean  $\pm$  S.E.M. of spike frequency during low- $Mg^{2+}$  treatment for various experimental conditions ( $n=5-7$ ). Spike frequency was determined as described in Materials and methods section. There were no statistical differences between the spike frequencies of these conditions (Student's *t*-test).

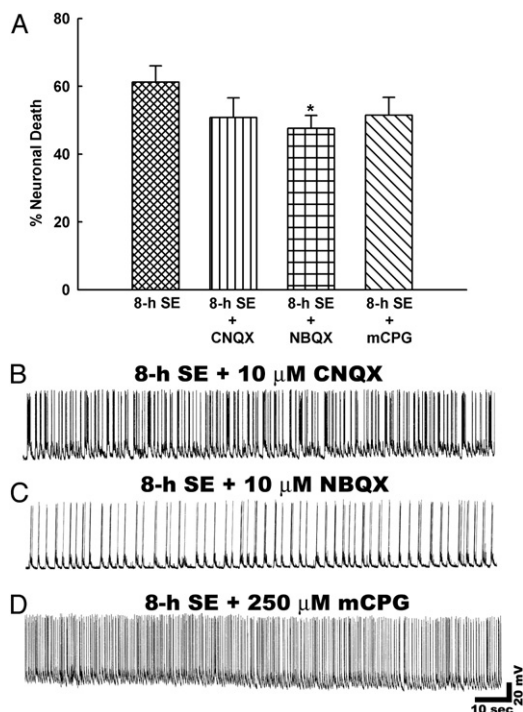


Fig. 4. Role of glutamate receptor subtypes in status epilepticus induced neuronal death. A. Neuronal death assessment revealed that there were no statistically significant differences between CNQX and mCPG treated cultures and 8-h status epilepticus alone cultures ( $n=6$ ,  $P=0.7$ ). However, a small but significant reduction in neuronal death was observed upon NBQX treatment ( $n=6$ ,  $*P<0.05$ , One-way ANOVA, post-hoc Tukey test). Data are represented as mean  $\pm$  S.E.M. B–D. Representative current clamp recordings from neurons subjected to treatment with various glutamate receptor antagonists during low  $Mg^{2+}$  induced status epilepticus like activity. Treatment with AMPA/KAR receptor antagonist CNQX (10  $\mu$ M) or NBQX (10  $\mu$ M) did slow down low  $Mg^{2+}$  induced high frequency spiking. But the spike frequency was still  $>3$  Hz that meets the criteria of status epilepticus. Similarly, the metabotropic glutamate receptor antagonist mCPG (250  $\mu$ M) also had no effects on status epilepticus like activity.

excitotoxicity (Deshpande et al., 2007a; Limbrick et al., 1995). Reduced extracellular  $Ca^{2+}$  (0.2 mM) during low  $Mg^{2+}$  treatment significantly attenuated net neuronal death when compared to 8-h status epilepticus controls (Fig. 3A). Status epilepticus durations of 8 h with 2 mM  $Ca^{2+}$  ( $n=9$ ) resulted in  $60.2 \pm 5.03\%$  neuronal death. In cultures treated with low  $Ca^{2+}$  solution for 8 h ( $n=6$ ), net neuronal death was determined to be  $4.05\% \pm 3.03\%$  when controlled for sham treatment. Thus, there was a significant ( $p<0.001$ ) attenuation of net neuronal death in the presence of low extracellular  $Ca^{2+}$  compared to the 8-h status epilepticus controls with normal extracellular  $Ca^{2+}$ . Lowering extracellular  $Ca^{2+}$  had no effects on the expression of low  $Mg^{2+}$  induced high frequency epileptiform discharges (Fig. 3B). These experiments indicate that status epilepticus induced neuronal death is dependent upon influx of extracellular  $Ca^{2+}$ .

In order to investigate the route of this  $Ca^{2+}$  entry, we used concentrations of well-characterized voltage-gated  $Ca^{2+}$  channels inhibitors shown to be effective under our physiological conditions (Deshpande et al., 2007a). Cadmium ( $Cd^{2+}$ ) is a non-selective  $Ca^{2+}$  channel blocker, however recent reports have indicated that this metal ion can initiate apoptosis and necrosis

in neuronal cultures (Lopez et al., 2003). This could have potentially confounded our cell death results. Therefore, we inhibited the  $Ca^{2+}$  channels with the more selective agents that would have been inhibited by the general effects of  $Cd^{2+}$  and avoided the possible toxic effects of this metal. We first blocked L-type  $Ca^{2+}$  channels that have the highest conductance amongst voltage-gated  $Ca^{2+}$  channels. Treatment with nifedipine (5  $\mu$ M,  $n=6$ ) appeared to slow down low  $Mg^{2+}$  induced high frequency spiking (Fig. 3C and Table 1), but it did not significantly reduce neuronal death compared to untreated 8-h status epilepticus controls (Fig. 3A). T type voltage-gated  $Ca^{2+}$  channels that are blocked by ethosuximide (Sun et al., 2004) are also reported to be upregulated following status epilepticus and have been implicated in epilepsy. Ethosuximide (1 mM,  $n=5$ ) did not significantly prevent cell death due to status epilepticus in this model (Fig. 3A). It was not possible to evaluate the effects of  $\omega$ -conotoxin because electrophysiological studies showed that this agent, similar to tetrodotoxin, effectively blocked synaptic transmission and also prevented low  $Mg^{2+}$  induced high frequency spiking (Table 1). Therefore the role of presynaptically located N or P/Q type  $Ca^{2+}$  channels cannot be completely dismissed. However, inhibition of status epilepticus using tetrodotoxin or  $\omega$ -conotoxin in the presence of 0  $Mg^{2+}$  demonstrated that low  $Mg^{2+}$  alone was not causing neuronal death. The lack of effects of nifedipine and

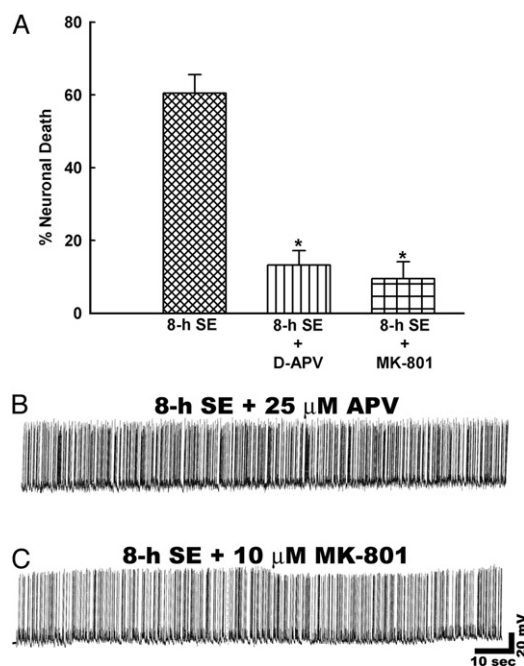


Fig. 5. Status epilepticus induced net neuronal death is NMDA receptor dependent. A. There was a significant reduction in net neuronal death when NMDA receptor antagonists APV (25  $\mu$ M) or MK-801 (10  $\mu$ M) were present throughout the duration of status epilepticus as compared to 8-h status epilepticus controls ( $n=6$ ,  $*P<0.001$ , one-way ANOVA, post-hoc Tukey test). Data are represented as mean  $\pm$  S.E.M. B–C. Representative current clamp recordings from neurons subjected to treatment with NMDA receptor antagonists during low  $Mg^{2+}$  induced status epilepticus. Treatment with APV (25  $\mu$ M) or MK-801 (10  $\mu$ M) had no effects on low  $Mg^{2+}$  induced high frequency spiking.



ethosuximide on reducing cell death from status epilepticus supports the interpretation that  $\text{Ca}^{2+}$  entry through voltage-gated  $\text{Ca}^{2+}$  channels is not playing a significant role in causing cell death.

### 3.4. Role of glutamate receptor subtypes in status epilepticus induced neuronal death

To examine the possibility that the excessive entry of extracellular  $\text{Ca}^{2+}$  through glutamate receptor activated channels was involved in status epilepticus induced neuronal death; in vitro status epilepticus was induced in the presence of various glutamate receptor subtype antagonists. Treatment with the AMPA/kainate glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10  $\mu\text{M}$ ,  $n=6$ ) had no significant effects on the percentage of neuronal death compared with 8-h status epilepticus cultures (Fig. 4A). Exposure to the metabotropic glutamate receptor antagonist (S)- $\alpha$ -methyl-4-carboxyphenylglycine (mCPG, 250  $\mu\text{M}$ ,  $n=6$ ) did not significantly reduce neuronal death after 8 h of status epilepticus (Fig. 4D). The AMPA/kainate glutamate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX, 10  $\mu\text{M}$ ,  $n=6$ ) did show a small decrease in neuronal death from 8 h of status epilepticus compared to controls ( $p<0.05$ ). In the current clamp studies, while treatment with CNQX and mCPG had no effects on status epilepticus associated

spiking, NBQX appeared to considerably slow down low  $\text{Mg}^{2+}$  induced high frequency epileptiform spiking (Fig. 4B–D and Table 1). This is in line with our previous observations that NBQX produced greater decreases in intracellular  $\text{Ca}^{2+}$  levels compared to CNQX during low  $\text{Mg}^{2+}$  induced status epilepticus (DeLorenzo et al., 1998). Thus, this reduction of the severity of electrographic epileptiform discharges may have contributed to its minor, but significant effect in reducing neuronal death.

### 3.5. Status epilepticus induced neuronal death is NMDA receptor dependent

Treatment with the NMDA receptor antagonist 5-methyl-10,11-dihydro-5-*H*-dibenzocyclohepten-5,10-imine maleate (MK-801, 10  $\mu\text{M}$ ,  $n=6$ ) produced a marked reduction in net neuronal death to  $9.5\pm3.36\%$ , a figure that represented approximately an 80% decrease in neuronal death compared to 8-h status epilepticus controls (Fig. 5A). Prolonged status epilepticus is associated with increases in extracellular glutamate that could depolarize the neurons. MK-801 is a voltage-dependent blocker of NMDA receptor ion channel and its ability to block is limited under the depolarized condition. Although our electrophysiological studies did not reveal depolarization in neurons undergoing prolonged status epilepticus (membrane potentials: control =  $-62.5\pm3.5$  mV, 2-h low  $\text{Mg}^{2+}$  =  $-60.2\pm4.2$  mV, 8-h low  $\text{Mg}^{2+}$  =  $-59.8\pm3.1$  mV), to confirm our findings with MK-

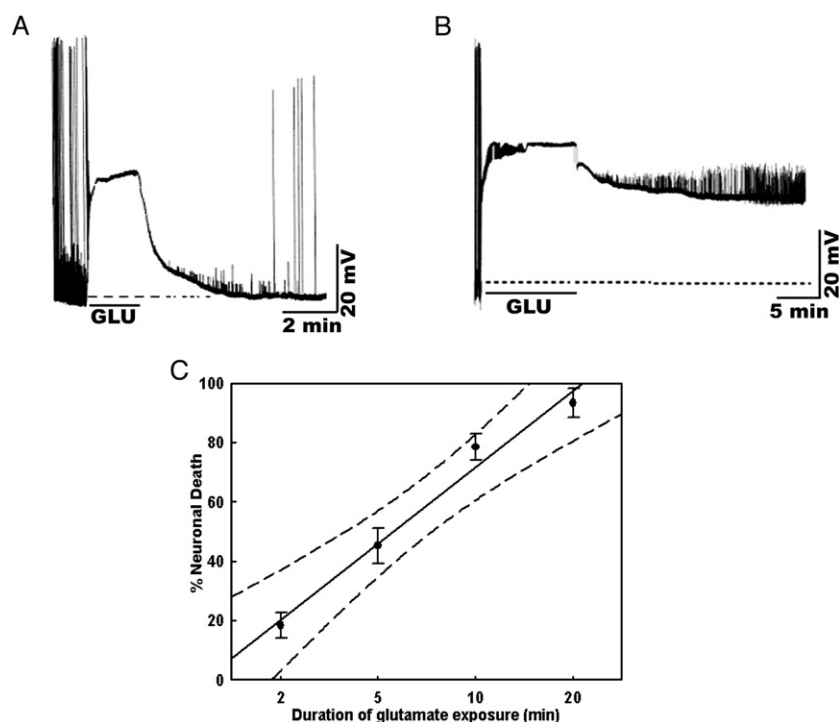


Fig. 6. Time course of neuronal cell death following stroke-like injury in vitro. A. Current clamp recording from a neuron subjected to glutamate (500  $\mu\text{M}$  + 10  $\mu\text{M}$  glycine) for a short duration (5 min). The neuron shows rapid depolarization when exposed to glutamate, stays depolarized as long as glutamate is present in the bath solution and after 5 min when washed with control recording solution regains its resting membrane potential. B. Current clamp recording from a neuron subjected to glutamate (500  $\mu\text{M}$  + 10  $\mu\text{M}$  glycine) for a long duration (10 min). This constitutes the excitotoxic insult. Despite removal of glutamate after the 10-min period, the neuron stays depolarized for as long as recording is continued. This constitutes the extended neuronal depolarization (END) phase. C. Increasing durations of glutamate exposure (500  $\mu\text{M}$  glutamate along with 10  $\mu\text{M}$  glycine dissolved in physiological recording solution containing 1 mM  $\text{Mg}^{2+}$ ) caused a corresponding increase in percent neuronal death as assessed using the FDA–PI intravital dye staining method. Linear regression analysis of neuronal cell death and duration of glutamate exposure gave an  $r^2$ -value of 0.98. Dotted lines represent 95% confidence interval limits.

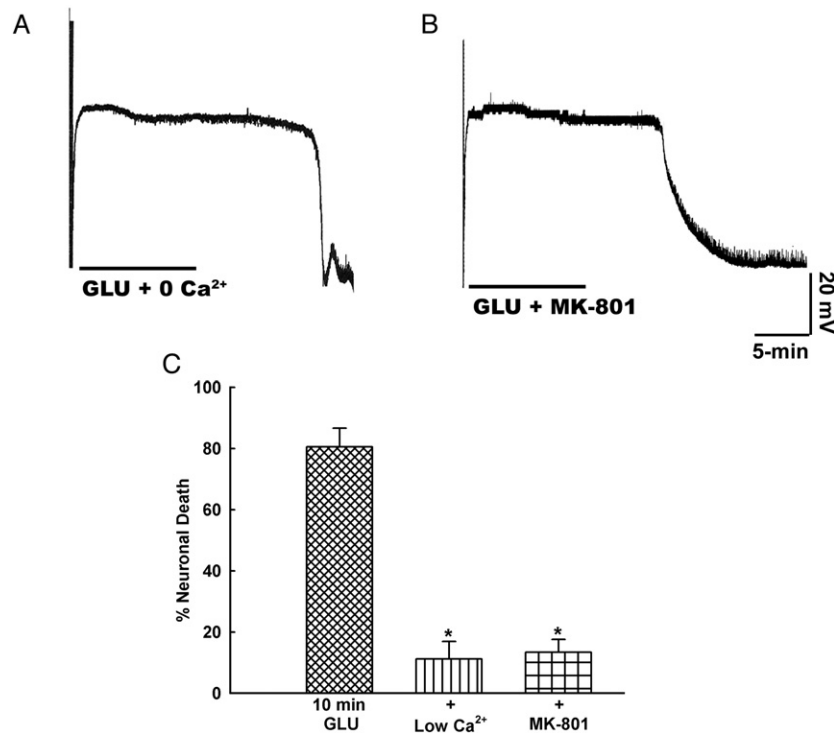


Fig. 7. Mechanism of neuronal cell death following stroke-like injury in vitro. Representative current clamp recordings from neurons demonstrating that excitotoxic glutamate injury (500  $\mu$ M glutamate, 10  $\mu$ M glycine, 10-min duration) produced in medium containing A, no added extracellular  $\text{Ca}^{2+}$  or B, in the presence of NMDA channel inhibitor MK-801 during glutamate exposure prevents development of END phase following termination of glutamate insult. C. Neuronal death following excitotoxic glutamate exposure (500  $\mu$ M glutamate, 10  $\mu$ M glycine, 10-min duration) was dependent upon activation of NMDA receptor- $\text{Ca}^{2+}$  dependent pathway. There was a significant reduction in neuronal death when glutamate injury was produced in the presence of low  $\text{Ca}^{2+}$  (0.2 mM). Similar reduction in neuronal death was also observed when NMDA receptor antagonist MK-801 (10  $\mu$ M) was present throughout the duration of glutamate exposure ( $n=5-7$ ,  $*P<0.001$ , one-way ANOVA, post-hoc Tukey test). Data are represented as mean  $\pm$  S.E.M.

801, we also performed experiments with a competitive NMDA receptor antagonist, D-APV. Treatment with 2-amino-5-phosphonovalerate (D-APV, 25  $\mu$ M,  $n=6$ ) also showed a marked decrease in net neuronal death (Fig. 5A). Co-incubation with D-APV throughout the duration of 8-h status epilepticus reduced net neuronal death to  $13.2 \pm 2.02\%$ . The reductions in neuronal death produced by D-APV and MK-801 were both statistically significant ( $p<0.001$ ). Treatments with APV or MK-801 had no effects on the expression of low  $\text{Mg}^{2+}$  induced high frequency epileptiform discharges (Fig. 5B and C and Table 1). These results demonstrate that during in vitro status epilepticus, extracellular  $\text{Ca}^{2+}$  that enters neurons through the NMDA receptor pathway is coupled to the molecular cascades that initiate neuronal death.

### 3.6. Time course and mechanism of cell death following stroke-like injury in vitro

Application of high concentrations of glutamate to hippocampal neuronal cultures is an established model for studying stroke-like injury in vitro (Choi et al., 1987; Deshpande et al., 2007a; Limbrick et al., 1995). We utilized this model to investigate the time course and mechanism of cell death following stroke-like injury in vitro. Following glutamate exposure, there was a massive depolarization of the neuronal membrane and the neuron stayed depolarized as long as glutamate was present in

the extracellular medium. Recovery from this depolarized state was contingent upon duration of glutamate exposure. Thus, following 2 min glutamate exposure neurons recovered to baseline membrane potentials (Fig. 6A). However, a 10 min glutamate exposure produced a state of irreversible membrane depolarization known as extended neuronal depolarization (END) (Fig. 6B). This END state correlates with impending neuronal cell death (Deshpande et al., 2007a; Limbrick et al., 1995). Indeed, as shown in Fig. 6C, bath application of glutamate (500  $\mu$ M glutamate, 10  $\mu$ M glycine,  $n=5-7$ ) produced a time-dependent increase in neuronal cell death. Thus, 2-min glutamate exposure produced around 20% cell death. Following 5-min glutamate stimulation approximately 50% cell death was observed. A 10-min glutamate exposure produced around 80% cell death and 20-min glutamate stimulation caused greater than 90% of cell death. Linear regression analysis revealed a strong correlation ( $r^2=0.98$ ) between duration of glutamate exposure and neuronal cell death (Fig. 6C).

Further, in agreement with previously established results (Choi et al., 1987; Deshpande et al., 2007a; Limbrick et al., 1995) we observed that stimulation of NMDA- $\text{Ca}^{2+}$  dependent pathway was primarily responsible for the glutamate induced cell death in vitro. Thus, glutamate application in the presence of low  $\text{Ca}^{2+}$  medium or MK 801 (10  $\mu$ M) didn't prevent the early depolarization but prevented development of END (Fig. 7A and B). Similarly, as shown in Fig. 7C, the cell death



was significantly ( $P < 0.001$ ,  $n = 5-7$ ) reduced when glutamate was applied in the presence of NMDA receptor antagonist MK-801 (10  $\mu\text{M}$ ) or when glutamate was applied in a medium containing low  $\text{Ca}^{2+}$  (0.2 mM). Thus, while neuronal cell death following status epilepticus like or stroke-like injury in vitro followed a similar mechanism, unlike status epilepticus induced cell death, the neuronal death following glutamate injury had a much quicker onset ranging in minutes rather than hours.

#### 4. Discussion

The results from this study indicate that electrographic epileptiform discharges in hippocampal neuronal cultures and the resultant excessive synaptic activity can induce cell death in a time-dependent manner in an in vitro model of status epilepticus that controls for any effects of environmental influences. We observed that the degree of neuronal death was linear and directly correlated with increasing durations of continuous high frequency epileptiform discharges induced by treatment with low  $\text{Mg}^{2+}$ . Status epilepticus induced neuronal death was also found to be dependent upon extracellular  $\text{Ca}^{2+}$ , since reducing the driving force for  $\text{Ca}^{2+}$  entry by lowering extracellular  $\text{Ca}^{2+}$  during status epilepticus significantly attenuated neuronal death. Status epilepticus mediated neuronal death was reduced by NMDA receptor channel inhibition, while neuroprotection afforded by inhibition of non-NMDA glutamate receptor subtypes or voltage-gated  $\text{Ca}^{2+}$  channels appeared to be minor. In contrast to glutamate excitotoxicity and stroke induced cell death, status epilepticus mediated activation of NMDA receptors required hours to induce significant cell death. Our results are consistent with data obtained from in vivo models and clinical studies demonstrating that the neuronal death is dependent upon duration of status epilepticus (Duncan, 2002; Holmes, 2002; Towne et al., 1994) and offers a unique model to investigate the time-dependent effects of status epilepticus on neuronal viability.

Neuronal injury secondary to status epilepticus is the consequence of excessive neuronal excitability and synaptic activity with high frequency epileptiform discharges, resulting in depolarizing shifts of membrane potential, free radical production, neuronal metabolic overload and elevations in extracellular glutamate (reviewed in: Delorenzo et al., 2005; Lipton, 1999; Lowenstein and Alldredge, 1998). Our data supports the previous research that showed neuronal death after status epilepticus to be excitotoxic in nature (Abele et al., 1990; Delorenzo et al., 2005; Fountain, 2000; Fujikawa, 2005; Meldrum, 1991; Peterson et al., 1989). Thus, glutamate mediated activation of NMDA receptors results in excessive influx of  $\text{Ca}^{2+}$  that triggers various NMDA receptor-coupled neurotoxic signaling cascades, some of which induce neuronal death, including the activation of proteases (Araujo et al., 2005), neuronal nitric oxide synthase (Chuang et al., 2007; Dawson and Dawson, 1996), ceramides (Mikati et al., 2003) and poly(ADP-ribose) polymerase-1 (Mandir et al., 2000). These variables may combine in a positive feed-forward cycle resulting in increased levels of intracellular  $\text{Ca}^{2+}$ , with subsequent energy failure and neuronal death (Fujikawa, 2005). The neuroprotection we observed upon

NMDA receptor antagonism or lowering extracellular  $\text{Ca}^{2+}$  could possibly result from inhibition of these neurotoxic cascades. It will be interesting to investigate these possibilities in future studies. These results are in line with earlier in vivo studies from our lab (Rice and DeLorenzo, 1998) and those reported by others (Fujikawa et al., 1994), that NMDA receptor inhibition did not stop on-going status epilepticus activity, but the blockade of NMDA receptor signaling pathways was sufficient to attenuate status epilepticus induced neuronal death.

Microdialysis studies in both animals and humans have documented that prolonged seizure activity during status epilepticus is associated with significant elevations in extracellular glutamate (Ueda et al., 2002; Wilson et al., 1996). Thus, it is important to determine if accumulation of extracellular glutamate in the culture solution contributes to the status epilepticus induced neuronal death in our hippocampal neuronal culture model. While continuous perfusion of fresh low  $\text{Mg}^{2+}$  solution throughout the duration of status epilepticus does not remove the rapidly generated synaptic glutamate during status epilepticus, it prevents build up of glutamate or other seizure-induced metabolites that diffuse out of the synaptic cleft and accumulate over time in the culture media. Despite the continuous perfusion of fresh extracellular media, there was no significant decrease in neuronal death during status epilepticus. This finding is further supported by the observation that sustained, prolonged status epilepticus caused by electrical stimulation of the perforant pathway alone can damage postsynaptic neurons (Olney et al., 1983; Sloviter, 1983). In addition, it has also been reported that increasing durations of kainic acid induced status epilepticus to the point of neuronal injury cause cell death in the absence of a measurable increase in extracellular glutamate concentration (Tanaka et al., 1996). Our results are also in agreement with animal (Fujikawa, 2005; Gorter et al., 2003; Holmes, 2002; Pitkanen et al., 2002) and human data (Duncan, 2002; Liu et al., 2005; Thom et al., 2005) that indicate neuronal death is associated with acute status epilepticus and not with the subsequent chronic spontaneous recurrent seizures. Indeed post-mortem studies in humans have shown that neuronal loss produced upon accidental ingestion of domoic acid induces status epilepticus in the absence of systemic complications or pre-existing epilepsy that produces neuronal loss, which is similar in distribution to what is observed in clinical status epilepticus patients or in animal models of status epilepticus (Fujikawa et al., 2000).

In concordance with previously published studies, status epilepticus induced neuronal death in this model was found to be dependent on extracellular  $\text{Ca}^{2+}$  and occurred via the activation of NMDA receptor pathway (Abele et al., 1990; Meldrum, 1991; Peterson et al., 1989). However, status epilepticus induced activation of the NMDA receptor pathway required approximately 8–10 h to produce over 80% cell death in comparison to glutamate induced cell death in cultured neurons that requires only 10–15 min to achieve similar amounts of cell death (Choi et al., 1987; Deshpande et al., 2007b; Hardingham et al., 2002). Sattler et al., have reported that synaptically and extrasynaptically activated NMDARs are equally capable of excitotoxicity. In addition, the relative contributions vary with

the location of extracellular excitotoxin accumulation (Sattler et al., 2000). Thus, further studies are needed to evaluate the time-dependent difference in cell death produced by these different injuries.

Development of the “excitotoxic hypothesis” in the 1980’s (Olney, 1985) led to the production of many NMDA receptor antagonists including some used in this study. However, later observations that these agents were neurotoxic resulted in a decreased interest towards NMDA receptor antagonism as a potential therapeutic avenue (Olney, 1994). While the interest has been rekindled in recent years with development of ketamine and memantine and other NMDA receptor antagonists, the progress has been slow (Chen and Lipton, 2006; Kohl and Dannhardt, 2001). The hippocampal neuronal culture model of in vitro status epilepticus provides a powerful model that could allow for rapid screening of neuroprotective agents and suggests that further investigations into the use of NMDA receptor antagonists should be considered in the treatment of status epilepticus. The pharmacological evidence used in this paper allowed us to selectively inhibit various ion channels and receptors without stopping status epilepticus. It was important to be able to demonstrate that NMDA receptor activation was an important mediator of cell death during status epilepticus. Inhibition of the status epilepticus spike discharges by using tetrodotoxin or  $\omega$ -conotoxin in the presence of 0  $\text{Mg}^{2+}$  demonstrated that 0  $\text{Mg}^{2+}$  alone was not causing neuronal death. This paper provides direct evidence that cell death can be produced by continuous electrographic epileptiform events through activation of the NMDA receptor system. The longer time course required to cause NMDA receptor- $\text{Ca}^{2+}$  dependent neuronal death due to in vitro status epilepticus offers a wide therapeutic window to develop novel therapeutic interventions to prevent brain injury due to status epilepticus.

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