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Spreading depolarizations mediate excitotoxicity in the development of acute cortical lesions☆

Jason M. Hinzman [a,*], Vince A. DiNapoli [a,b], Eric J. Mahoney [a], Greg A. Gerhardt [c], Jed A. Hartings [a,b]

[a] Department of Neurosurgery, University of Cincinnati (UC) College of Medicine and Neurotrauma Center at UC Neuroscience Institute, Cincinnati, OH, USA
[b] Mayfield Clinic, Cincinnati, OH, USA
[c] Department of Anatomy and Neurobiology, University of Kentucky Chandler Medical Center, Morris K. Udall Parkinson's Disease Research Center of Excellence, Center for Microelectrode Technology, Spinal Cord and Brain Injury Research Center, Lexington, KY, USA

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ABSTRACT

Spreading depolarizations (SD) are mass depolarizations of neurons and astrocytes that occur spontaneously in acute brain injury and mediate time-dependent lesion growth. Glutamate excitotoxicity has also been extensively studied as a mechanism of neuronal injury, although its relevance to in vivo pathology remains unclear. Here we hypothesized that excitotoxicity in acute lesion development occurs only as a consequence of SD. Using glutamate-sensitive microelectrodes, we found that SD induced by KCl in normal rat cortex elicits increases in extracellular glutamate (11.6 ± 1.3 μM) that are synchronous with the onset, sustainment, and resolution of the extracellular direct-current shift of SD. Inhibition of glutamate uptake with D,L-*threo*-β-benzyloxyaspartate (TBOA, 0.5 and 1 mM) significantly prolonged the duration of the direct-current shift (148% and 426%, respectively) and the glutamate increase (167% and 374%, respectively) in a dose-dependent manner ($P < 0.05$). These prolonged events produced significant cortical lesions as indicated by Fluoro-Jade staining ($P < 0.05$), while no lesions were observed after SD in control conditions or after cortical injection of 1 mM glutamate (extracellular increase: 243 ± 50.8 μM) or 0.5 mM TBOA (glutamate increase: 8.5 ± 1.6 μM) without SD. We then used an embolic focal ischemia model to determine whether glutamate elevations occur independent of SD in the natural evolution of a cortical lesion. In both the ischemic core and penumbra, glutamate increased only in synchrony with anoxic terminal SD (6.1 ± 1.1 μM) and transient SDs (11.8 ± 2.4 μM), and not otherwise. Delayed terminal SDs were also observed in two animals at 98 and 150 min after ischemic onset and induced similar glutamate elevations. Durations of SDs and glutamate increases were significantly correlated in both normal and ischemic animals ($P < 0.05$). These data suggest that pathologically prolonged SDs are a required mechanism of acute cortical lesion development and that glutamate elevations and the mass electrochemical changes of SD and are merely different facets of the same pathophysiologic process.

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Introduction

In 1954, Hayashi observed that elevations in glutamate were capable of inducing convulsions and seizures (Hayashi, 1954). Fifteen years later, Olney discovered that high concentrations of glutamate produced lesions in the brain, coining the term "excitotoxicity" (Olney, 1969). This toxic process was found to be dependent on an overwhelming influx of calcium from activated N-methyl-D-aspartate receptors (NMDARs) (Choi, 1987; Rothman, 1984). The discovery of elevations in extracellular glutamate after traumatic brain injury (TBI) and stroke (Benveniste et al., 1984; Bullock et al., 1995, 1998; Hillered et al., 1989; Nilsson et al., 1990) implicated excitotoxicity as a principal mechanism of secondary brain injury and identified NMDARs as a lead candidate for therapeutic targeting. Following promising preclinical studies (Gill et al., 1987, 1988; Hayes et al., 1988), multiple NMDAR antagonists were tested in clinical trials (Muir, 2006). However, all of these trials failed to show clinical efficacy (Muir, 2006). Doubts about glutamate's role as the primary driver of excitotoxicity (Obeidat et al., 2000; Obrenovitch and Urenjak, 1997) were reinforced by reports that endogenous elevations in glutamate alone are insufficient to induce significant neuronal damage (Massieu et al., 1995; Obrenovitch et al., 1997) and that supra-physiological concentrations of glutamate (>20 mM) are required for the development of focal lesions in vivo (Landolt et al., 1998).

Abbreviations: ATSD, anoxic terminal spreading depolarization; CCA, common carotid artery; EAAT, excitatory amino acid transporter; ECA, external carotid artery; ECoG, electrocorticography; ICA, internal carotid artery; MEA, microelectrode arrays; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; NMDAR, N-methyl-D-aspartate receptor; PBS, phosphate-buffered saline; SD, spreading depolarization; TBOA, D,L-*threo*-β-benzyloxyaspartate; TTC, 2,3,5 triphenyltetrazolium.

☆ J.M.H. and V.A.D. contributed equally to this work.

* Corresponding author at: Department of Neurosurgery, University of Cincinnati, 231 Albert Sabin Way, Cincinnati, OH 45267, USA. Fax: +1 513 558 7702.
E-mail address: jmhinz2@gmail.com (J.M. Hinzman).

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An alternative role for glutamate in brain injury is based on Van Harreveld's hypothesis that accumulation of extracellular glutamate is responsible for the initiation and propagation of spreading depolarization (SD) (Van Harreveld, 1959). SDs are waves of mass depolarization of neurons and astrocytes that propagate across the cerebral cortex at 2–5 mm/min (Dreier, 2011; Somjen, 2001). Recent studies have shown that SDs are a key pathophysiological process of secondary brain injury that occur spontaneously and frequently in many patients with acute neurological insults (Bosche et al., 2010; Dreier et al., 2006, 2009; Hartings et al., 2009, 2011a; Lauritzen et al., 2011) and are associated with neurological deterioration and poor outcomes (Dreier et al., 2006, 2009; Hartings et al., 2011b; Oliveira-Ferreira et al., 2010). In animals, prolonged SDs are a causal mechanism of ischemic lesion growth (Back et al., 1996; Busch et al., 1996; Nakamura et al., 2010).

Recent ex vivo experiments using glutamate-sensitive microelectrode arrays (MEAs) have supported Van Harreveld's hypothesis, showing that regenerative glutamate release and presynaptic NMDAR activation are critical mechanisms of SD initiation and propagation (Zhou et al., 2013). The loss of electrochemical membrane gradients during SD (Hablitz and Heinemann, 1989; Hansen and Lauritzen, 1984; Kraig and Nicholson, 1978) may also imply that SD is a primary contributor to excitotoxic processes. Microdialysis studies have shown increases in extracellular glutamate in association with both SD and the related phenomenon of anoxic terminal spreading depolarization (ATSD) (Fabricius et al., 1993; Iijima et al., 1998; Kunimatsu et al., 1999; Satoh et al., 1999; Ueda et al., 1992). During sustained depolarizations, neurons may be particularly vulnerable to glutamate since the Mg^{2+} block of NMDARs is removed and the sodium- and voltage-dependence of excitatory amino acid transporters (EAATs) would limit glutamate clearance (Danbolt, 2001; Sarantis and Attwell, 1990; Szatkowski et al., 1990). Indeed, it was recently shown that neuronal death induced by SD is mediated by irrecoverable calcium influx through NMDARs, as typically observed with glutamate excitotoxicity (Aiba and Shuttleworth, 2012).

The mechanisms underlying glutamate increases in the development of acute brain lesions remain unclear. Similarly, it is unknown whether excitotoxicity is a process that occurs independent of SD, in strict association with SD, or some combination; no studies have examined glutamate dynamics in relation to SD in a model of focal brain lesion development. Therefore, here we used glutamate-sensitive electrodes to determine whether SD is a required mechanism for the development of excitotoxic lesions and to determine whether glutamate increases are a cause or consequence of SD in the natural development of focal cerebral infarction. Our results indicate that excitotoxic processes are confined to the phenomenon of SD and have implications for neuroprotection clinical trials.

Methods

Animals

Forty-five male Sprague–Dawley rats weighing 325–400 g (Harlan Laboratories Inc.) were used in the experiments. Animals were housed singularly in polycarbonate cage (10.25 × 18.75 × 8 in.) with bed-o-cob bedding in a pathogen free room. Animals were in a 12 h light/dark cycle with food and water available ad libitum according to standards of the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal procedures and care occurred during the light cycle and were approved by the University of Cincinnati Institutional Animal Care and Use Committee and conformed to the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council).

Glutamate microelectrode array fabrication

Microelectrode arrays (MEAs) with four platinum recording sites (50 × 150 μm) arranged vertically with 50 μm spacing between sites, spanning a total distance of 750 μm, were prepared and selected for in vivo recordings as follows. Briefly, a glutamate-oxidase solution covered the distal pair of recording sites to allow for the enzymatic conversion of glutamate to α-ketoglutarate and the generation of the reporter molecule, H_2O_2. A small drop (~0.1 μL) of the glutamate-oxidase solution, consisting of 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 0.125% glutaraldehyde (Sigma-Aldrich), and 1% glutamate-oxidase (Sigma-Aldrich) was manually applied using a dissecting microscope and a microsyringe. Two enzyme coats were applied with a 1-minute drying period between each coat. The two proximal recording sites served as sentinels and were coated with a solution containing 1% bovine serum albumin and 0.125% glutaraldehyde using the same procedures. After coating, the MEAs were cured for at least 48 h in a low humidity environment. The two different coatings allowed the use of a self-referencing technique, whereby the background current of the sentinel (− glutamate oxidase) sites was subtracted from the current of the (+ glutamate-oxidase) sites, thus producing a more selective glutamate measure and allowing the determination of basal levels of glutamate.

A size exclusion layer of 1,3-phenylenediamine was electroplated onto the platinum recording sites to restrict the passage of large molecule interferents (e.g., ascorbic acid). For electroplating, the enzyme-coated MEAs were connected to the FAST-16 mkI system (Fast Analytical Sensor Technology Mark I; Quanteon, L.L.C., Nicholasville, KY) and the tip of the MEA was placed in a 5 mM 1,3-phenylenediamine solution (Acros Organics, Morris Plains, NJ). A triangular potential wave with ± 0.25 V peak amplitude, offset of − 0.5 V, and frequency of 0.05 Hz was applied for 20 min (Hinzman et al., 2010, 2012).

Glutamate MEA calibration

Calibrations were conducted to test the capability of the MEAs to measure glutamate and generate a standard curve for the conversion of current to glutamate concentration. Constant potential amperometry was performed using a specialized grounded head stage that permitted simultaneous electrophysiological measures. A potential of 0.7 V versus a Ag/AgCl reference electrode was applied to oxidize the reporter molecule, H_2O_2, which is a two-electron oxidation reaction that occurs at the platinum recording sites. The resulting current was amplified and digitized by the FAST-16 mkI system. The MEAs were placed in a continuously-stirred 40-mL solution of 0.05 M phosphate-buffered saline maintained at 37 °C with a re-circulating water bath (Gaymar Industries Inc., Orchard Park, NY). The MEAs were exposed to final concentrations of 250 μM ascorbic acid, 20, 40, and 60 μM glutamate, and 8.8 μM H_2O_2. Parameters tested were limit of detection (three times the relative standard deviation of the baseline noise), selectivity (ability of the MEA to measure glutamate compared to ascorbic acid), and slope of the electrode (the linear increase in current due to additions of glutamate). The average limit of detection was 0.98 ± 0.1 μM, selectivity was 29 ± 3.3 (glutamate: ascorbic acid), and slope was 13.8 ± 0.01 pA/μM. Calibrations were made before in vivo experimentation to ensure that the MEAs were functional before implantation. Previous studies have shown that these arrays have rapid glutamate response times (≤1 s) and perform similarly pre- and post-implantation (Burmeister and Gerhardt, 2001; Burmeister et al., 2002; Hinzman et al., 2010, 2012).

Electrophysiology and drug delivery

For electrophysiology, borosilicate glass micropipettes (1.5 mm o.d. 0.86 mm i.d., Sutter Instrument, Novato, CA) were pulled (P-97, Sutter Instruments) with a 2–3-μm tip diameter and average impedance of 1.7 ± 0.1 MΩ. To record both DC and AC signals, pipettes were filled with saline and connected to a differential amplifier (DP-304, Warner instrument, Hamden, CT) versus a Ag/AgCl reference. To measure extracellular glutamate and electrophysiology at the same location, a micropipette was connected to the surface of the MEA with wax so that the tip

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