Electrophysiological Mechanisms of Delayed Excitotoxicity: Positive Feedback Loop between NMDA Receptor Current and Depolarization-Mediated Glutamate Release

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ABSTRACT

Delayed excitotoxic neuronal death following insult from exposure to high glutamate concentrations appears important in several CNS disorders. Although delayed excitotoxicity is known to depend on NMDA receptor (NMDAR) activity and Ca\(^{2+}\) elevation, the electrophysiological mechanisms underlying post-insult persistence of NMDAR activation are not well understood. Membrane depolarization and non-specific cationic current in the post-insult period have been reported previously, but were not sensitive to NMDAR antagonists. Here, we analyzed mechanisms of the post-insult period using parallel current- and voltage-clamp recording and Ca\(^{2+}\) imaging in primary hippocampal cultured neurons. We also compared more vulnerable older neurons (~22 days in vitro, DIV) to more resistant younger (~15 DIV) neurons, to identify processes selectively associated with cell death in older neurons. During exposure to a modest glutamate insult (20 \(\mu\)M, 5 min), similar degrees of Ca\(^{2+}\) elevation, membrane depolarization, action potential block, and increased inward current occurred in younger and older neurons. However, following glutamate withdrawal, these processes recovered rapidly in younger but not in older neurons. The latter also exhibited a concurrent post-insult increase in spontaneous miniature excitatory post-synaptic currents, reflecting glutamate release. Importantly, post-insult NMDAR antagonist administration reversed all of these persisting responses in older cells. Conversely, repolarization of the membrane by voltage clamp immediately after glutamate exposure reversed the NMDAR-dependent Ca\(^{2+}\) elevation. Together, these data suggest that, in
vulnerable neurons, excitotoxic insult induces a sustained positive feedback loop between NMDAR-dependent current and depolarization-mediated glutamate release which persists after withdrawal of exogenous glutamate and drives Ca\textsuperscript{2+} elevation and delayed excitotoxicity.

**Key Words:** age, hippocampal neurons, Ca\textsuperscript{2+} imaging, culture
INTRODUCTION

Excitotoxic neuronal death (Olney 1986) can be induced by excessive activation of glutamate receptors during exposure to high glutamate concentrations. The induction of such excitotoxicity depends on Ca\(^{2+}\) influx through \(N\)-methyl-D-aspartate type glutamate receptors (NMDARs) (Choi 1992; Hartley et al. 1993; Limbrick et al. 2001; Lipton, 2004; Nicotera and Orrenius 1998; Randall and Thayer 1992; Tymianski et al. 1993) and is widely thought to play an important role in several neuropathological conditions, including ischemia, stroke, trauma, HIV-related neurotoxicity and, possibly, neurodegenerative disease (Choi 1995; Lipton and Rosenberg 1994; Olney 1986).

However, excitotoxic neuronal death is often delayed for some hours after the termination of a glutamate insult. Several lines of evidence indicate that continuing activity of NMDARs during the post-insult phase is essential for the expression of this delayed excitotoxicity. Perhaps most importantly, it has been found that NMDAR antagonists administered up to an hour or longer after glutamate exposure can be fully neuroprotective (Choi et al. 1988; Levy and Lipton, 1990; Munir et al. 1995; Okiyama et al. 1998; Rothman et al. 1987; Shalaby et al. 1992; Smith et al. 1993). Delayed excitotoxicity also appears to be Ca\(^{2+}\)-dependent and has been linked to a persistent Ca\(^{2+}\) elevation (PCE) that follows termination of glutamate insult. This PCE has been sensitive to block by NMDAR antagonists in some studies, but not in others (Attucci et al. 2002; Cheng et al. 1999; Dubinsky 1993; Limbrick et al. 2001; Rajdev and Reynolds...
1994; Randall and Thayer 1992; Vergun et al. 1999), suggesting there may be multiple phases or sources of Ca\textsuperscript{2+} elevation.

Despite the critical importance of the post-insult period, however, little is known about the mechanisms that sustain NMDAR activity or the PCE following glutamate withdrawal, in part because of a paucity of electrophysiological studies on delayed excitotoxicity and the sequence of events unfolding during the post-excitotoxic insult period. The few studies on this phase have identified several membrane responses as potential mechanistic mediators of delayed excitotoxicity, including extended neuronal depolarization (Coulter et al. 1992; Limbrick et al. 2001) and an inward non-specific cationic current (post-exposure current, $I_{PE}$) (Chen et al. 1997; Chen et al. 1998). However, once induced, neither of these membrane responses could be interrupted by NMDAR antagonists, indicating that they do not mediate the toxic actions of persistent NMDAR activity.

Here, we combined parallel current-clamp, voltage-clamp and Ca\textsuperscript{2+} imaging techniques, to analyze events during and following excitotoxic exposure. Further, to elucidate which post-insult responses are most closely associated with cell death, we studied primary hippocampal cultured neurons at different ages, as their vulnerability to excitotoxicity increases substantially with developmental age (Adamec et al. 1998; Attucci et al. 2002; Cheng et al. 1999; Choi 1992; Clodfelter et al. 2002; Frandsen and Schousboe 1991; Toescu and Verkhratsky 2000; Xia et al. 1995). Together, the results appear to identify a novel positive feedback loop that sustains NMDAR current and Ca\textsuperscript{2+} elevation following excitotoxic insult.
METHODS

Cell Culture: Primary hippocampal mixed neuronal-glial cell cultures were prepared from fetal pup tissue (embryonic day 18) obtained from pregnant Sprague-Dawley rats using slight modifications of the Banker method (Banker and Cowan 1977) as previously described (Porter et al. 1997). All protocols were approved by the institutional animal care and use committee. Fetal hippocampal tissue was isolated, and neurons were dissociated and plated on 35 mm poly-L-lysine-coated dishes and Thermanox plastic coverlips (Nunc, Naperville, IL) as described previously (Blalock et al. 1999). In most studies, age comparisons were performed in cultures of younger neurons (11-17 days in vitro, DIV) against sister cultures of older neurons (21-24 DIV), though in some experiments (see Figure 1), somewhat different age ranges (younger, 7-14 DIV; older, 28-35 DIV) were used. All experiments were conducted at room temperature.

Solutions: For all experiments the external recording/imaging solution contained (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 10 D-glucose, 2 CaCl₂, 1 MgCl₂, 0.01 glycine. pH was adjusted to 7.35 using NaOH. Osmolality was adjusted to 310 mOsm with sucrose. The recording pipette solution contained (in mM): 150 KCH₃SO₄, 5 HEPES, 4 Tris-ATP, 0.3 Tris-GTP, 1.4 Tris-phospho-creatine, and 0.1 leupeptin. pH was adjusted to 7.35 using KOH and osmolality was adjusted to 290 mOsm by dilution with dH₂O. All solutions were sterile-filtered.
Electrophysiology: Recording pipettes made from glass capillary tubes (Drummond Scientific, Broomall, PA) were pulled on a horizontal micropipette puller (model P-87; Sutter Instruments, Novato, CA). All pipettes were coated with polystyrene Q-dope and were fire-polished immediately before recording (Corey and Stevens 1983). Tip resistances were similar across the two age groups and ranged between 2.4 and 4.5 MΩ (mean = 3.3 ± 0.09 MΩ). Plastic coverslips layered with hippocampal neurons, were cut into fragments and transferred to a perfusion chamber (Warner Instruments Inc., Hamden, CT). Spontaneous synaptic activity was monitored in individual neurons with current-clamp or voltage-clamp techniques using Axopatch 200 amplifiers and pClamp 8.0 software (Axon Instruments, Foster City CA). Cells unable to maintain a resting $V_m$ of at least -50 mV for five min without current injection through the recording electrode were discarded. Spontaneous excitatory postsynaptic potentials (EPSPs) and action potentials (APs) were filtered at 10 kHz and digitized at 1 kHz. In voltage-clamp experiments, neurons were clamped at -80 mV throughout the experiment. Spontaneous excitatory postsynaptic currents (EPSCs) were filtered at 1 kHz and digitized at 2 kHz.

At the beginning of each experiment, junction potentials were nulled in the bath and pipette capacitance was compensated. Whole-cell membrane capacitance was determined offline by integrating the area of the capacitive transient generated during a 10 ms, 5 mV hyperpolarizing step from -70 mV. Membrane capacitance was 33.8 ± 1.9 pF for younger neurons ($n = 47$) and 40.1 ± 3.5 pF for older neurons ($n = 61$) ($p < 0.05$). Access resistances were 6.9 ±
0.46 and 5.59 ± 0.3 MΩ (p < 0.05), respectively. These values and the differences observed between younger and older cells were similar to those reported earlier (Blalock et al. 1999; Porter et al. 1997).

Indo-1 imaging: For some Ca²⁺ imaging studies, Indo-1 was used as described previously (Attucci et al. 2002; Clodfelter et al. 2002). Briefly, cultures were placed at room temperature in the dark for a 20 min incubation period in 2 µM of the acetoxyethyl ester of Indo-1, AM (Molecular Probes, Eugene, OR). Cells were then washed three times with indicator-free recording solution, and incubated for an additional 15 min to allow de-esterification of the intracellular indicator.

Ca²⁺ transients were acquired on an RCM 8000 UV-compatible confocal laser-scanning microscope (Nikon, Inc., NY) equipped with a Nikon Diaphot 300 and a 40 X water immersion objective. Two emitted wavelengths (<400 nm and >500 nm) were acquired simultaneously through a dichroic mirror centered at 445 nm. Excitation was provided by an argon-laser (351-364 nm). All signals were background-subtracted from a cell-free area adjacent to the cells of interest. The area of interest was outlined by hand using Metamorph imaging software (Universal Imaging Corporation, West Chester, PA), and consisted of the somatic region. A ratio of the two wavelength images was constructed in Metamorph and analyzed based on an in situ calibration (see below). Ratios were acquired immediately prior to glutamate (Glu) application (rest), and at 2, 10, 30 s and 5
min during glutamate application, as well as at 1, 5, 15, 30, and 45 min post-glutamate.

*In situ* Indo-1 calibration: Ca$^{2+}$ concentrations were calibrated *in situ* using hippocampal neurons prepared and maintained as above. Neurons were dialyzed with Indo-1 (pentapotassium salt, Molecular Probes, Eugene, OR) at two concentrations (50 or 100 µM) using patch pipettes, to determine the extent of intracellular Indo-1-loading. The bathing solution consisted of (in mM): 130 LiCl$_2$, 5 CsCl, 2 MgCl$_2$, 10 glucose, 10 Heps, 0.001 TTX and 0.1 D(-)-2-amino-4-phosphonopentanoic acid (D-APV). Ratios were converted to [Ca$^{2+}$] by the equation [Ca$^{2+}$]=Kdβ(R-R$_{min}$)/(R$_{max}$-R), where R is the 400/500 nm fluorescence emission ratio of a particular cell and R$_{max}$, R$_{min}$, and Kdβ were determined from the *in situ* calibration (Gryniewicz et al. 1985; Poenie 1990). Separate calibration curves were developed for younger and older cells and no differences in the parameters were found. The *in situ* calibration yielded values of 1.3 for R$_{min}$, 4.3 for R$_{max}$ and 0.8 for Kdβ (in µM).

*Fura-2 imaging and concomitant electrophysiology:* Axon Imaging Workbench 2.2 (AIW, Axon Instruments) and pClamp 7 were used for concomitant acquisition of Ca$^{2+}$ imaging and electrophysiology data. Briefly, glass pieces (broken glass-bottom 35 mm culture dishes) containing primary cultures of hippocampal cells were transferred to a RC-22 perfusion chamber (Warner Instruments Inc.) and individual cells were loaded with 10 µM Fura-2
pentapotassium salt (Molecular Probes) via the recording patch pipette. Although this relatively low Fura 2 concentration minimized the intracellular buffering of Ca\(^{2+}\) (Helmchen et al., 1996), it also made it difficult to reliably image Ca\(^{2+}\) dynamics in neuronal processes. As such, all measurements reported here refer to somatic Ca\(^{2+}\) levels.

A CCD camera (Princeton Instruments, MicroMax 5 MHz) in combination with an illumination controller (Sutter Instruments, Lambda DG4) was used to acquire emitted light (510 nm) and excite the fluorophore (340 and 380 nm) through a dichroic mirror. A Nikon E600FN microscope hosted the recording and imaging setup. Exposure times were kept to a minimum (0.5-1 sec) and a ratio was acquired every 20 sec starting two minutes before glutamate application. Background-subtracted 340 and 380 nm wavelengths were ratioed and analyzed using AlW. Calibration of Fura-2 ratiometric values was accomplished using a series of increasing free Ca\(^{2+}\) concentrations (Molecular Probes, 1mM Mg\(^{2+}\) kit). Values for \(R_{\text{min}}, R_{\text{max}}\) and \(K_D\) were determined from the calibration curve and used to calculate free [Ca\(^{2+}\)] using the formula described above for Indo-1. For this calibration set, \(R_{\text{min}}, R_{\text{max}}\) and \(K_D\) were 0.6, 7.9 and 1.3 \(\mu\)M, respectively.

In experiments in which a hybrid current clamp/voltage clamp protocol was used, the Axopatch 1D (Axon Instruments) was switched from current clamp to voltage clamp mode and a command potential of −30 mV was immediately imposed on the cell membrane. Then, over the course of one minute, \(V_m\) was ramped linearly to a final value of −80 mV.
**Drug Delivery:** All drugs, including L-glutamate, D-APV, the AMPA receptor blocker, CNQX, the NMDA receptor antagonist MK-801, and the Na\(^+\) channel blocker, TTX, were dissolved in HPLC grade H\(_2\)O and stored as stock solutions (\(+4^\circ\) C). When used, these compounds were diluted 1000x in recording solution.

QX-314 (1 mM) (Tocris, Ellisville, MO) was present in the recording pipette in a subset of voltage-clamp experiments to prevent the possible generation of Na\(^+\)-driven “action potential” currents in the recorded cell. In each experiment, the control condition consisted of adding H\(_2\)O vehicle to the recording medium at a 1:1000 dilution. All external solutions were perfused through the recording chamber at 1 to 1.5 mL/min.

**Data Analysis:** Effects of age and/or treatment were assessed by repeated measures ANOVA. Maximal [Ca\(^{2+}\)], \(V_m\), and \(I\) values during the glutamate application were substantially larger than pre- and post-glutamate values and therefore analyzed separately by one-way ANOVA.

Properties of spontaneous membrane events (*i.e.* currents and potentials) including amplitude and total number of events were analyzed using Minianalysis software (Synaptosoft) and pClamp 7. The number of EPSCs was counted over a two minute period immediately before and fifteen minutes after glutamate exposure and the EPSCs were summed and binned according to amplitude. Grouping the data in this manner revealed that the majority of events in each treatment group fell between 15 and 20 pA (*i.e.* the peak of the distribution). After the peak, the number of events fell exponentially as the event amplitude
increased to approximately 300 pA. This decay was fit well with a single exponential (black line) and the resulting tau values were compared between groups using a Z-test. z-scores > |2| were considered significant. Events >300 pA (i.e. compound events) were excluded from these histograms and analyzed separately, primarily because their amplitudes were narrowly distributed and easily discriminated from small events. The mean number of these large compound events in a two minute window before and 15 minutes after glutamate exposure was compared across groups using repeated measures ANOVA. Scheffe’s F-test was used for all post hoc analyses. All statistical tests were conducted with SigmaStat (2.0.3) or Statview (5.0.1). For all statistical tests, significance was set at p < 0.05.

To compare membrane repolarization and [Ca^{2+}]i recovery kinetics, $V_m$ and [Ca^{2+}]i values recorded during the post-insult period were normalized to their respective peak values obtained immediately before glutamate removal. These normalized values were then fit with a Boltzman equation and the time required for $V_m$ and [Ca^{2+}]i to recover to half maximal values was compared using a z-test.
RESULTS

NMDAR activity was required for persistent Ca\(^{2+}\) elevation

Figure 1 illustrates experiments in which cultures were exposed for 5 minutes to glutamate, and then imaged for the remainder of one hour. As cells age in culture they exhibit a greater amplitude and more prolonged duration of Ca\(^{2+}\) elevation after a glutamate insult. The prolonged Ca\(^{2+}\) elevation (PCE) in older neurons following glutamate exposure is not attributable to a larger initial peak in Ca\(^{2+}\), since younger neurons do not reliably show a substantial PCE, even when glutamate concentrations are adjusted to generate similar maximal Ca\(^{2+}\) elevations (Attucci et al. 2002).

Age-dependent changes and NMDAR sensitivity of the PCE are shown in Figure 1. As reported previously (Attucci et al. 2002), a 5-min glutamate application (100 µM) to younger (7-14 DIV) neurons produced a rise in [Ca\(^{2+}\)]\(_i\) that endured for the duration of glutamate application, but then began to return gradually to basal levels soon after removal of glutamate (Figs. 1A, C). In older cells (28-35 DIV), [Ca\(^{2+}\)]\(_i\) also increased during glutamate (10 µM) application (Figure 1B). However, in contrast to younger neurons, [Ca\(^{2+}\)]\(_i\) remained elevated after glutamate removal and did not recover to basal levels.

In parallel experiments, we confirmed the regulation of this age-dependent PCE by post-insult NMDAR activity, exposing another group of cells to the competitive NMDAR antagonist, D-APV (100 µM) immediately after the termination of the 5-min glutamate insult (Figure 1A, B). Other cells were exposed post-insult to the AMPA receptor antagonist, CNQX (10 µM). Neither
antagonist had a significant effect on younger cultures, in which the PCE was small. However, in older cells, the post-insult application of D-APV markedly facilitated the recovery of $[\text{Ca}^{2+}]$. In contrast, CNQX had no effect on the PCE in older cells.

Because there appear to be some differences in neuroprotective actions of competitive and non-competitive NMDAR antagonists (Levy and Lipton, 1990), we also tested the effects of the non-competitive NMDAR antagonist, MK-801, in a similar experiment. When given immediately in the post insult period, MK-801 also reversed the PCE in older cells (Figs. 1C, D). These results indicate that the age-dependent PCE is maintained by post-insult activity of NMDARs.

**Membrane depolarization was age-related and NMDAR-dependent**

To determine whether post-insult membrane depolarization and spontaneous action potential firing also exhibit age-and/or NMDAR-dependence, younger (11-17 DIV) and older (21-24 DIV) neurons were examined under current clamp conditions with whole-cell patch pipettes before, during, and after a 5 min glutamate application (20 $\mu$M) (Figure 2). In order to reduce synaptic connectivity differences between age-in-culture groups during the electrophysiological studies, the younger neurons in this experiment and all subsequent experiments were slightly older (11-17 DIV), and the older neurons were somewhat younger (21-24 DIV), than those used in the preceding experiment (see Fig 1). Cells $< 11$ DIV exhibit sparse synaptic connectivity (Porter et al., 1997) and little spontaneous action potential activity (data not shown), whereas 11-17 DIV
neurons exhibit more extensive synaptic connectivity and increasing spontaneous network activity (see Figs 2 and 3). Moreover, the degree of Ca\(^{2+}\) elevation in younger (11-17 DIV) and older (> 21 DIV) neurons during a 5 min application of 20 \(\mu\)M glutamate is more nearly comparable (see Figure 6), although the PCE and probability of cell death are still substantially greater in the older neurons (Attucci et al. 2002).

Prior to glutamate treatment, resting \(V_m\) was slightly but significantly more depolarized in younger vs older neurons (-55.23 ± 2.08 mV \(n = 17\), vs -62.19 ± 2.44 mV \(n = 16\), \(p < 0.05\)). In both age groups, neuronal \(V_m\) was permitted to fluctuate freely and no holding current was applied during the course of the experiment. Representative examples of these experiments are illustrated in Figure 2A-C and group mean data are shown in Figure 2D. Bath perfusion of glutamate induced substantial membrane depolarization (denoted as \(\Delta V_m\), or difference from resting \(V_m\)), which was maintained for the 5 min duration of glutamate exposure, and did not differ between age groups (younger, 51 ± 4.5 mV, \(n = 17\); older, 55 ± 3.5 mV, \(n = 16\); \(p = 0.55\)) (Figs. 2A, B, D, Glu).

Upon depolarization, all spontaneous action potential (AP) activity, which tended to be greater in older cultures, was blocked (Figs. 2A, B). Similar to the pattern of [Ca\(^{2+}\)] changes that occurred following glutamate exposure (Figure 1A, B), younger neurons repolarized quickly after washout of glutamate (Figs. 2A, D, 5 min post). In older neurons, repolarization generally failed, resulting in persistent depolarization (Figure 2B). Repolarization in the younger group, which generally occurred within five minutes of glutamate removal (Figure 2D, 5 min, p
< 0.005), was typically accompanied by reemergence of spontaneous APs and EPSPs at pre-insult or greater frequency (Figure 2A). AP activity generally did not resume in depolarized older neurons (Figure 2B).

To test whether the age-related depolarization following glutamate exposure was dependent on post-insult NMDAR activity, a subset of older neurons \( (n = 8) \) was subjected to the same 5 min, 20 µM glutamate insult, followed immediately by perfusion with normal recording medium containing 10 µM MK-801. Similar to its effects on the PCE (Figure 1D), post-insult application of MK-801 reduced or prevented the development of sustained depolarization (Figure 2C, D, \( p < 0.005, 15 \) min post). MK-801- treated older neurons behaved similarly to younger neurons and were able to restore membrane potential and spontaneous APs within minutes of the removal of glutamate, indicating that this depolarization, like the PCE, was both age- and NMDAR activity-dependent.

**Persistent post-insult current was age- and NMDAR-dependent**

In a series of voltage-clamp experiments, paralleling current-clamp studies in Figure 2, younger (~15 DIV, \( n = 25 \)) and older (~22 DIV, \( n = 23 \)) neurons were voltage-clamped at –80 mV before, during and after 5 min glutamate exposure (20 µM). A subset \( (n = 9) \) of older cells also was treated in the post-insult period with MK-801 (see below). Representative examples of these experiments are illustrated in Figure 3.

No age-related differences were found in holding current \( (I) \) during the pre-glutamate period (younger, -86.5 + 69.6 pA; older, -123.11 + 91.9 pA). Bath
perfusion of glutamate (5 min) induced a substantial increase in current ($\Delta I$) for both age groups ($p < 0.0001$), which tended non-significantly to be greater in the older group (-939 ± 110 pA) relative to the younger group (-728 ± 61 pA, $p > 0.05$) (Figs. 3A, B, D, Glu). During the post insult period, $\Delta I$ was significantly larger in older neurons ($p = 0.01$) (Figure 3 B, D, 5 min post). However, under these hyperpolarized conditions, the post-insult current in older neurons declined considerably during the next 15 min.

MK-801 treatment blocked or reversed the post insult current in older neurons (5 min, $p < 0.01$; 15 min, $p < 0.05$), indicating dependence on persistent NMDAR activity. Because NMDAR current is much attenuated at negative holding potentials (-80 mV here), it seems highly likely that the post-insult NMDAR-mediated current would have been substantially larger and lasted longer if the membrane had been clamped to a more positive potential. Nonetheless, even at these hyperpolarized levels, there was substantially greater NMDAR-dependent current in older neurons during the post-insult period.

**Changes in network activity following glutamate exposure**

The voltage-clamp mode provides an advantageous configuration for monitoring synaptic activity and transmitter release of neighboring connected cells, and for gauging network-wide behavior during and following glutamate exposure. That is, neurons clamped at a constant hyperpolarized potential are relatively protected against the subsequent post-insult $[Ca^{2+}]$ elevation experienced by neighboring unclamped cells (see below, Figure 4). Moreover, because the clamped neuronal
membrane does not fluctuate, driving force on excitatory postsynaptic currents (EPSCs) is constant and the clamped neuron can serve as a sensitive monitor of alterations in presynaptic glutamate release by assessing spontaneous network EPSCs before, during, or after toxic glutamate exposure.

Pharmacological approaches were used to define and identify the sources of EPSCs observed in the postsynaptic, voltage-clamped neuron (Figure 4). Spontaneous EPSCs could be divided roughly into two main categories (Figure 4A), based on their amplitudes: Smaller (<40 pA) EPSCs, most of which are likely unitary or mini-EPSCs, and larger compound inward currents (>300 pA) that often lasted for more than 100 ms ("compound EPSCs") (Figure 4A, right panel). Both small and compound EPSCs were blocked by CNQX, a specific AMPA receptor AMPAR) blocker, showing that they were generated by presynaptic glutamate release and mediated by AMPARs (Figure 4B). NMDARs appeared to make little contribution to spontaneous EPSC activity under these hyperpolarized clamped conditions, as MK-801 application did not alter the total number of small or large EPSCs (Figure 4C). The Na+ channel blocker, TTX, blocked only the larger burst-like compound EPSCs, while small unitary EPSCs were little affected by blockade of Na+ channels (Figure 4D). Large compound currents also were not inhibited by intracellular dialysis with the Na+ channel blocker, QX-314 (1 mM) (data not shown). Thus, the large compound EPSCs result from synchronous release of transmitter quanta evoked by (unseen) presynaptic action potentials.
To quantify the age-related changes in synaptic activity following glutamate (Figure 3), histograms of EPSC amplitudes were constructed before and after exposure to glutamate (Figure 5). The numbers of small and compound EPSCs were greater in older than younger neurons in the pre-glutamate period (number of small EPSCs, \( p < 0.001 \); number of compound EPSCs, \( p < 0.05 \)). In the post-glutamate period, the clearest change for younger neurons was a general increase in the number of EPSCs in the 15 to 60 pA range (Figure 5A). Exponential regression analysis revealed a post-insult increase in the histogram \( \tau \) (from pre-Glu, 10.03 pA to post-Glu, 14.45 pA, \( p < 0.05 \)), reflecting a shift to slightly larger EPSCs and a non-significant increase in the peak of the distribution (pre-Glu, 126 observations vs post-Glu, 190 observations, \( p > 0.05 \)). The number of compound EPSCs (i.e. \( \geq 300 \) pA), which reflected impinging action potentials, also tended to increase in the post-glutamate period, although this effect was not significant (Figure 5A, inset). Nevertheless, for many younger neurons (e.g., the one illustrated in Figure 3A), the number of compound EPSCs increased dramatically after glutamate exposure.

In contrast, the post-insult period for older neurons (Figure 5b) was characterized by a dramatic and selective increase in the number of smaller EPSCs, primarily in the range of 10-25 pA, with little change in the number of EPSCs above 30 pA. Regression analysis revealed a post-glutamate increase in distribution peak height (pre-Glu, 187 observations vs post-Glu, 331 observations, \( p < 0.05 \)) along with a reduction in \( \tau \) (from pre-Glu, 21.05 pA to post-Glu, 14.86 pA, \( p < 0.05 \)). Moreover, compound EPSCs, which were
prominent in older neurons during the pre-glutamate period (see Figs. 3B, C), essentially disappeared ($p < 0.01$) in the post-glutamate period (Figure 3B and 5B, inset), reflecting the loss of action potential activity in depolarized, unclamped neighboring neurons (Figure 2B) and the resulting lack of coordinated, synchronous glutamate release from multiple terminals in axonal ramifications within the neuronal network. These changes in the EPSC amplitude distributions for both age groups were maintained for the duration of recording (i.e. up to 30-45 min after glutamate washout). Together these alterations indicate that post-glutamate EPSCs and action potentials (and synchronous multiple EPSC bursts) are unchanged or increased somewhat in younger neurons, whereas, in older neurons, there is a complete loss of action potential-related compound EPSC events, along with a substantial increase in small EPSCs.

In older neurons treated with MK-801 post glutamate, however, synaptic activity was qualitatively and quantitatively similar across the pre- (peak of 170 observations, $\tau = 21.55$) and post- (peak of 168 observations, $\tau = 21.46$) glutamate periods (Figure 5C), much as in younger neurons. Also, compound EPSCs remained constant in the post-insult period for older cells treated with MK-801 (Fig 5C, inset). Thus, changes in network activity in the post-insult period were age-related and critically dependent on the ongoing activity of NMDARs, similar to the changes observed in intracellular $\text{Ca}^{2+}$, membrane depolarization, and inward current.

**Repolarization of neuronal $V_m$ in the post-insult period reverses the PCE**
To test whether correlative and/or causative relationships exist between the PCE and depolarization, we assessed \([\text{Ca}^{2+}]_i\) and \(V_m\) simultaneously in a subset of neurons before, during, and after a glutamate insult. In these neurons, glutamate induced an elevation in \(\text{Ca}^{2+}\) and a depolarization of the \(V_m\) that usually recovered soon after removal of glutamate in younger \((n = 5)\) (Figs. 6 A, C) but not older \((n = 7)\) (Figs. 6B, D) neurons. Across all cells (younger and older), the magnitude of the depolarization measured 15 min after glutamate washout exhibited a strong positive correlation with the magnitude of the PCE at 15 min \((p < 0.01)\) (Figure 6E). Moreover, for six neurons (all 5 younger neurons and one older neuron) in which the \([\text{Ca}^{2+}]_i\) and the \(V_m\) spontaneously recovered to near baseline values, the repolarization of \(V_m\) preceded the recovery of \([\text{Ca}^{2+}]_i\) (Figure 6F). \(\Delta V_m\) declined to half of its maximal value in 78.6 ± 6.67 sec, while \(\Delta[\text{Ca}^{2+}]_i\) fell to its half maximal value in 123.8 ± 6.92 sec \((z = 4.71, p < 0.05)\).

The more rapid recovery of \(V_m\) compared to \([\text{Ca}^{2+}]_i\), following glutamate withdrawal suggests that restoration of \(V_m\) may be a requisite for reestablishing \([\text{Ca}^{2+}]_i\) homeostasis during the post insult period. Thus, to test the hypothesis that the PCE depends on \(V_m\), the \(V_m\) of another subset of older neurons \((n = 7)\) in the experiment above was directly controlled in a hybrid current clamp-voltage clamp experiment in which \(V_m\) was monitored in current-clamp before and during glutamate exposure, but was then ramped to −80 mV by switching to voltage clamp immediately after glutamate washout (Figure 7B). Upon imposition of voltage clamp, \(I\) showed a steep reduction within two minutes, which was generally followed by the restoration of \([\text{Ca}^{2+}]_i\) to near pre-glutamate levels. There
were no differences in \([Ca^{2+}]_i\), between younger and voltage-clamped older neurons in the post-insult period, and at 15 min after glutamate removal, \([Ca^{2+}]_i\), in voltage-clamped older neurons was significantly lower than that in older neurons recorded in current clamp throughout (Figure 7C). Although some space-clamp problems may occur in these older neurons, voltage-clamp of slow potentials even in ramified neurons is generally effective after a delay. Therefore, in the present study, the hybrid clamp likely established relatively good control of membrane voltage, at least in the soma (Brown and Johnston, 1983).

Thus, \([Ca^{2+}]_i\), in younger neurons and older voltage-clamped neurons was indistinguishable during the post-insult period. These effects of clamping the membrane at \(-80\text{mV}\) following the insult on \([Ca^{2+}]_i\), were similar to those of NMDAR blockade in reversing the PCE in older neurons (Figure 1D), although the PCE reversal occurred more rapidly with voltage-clamp (Figure 7). However, the time course differences were likely the result of several methodological differences. The cells shown in Fig. 1 were considerably older than those in Fig. 7, making it more difficult to reverse the PCE. Moreover, the cells in Fig. 7 were studied under whole cell patch recording conditions and were also loaded with a lower concentration of \(Ca^{2+}\) buffering indicator, factors that may have accelerated cytosolic decay kinetics.

**DISCUSSION**

The results here show that multiple electrophysiological and \([Ca^{2+}]_i\), responses persist during the post-glutamate exposure period in older, vulnerable
neurons. These persisting responses include $[\text{Ca}^{2+}]_i$ elevation, inward current, membrane depolarization, block of action potentials, and increased frequency of miniature glutamate EPSCs (presumably driving NMDAR activity in depolarized neurons). Importantly, these processes appear to be interrelated, as each could be attenuated by MK-801 blockade of NMDAR current in the post-insult period. Conversely, repolarization of the membrane by voltage clamp was followed by termination of inward current and a return to near baseline of the elevated $[\text{Ca}^{2+}]_i$ (Figure 7). These reciprocal dependences suggest the operation of a positive feedback loop, as discussed further below.

**Conflicting results on the NMDAR dependence of post-insult responses**

As noted, prior studies of the post-insult phase also have seen extended depolarization (Coulter et al. 1992; Limbrick et al. 2001) and persistent inward current (Ipe, Chen et al. 1998). However, once induced, those responses were resistant to NMDAR antagonists. Conflicting results on NMDAR dependence could arise from several factors, including, of course, activation of different membrane currents in those studies than in the present study. Alternatively, the results could reflect differences in insult intensity. Substantially more intense excitotoxic insults were used in most prior studies (e.g., 500 $\mu$M glutamate for 10 min by Limbrick et al. (2001)) than here. Conceivably, more intense insults could trigger irreversible sequelae or processes that are independent of NMDARs, such as mitochondrial depolarization and disruption of electron transport (Vergun et al. 1999). In addition, irreversible NMDAR-independent changes might
accumulate with time after the insult, but the present study only examined the effects of NMDAR antagonists delivered immediately upon glutamate washout. Nonetheless, although additional dosage and time course studies will be needed to resolve these discrepancies, the present data clearly indicate that major post-insult electrophysiological and \([\text{Ca}^{2+}]_i\) responses are, at least initially, NMDAR dependent.

**Altered network activity and sources of post-insult glutamate**

An important and longstanding question regarding delayed excitotoxicity has been how NMDAR activity is maintained in the post-insult, glutamate washout period. One prominent hypothesis addressing this question proposes that high glutamate insult induces persistent neuronal hyperexcitability, resulting in amplified network firing frequencies and increased release/accumulation of endogenous glutamate (Choi 1992; Olney 1986). However, our findings from voltage-and current-clamped neurons showed that instead, insulted older neurons were depolarized and quiescent, exhibiting marked reductions in action potential activity and related synchronous compound EPSCs (Figs. 2B and 3B, 5B, inset). Nonetheless, despite the blockade of synchronized compound release, overall network glutamate release may well have been maintained or even increased, because, concomitantly, there was a substantial increase in the frequency of smaller (< 40 pA), TTX-insensitive AMPAR-mediated mini-EPSCs (Figure 5). Interestingly, this conclusion is consistent with studies employing
direct measurement of glutamate, that found greater release in older neurons (Fogal et al, 2005).

However, it should be emphasized that the critical issue is not whether total release is elevated after insult, but whether NMDAR activation is increased. NMDAR-mediated EPSCs are not readily observed in neurons voltage clamped at these hyperpolarized levels (Figure 4), and therefore, the voltage-clamped neuron serves as a monitor for glutamate release but not for NMDAR activation. In the absence of voltage clamp, however, the Mg$^{2+}$ block would be relieved by the extended depolarization in depolarized older neurons following insult (Fig. 2), allowing the glutamate release reflected in the mini-EPSCs to strongly activate NMDARs. Therefore, regardless of whether total release is increased, its impact on NMDAR activation should be substantially greater post-insult, during extended depolarization, than prior to insult, when depolarization occurs primarily during brief 1-3 ms action potentials. Moreover, spontaneous release from numerous depolarized cells in a network presumably activates NMDARs over a larger extent of the dendritic arborization and soma than does the compound release from the ramifications of a single axon.

One caveat to the above interpretation is that NMDARs are modulated by both desensitization and Ca$^{2+}$-dependent inactivation (Erreger and Traynelis, 2005; Krupp et al, 1999; Vicini et al, 1998). These inhibitory processes could reduce NMDAR sensitivity in depolarized neurons. However, the effectiveness seen here of an NMDAR antagonist in reversing post-insult changes in
depolarized neurons (Figs. 1, 2, 3, 5) indicates that NMDARs retain activity under these conditions.

In the absence of action potentials, it seems likely that the high rate of EPSCs in the older neurons resulted from sustained depolarization of the neuronal network and asynchronous glutamate release from presynaptic terminals. As noted, slow shifts in membrane potential, such as the prolonged depolarization seen here, can spread for considerable electrotonic distances and should reach axon terminals from initial sources in dendrites and soma (Johnston and Brown 1983; Alle et al., 2006; Shu et al., 2006).

An alternative explanation for increased EPSCs is that glutamate treatment might have altered dendritic electrotonic structure. If dendritic electrotonic distance were reduced in the older group, small EPSCs might emerge from the noise more often after glutamate insult (Brown and Johnston 1983; Johnston and Brown 1983). In the present studies, an increase in smaller amplitude EPSCs after glutamate was observed. Nevertheless, the effects of glutamate insult did not shift the overall amplitude distribution (Figure 5B), suggesting that altered electrotonic structure was not a major factor in the results.

In addition, a possible alternative to EPSCs as a primary source of depolarizing post-insult glutamate might be reverse glutamate transport from neurons or glia, a mechanism previously implicated in some forms of excitotoxicity (Billups and Atwell, 1996). Reverse transport can occur via several distinct high affinity transporters when extracellular K⁺ levels are exceedingly
high (e.g. after a glutamate insult), resulting in higher levels of extracellular glutamate and greater toxicity (Szatkowski and Attwell, 1994; Billups and Attwell, 1996). In this view, transporters in neurons or glia that take up glutamate during the insult could reverse their glutamate transport during the post-insult period, providing a source of glutamate release for post-insult NMDAR activation. However, it is unlikely that reverse transport can account for the increased EPSC activity, as the size, shape, and duration of mini-EPSCs following glutamate exposure appear to reflect vesicular, or quantal release from axon terminals (or surrounding astrocytes, e.g. Fiacco and McCarthy, 2004) rather than extrusion by a membrane pump. In addition, glutamate release was recently found elevated in older cortical cultures compared to younger cultures, even though a major source of reverse glutamate transport was blocked by DL-threo-β-benzylxoyaspartate (TBOA). The enhanced release was nearly restored to basal levels by application of MK-801 (Fogal et al., 2005). It is also important to note that several post-insult changes, highly similar to those described in the present study, including the PCE (Hartley et al., 1993), extended depolarization (Sombati et al., 1991) and increased inward current (Chen et al., 1997, 1998), can also be induced by application of NMDA, which, unlike glutamate, is non-transportable. Consequently, although reverse transport of glutamate may contribute, it seems likely that the major source of post-insult glutamate driving the NMDAR-dependent changes is persistent release from depolarized presynaptic terminals.

**Ca^{2+} sources of the persistent Ca^{2+} elevation**
As noted, all PCEs may not reflect the same process. The sources of Ca\(^{2+}\) in excitotoxicity apparently vary under different conditions, and have been suggested to include, in addition to NMDARs, release from, or impaired uptake into, the mitochondria (Nicholls and Budd 2000; Vergun et al. 1999), influx through voltage-gated calcium channels (VGCCs) (Sucher et al, 1991; Blalock et al. 1999; Porter et al. 1997) or non-specific cation channels (Chen et al. 1997), group 1 mGluR activation of intracellular Ca\(^{2+}\) release from IP\(_3\)-sensitive stores (Conn and Pin, 1997; Moroni et al., 1998; Attucci et al., 2002) or impaired Ca\(^{2+}\) extrusion (Crespo et al. 1990; Khodorov et al. 1993; Limbrick et al. 2001; Sanchez-Armass and Blaustein 1987). Moreover, increasing evidence indicates that the PCE or excitotoxicity in older neurons can be blocked by inhibiting Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from ryanodine receptors (Clodfelter et al. 2002; Frandson and Schousboe; Lei et. al., 1992), which, together with similar effects of NMDAR and VGCC antagonists, suggests that the PCE seen here may be generated by the interplay of Ca\(^{2+}\) influx through NMDARs/VGCCs and consequent CICR.

**Possible relevance to brain aging and Alzheimer’s Disease (AD)**

There is mounting evidence that Ca\(^{2+}\) dysregulation also plays a role in brain aging and neurodegenerative conditions like Alzheimer’s disease (Disterhoft et al. 2004; LaFerla 2002; Mattson et al. 2000; Thibault et al. 1998; Toescu et al. 2004). Although aging in cell culture models is clearly very different from aging in animal models, some intriguing similarities in Ca\(^{2+}\) regulation have
been found. These include aging-related increases in neuronal L-type VGCC activity (Thibault and Landfield 1996; Porter et al. 1997), alterations in the clearance of Ca\(^{2+}\) (Korkotian and Segal, 1996; Adamec et al. 1998; Cheng et al. 1999; Toescu and Verkhratsky, 2000), changes in CICR (Lei et al, 1992; Clodfelter et al 2002; Gant et al 2006) and increased vulnerability to degeneration (Choi et al. 1987; McDonald et al. 1997; Attucci et al. 2002). Given these parallels, cells aging in mixed neuronal cultures may be a useful tool for studying alterations in Ca\(^{2+}\) homeostasis relevant to aging and AD.

However, one major difference in Ca\(^{2+}\) regulation between aging-in-culture and aging in animals is that NMDAR activity appears to decrease with aging (Barnes et al. 1997; Clayton et al. 2002; Magnusson 1998). Nevertheless, increased Ca\(^{2+}\) levels arising from other sources that do change with aging (e.g., L-type Ca\(^{2+}\) channels and ryanodine sensitive Ca\(^{2+}\) stores; Gant et al. 2006; Thibault and Landfield 1996; Thibault et al. 2001) could amplify the impact of episodes of excessive NMDAR activity, providing a mechanism for excitotoxic contributions to age-related neurodegenerative conditions and for positive results with the low-affinity, non-competitive NMDAR antagonist, memantine, in treating Alzheimer’s disease (Ferris 2003; Le and Lipton 2001).

**Positive feedback model of NMDAR current maintenance**

The well-established dependence of NMDAR current on both depolarization and the presence of glutamate (Mayer and Westbrook 1987) indicates that post-insult NMDAR current (Figure 3) likely is maintained by the
combination of prolonged depolarization (Figure 2) and persisting glutamate release (asynchronous mini-EPSC activity) (Figs. 4, 5). Conversely, the prolonged depolarization and asynchronous presynaptic release appear to depend on NMDAR activity (Figure 2C). The NMDAR current may modulate the membrane potential directly, or indirectly, via activation of a secondary conductance (e.g., Chen et al., 1997, 1998). Furthermore, the PCE depends both on NMDAR activity (Figure 1) and on membrane depolarization (Figure 7). Taken together, these data are consistent with a model of delayed excitotoxicity in which large NMDAR current (and depolarization) is initially triggered by excessive glutamate release, as might occur in a traumatic head injury or ischemic incident. Upon clearance (or washout) of excessive glutamate, outward currents are not large enough to repolarize the membrane against the persisting inward currents that are sustained by glutamate release from depolarized terminals. NMDAR current is likely a major component of the inward current, particularly given the slow kinetics and long decay constants of NMDARs (Clements et al. 1992). These reciprocal interactions lead to establishment of a positive feedback loop between the depolarizing NMDAR current on one hand, and the enabling depolarization and resulting glutamate release on the other. In turn, the sustained NMDAR activity appears to generate persistent \([Ca^{2+}]_i\) elevation through a combination of direct \(Ca^{2+}\) influx via NMDARs (and possibly, VGCCs) and CICR, resulting in excitotoxic neuronal death.

Interruption of this putative loop requires activation of outward currents sufficient to repolarize the membrane upon termination of excessive glutamate
exposure. The high density of K⁺ channels throughout the soma and, particularly the dendrites of pyramidal and other neurons (Johnston et al. 2000) apparently can repolarize the membrane in younger, but not older neurons, perhaps because of age-dependent increases in NMDAR current (Brewer et al. 2001; Cheng et al. 1999; Priestley et al. 1996; Xia et al. 1995). However, other mechanisms also might account for the inability of older neurons to repolarize, including changes in the relative number of inhibitory synapses, neuronal or glial energy metabolism, or levels of reactive oxygen species, and will require further investigation.

Summary and Conclusions

The present work provides the first comprehensive electrophysiological and Ca²⁺ imaging overview of the critical post-insult phase of excitotoxicity, and suggests a model in which positive feedback interactions between NMDAR activation on one hand and membrane depolarization/glutamate release on the other, maintain NMDAR current after the initial high glutamate exposure is terminated. The sustained NMDAR current, in turn, drives [Ca²⁺]i elevation, resulting in toxicity. This positive feedback model of excitotoxicity, if supported in future studies, might well have important therapeutic implications. A positive feedback loop can be disrupted by damping any of its elements and, therefore, elucidating reciprocal feedback mechanisms in delayed excitotoxicity may reveal new targets for intervention in a wide range of neuropathologies.
GRANTS

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FIGURE LEGENDS

Figure 1. Inhibition of NMDARs in the post-insult period reduced the Prolonged Ca\(^{2+}\) Elevation (PCE). A and B: Mean ± SEM [Ca\(^{2+}\)] \(_i\) levels obtained using Indo-1 confocal laser scanning microscopy are plotted before, during, and after a 5 min glutamate insult (100 \(\mu\)M) in 7-14 DIV (A) and (10 \(\mu\)M) in 28-35 DIV (B) neurons. Immediately after glutamate exposure, medium containing vehicle (open circles), 10 \(\mu\)M CNQX (filled triangles), or 100 \(\mu\)M D-APV (filled circles) was perfused through the culture dish. C and D: Similar experiment with a non-competitive NMDAR antagonist showing [Ca\(^{2+}\)] \(_i\) before, during, and after a 5 min glutamate insult in younger (C) and older (D) neurons. Immediately after glutamate exposure, medium containing vehicle (open circles) or 10 \(\mu\)M MK-801 (filled circles) was perfused through the culture dish. For A-D, Bars indicate the periods in which glutamate and glutamate receptor antagonists were applied. \(n = 5 – 10\) cells per group [Two-way ANOVA on repeated measures; post hoc Tukey's pairwise comparisons; B- Veh and CNQX both significantly different from D-APV at indicated time points (p < 0.05, *); D- Veh significantly different from MK-801 at indicated time points (p < 0.05, *)].

Figure 2. Glutamate exposure induces an age-dependent depolarization that requires post-insult NMDAR activity. A-C: Representative experiments illustrating the effects of a five minute glutamate exposure (Glu, 20 \(\mu\)M) on action potentials and \(V_m\) in Younger cultures (\(n = 17\)) (A), Older cultures (\(n = 8\)) (B), and Older cultures perfused post-glutamate with 10 \(\mu\)M MK-801 (\(n = 8\)) (C). D, Extent of depolarization (mean ± SEM) measured in Younger (hatched columns), Older
(filled columns), and Older MK-801-treated neurons (gray columns) during (Glu) and at 5 and 15 minutes post-glutamate exposure. * p < 0.05. Note that the Younger neurons and Older MK-801-treated neurons were generally able to repolarize and resume action potential activity.

Figure 3. Glutamate exposure induces persistent inward current that is age and NMDA receptor-dependent. A-C: Representative examples of spontaneous EPSC activity and inward current in neurons voltage-clamped at −80mV, recorded before, during, and after a five minute application of 20 µM glutamate (Glu). The dashed line shows baseline holding current (Ih). In Younger neurons (n = 25) (A), glutamate application resulted in a large increase in inward current followed by rapid restoration of baseline Ih following glutamate termination. B Older neurons (n = 14) exhibited a somewhat larger (but variable and nonsignificant) increase in inward current during glutamate exposure. After glutamate exposure, holding current remained elevated above pre-insult levels for more than five minutes before eventually recovering. The post insult period in Older neurons was also characterized by an increase in the appearance of smaller EPSCs and an almost complete loss of larger (>300 pA) EPSCs. C: The post-insult increase in inward holding current and changes in EPSC activity were completely reversed in older neurons by applying MK-801 (10 µM) immediately after the glutamate insult (n = 9). D: Mean ± SEM change in holding current (ΔI) during glutamate exposure and at 5 and 15 minutes after glutamate application
(relative to pre-insult levels) in Younger (white columns), Older (filled columns), and Older MK-801-treated (gray columns) neurons. * $p < 0.05$.

**Figure 4.** Pharmacological characterization of spontaneous EPSCs in hippocampal neuronal cultures. **A:** Typical record of spontaneous, ongoing synaptic activity (EPSCs) in a voltage-clamped cultured hippocampal neuron (*left panel*). Activity recorded at time points 1 and 2 are magnified in the *right panel* to illustrate small (A1) and compound (A2) EPSCs. **B:** Nearly all synaptic activity was abolished by the AMPA receptor antagonist CNQX (10 µM, $n = 12$). **C:** The NMDAR antagonist MK-801 (10 µM, $n = 10$) had little effect on EPSC frequency. **D:** The voltage-sensitive Na$^+$ channel blocker TTX (1 µM, $n = 5$) did not affect spontaneous small EPSCs, but eliminated compound EPSCs, suggesting that these large events were the result of presynaptic action potentials.

**Figure 5.** Glutamate exposure alters the distribution of EPSC amplitudes in an age- and NMDAR-dependent manner. EPSC activity exhibited a bimodal distribution. **A-C,** The first mode consisted of TTX-insensitive, small EPSCs (<100 pA). The number of small EPSCs observed during two 2 minute windows, one before and one 15 min after, glutamate perfusion were binned as a function of amplitude (in pA) and averaged across cells. Changes in distribution were assessed by exponential regression (*bold lines*). **Insets,** The second mode consisted of TTX-sensitive (see Figure 4) compound EPSCs (>300 pA). Mean number of compound EPSCs before (*hatched columns*) and after (*gray columns*)
glutamate exposure are illustrated in the *inset* of each panel. A: In younger neurons ($n = 25$), the number and amplitude of small EPSCs increased after glutamate application, and there was a non-significant trend for compound EPSCs to increase (*inset*). B: In older neurons ($n = 14$), the number of events in the lowest amplitude bins ($<40$ pA) increased substantially at 15 min after glutamate exposure, whereas compound EPSCs were nearly eliminated (*inset*). C: Post-insult application of MK-801 to older neurons ($n = 9$), produced a remarkable stabilizing effect. Both the amplitude/frequency histogram and the number of compound EPSCs (*inset*) were unchanged after glutamate exposure (see also Figure 2C and 3C). *Difference from pre-glutamate baseline, $p < 0.05$. See text for analyses of # events.

*Figure 6.* The PCE and extended neuronal depolarization occur within the same neurons and exhibit strong quantitative and temporal relationships. Simultaneous $[\text{Ca}^{2+}]_i$ imaging (with Fura-2) and electrophysiological recording in an individual younger ($n = 5$, A and C) and an older ($n = 7$, B and D) neuron before, during, and after 5-min exposure to 20 µM glutamate (Glu). Note that, for illustration purposes, recording electrodes have been subtracted from images. A and B: Representative pseudocolor Fura-2 ratiometric images taken immediately before (Pre-Glu), during the first minute of glutamate exposure (1 min Glu), and 15 min after glutamate application (15 min Post Glu). C and D: Time course plots of $[\text{Ca}^{2+}]_i$ measurements (*Top*) and continuous $V_m$ measurements in current-clamp (*bottom*) within the same neurons. Similar to the results in Figs 1 and 2, the
younger neuron was able to recover its $[Ca^{2+}]_i$ and $V_m$ to near basal levels, whereas the older neuron was not. $E$: Correlation analysis across all younger and older cells between the post-insult change in $[Ca^{2+}]_i$ ($\Delta Ca^{2+}_i$) and the simultaneously-recorded post-insult depolarization, indicated a close quantitative relationship between $V_m$ and $[Ca^{2+}]_i$ in the post-insult period (measured at 15 min post Glu). $F$, Recovery kinetics for $[Ca^{2+}]_i$ and $V_m$ immediately after washout of glutamate. Only data from cells in which either the $V_m$ or the $[Ca^{2+}]_i$ returned to near pre-glutamate levels were included (all younger neurons and one older neuron). In all cases, $Ca^{2+}$ recovery followed the repolarization of the $V_m$.

*Figure 7.* Active repolarization of the membrane immediately after glutamate exposure restores $[Ca^{2+}]_i$ to pre-insult levels. $A$ and $B$: $[Ca^{2+}]_i$ imaging (with Fura-2) and electrophysiological recording in an individual older neuron studied by hybrid current-clamp/voltage-clamp. Note that, for illustration purposes, the recording electrode has been subtracted from images. The $V_m$ was recorded in current-clamp mode before and during glutamate application (20 $\mu$M, 5 min), then voltage-clamped to $-80$ mV immediately after glutamate application. $A$: Representative pseudocolor Fura-2 ratiometric images taken immediately before (Pre-Glu), during the first minute of glutamate exposure (1 min Glu), and 15 min after glutamate application (15 min Post Glu). $B$: Time course plots of $[Ca^{2+}]_i$ measurements. (*top*) and continuous $V_m$ and current measurements (*bottom*) in the neuron illustrated in $A$. Plot symbols and traces in $B$ that correspond to current clamp conditions are in red, whereas those following the switch to voltage
clamp are shown in green. The point at the end of the 5-min glutamate exposure (Glu) at which the \( V_m \) was ramped to –80 mV is shown by the arrow. **D**, Mean ± SEM change in \([Ca^{2+}]_i\) (\(\Delta[Ca^{2+}]_i\)) at 5 and 15 min post-glutamate application in younger \((n = 5, \text{ hatched columns})\) and older \((n = 7, \text{ filled columns})\) neurons held under current clamp only, and older neurons switched from current clamp (CC) to voltage clamp (VC) in the post-glutamate period \((n = 7, \text{ gray columns})\). Similarly to younger neurons, older neurons hyperpolarized in the post-insult period were able to restore \([Ca^{2+}]_i\) to near basal levels. Older neurons held under current clamp throughout the experiment were not. * \(p \leq 0.05\). ** \(p \leq 0.01\).
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Figure 7. Active repolarization of the membrane immediately after glutamate exposure restores [Ca^{2+}] to pre-insult levels. A and B: [Ca^{2+}] imaging (with Fura-2) and electrophysiological recording in an individual older neuron studied by hybrid current-clamp/voltage-clamp. Note that, for illustration purposes, the recording electrode has been subtracted from images. The V_m was recorded in current-clamp mode before and during glutamate application (20 μM, 5 min), then voltage-clamped to -80 mV immediately after glutamate application. A: Representative pseudocolor Fura-2 ratiometric images taken immediately before (Pre-Glu), during the first minute of glutamate exposure (1 min Glu), and 15 min after glutamate application (15 min Post Glu). B: Time course plots of [Ca^{2+}] measurements. (top) and continuous V_m and current measurements (bottom) in the neuron illustrated in A. Plot symbols and traces in B that correspond to current clamp conditions are in red, whereas those following the switch to...
voltage clamp are shown in green. The point at the end of the 5-min glutamate exposure (Glu) at which the \( V_m \) was ramped to -80 mV is shown by the arrow. D, Mean ± SEM change in \([\text{Ca}^{2+}]_i\) (\( \Delta [\text{Ca}^{2+}]_i \)) at 5 and 15 min post-glutamate application in younger (n = 5, hatched columns) and older (n = 7, filled columns) neurons held under current clamp only, and older neurons switched from current clamp (CC) to voltage clamp (VC) in the post-glutamate period (n = 7, gray columns). Similarly to younger neurons, older neurons hyperpolarized in the post-insult period were able to restore \([\text{Ca}^{2+}]_i\) to near basal levels. Older neurons held under current clamp throughout the experiment were not. * \( p \leq 0.05 \). ** \( p \leq 0.01 \).
Figure 1. Inhibition of NMDARs in the post-insult period reduced the Prolonged Ca$^{2+}$ Elevation (PCE). A and B: Mean ± SEM [Ca$^{2+}$]$_i$ levels obtained using Indo-1 confocal laser scanning microscopy are plotted before, during, and after a 5 min glutamate insult (100 µM) in 7-14 DIV (A) and 10 (100 µM) in 28-35 DIV (B) neurons. Immediately after glutamate exposure, medium containing vehicle (open circles), 10 µM CNQX (filled triangles), or 100 µM D-APV (filled circles) was perfused through the culture dish. C and D: Similar experiment with a non-competitive NMDAR antagonist showing [Ca$^{2+}$]$_i$ before, during, and after a 5 min glutamate insult in younger (C) and older (D) neurons. Immediately after glutamate exposure, medium containing vehicle (open circles) or 10 µM MK-801 (filled circles) was perfused through the culture dish. For A-D, Bars indicate the periods in which glutamate and glutamate receptor antagonists were applied. n = 5-10 cells per group [Two-way ANOVA on repeated measures; post hoc Tukey's pairwise comparisons; B- Veh and CNQX both significantly different from D-APV at indicated time points (p < 0.05, *); D- Veh significantly different from MK-801 at indicated time points (p < 0.05, *)].
Figure 2. Glutamate exposure induces an age-dependent depolarization that requires post-insult NMDAR activity. A–C: Representative experiments illustrating the effects of a five minute glutamate exposure (Glu, $20 \mu$M) on action potentials and $V_m$ in Younger cultures ($n = 17$) (A), Older cultures ($n = 8$) (B), and Older cultures perfused post-glutamate with $10 \mu$M MK-801 ($n = 8$) (C). D, Extent of depolarization (mean ± SEM) measured in Younger (hatched columns), Older (filled columns), and Older MK-801-treated neurons (gray columns) during (Glu) and at 5 and 15 minutes post-glutamate exposure. * $p < 0.05$. Note that the Younger neurons and Older MK-801-treated neurons were generally able to repolarize and resume action potential activity.
Figure 3. Glutamate exposure induces persistent inward current that is age and NMDA receptor-dependent. A-C: Representative examples of spontaneous EPSC activity and inward current in neurons voltage-clamped at -80mV, recorded before, during, and after a five minute application of 20 μM glutamate (Glu). The dashed line shows baseline holding current (I_h). In Younger neurons (n = 25) (A), glutamate application resulted in a large increase in inward current followed by rapid restoration of baseline I_h following glutamate termination. B Older neurons (n = 14) exhibited a somewhat larger, but variable (and nonsignificant compared to young) increase in inward current during glutamate exposure. After glutamate exposure, holding current remained elevated above pre-insult levels for more than five minutes before eventually recovering. The post insult period in Older neurons was also characterized by an increase in the appearance of smaller EPSCs and an almost complete loss of larger (>300 pA) EPSCs. C: The post-insult
increase in inward holding current and changes in EPSC activity were completely reversed in older neurons by applying MK-801 (10 μM) immediately after the glutamate insult (n = 9). D: Mean ± SEM change in holding current (ΔI) during glutamate exposure and at 5 and 15 minutes after glutamate application (relative to pre-insult levels) in Younger (white columns), Older (filled columns), and Older MK-801-treated (gray columns) neurons. * p < 0.05.
Figure 4. Pharmacological characterization of spontaneous EPSCs in hippocampal neuronal cultures. A: Typical record of spontaneous, ongoing synaptic activity (EPSCs) in a voltage-clamped cultured hippocampal neuron (left panel). Activity recorded at time points 1 and 2 are magnified in the right panel to illustrate small (A1) and compound (A2) EPSCs. B: Nearly all synaptic activity was abolished by the AMPA receptor antagonist CNQX (10 μM, n = 12). C: The NMDAR antagonist MK-801 (10 μM, n = 10) had little effect on EPSC frequency. D: The voltage-sensitive Na⁺ channel blocker TTX (1 μM, n = 5) did not affect spontaneous small EPSCs, but eliminated compound EPSCs, suggesting that these large events were the result of presynaptic action potentials.
Figure 5. Glutamate exposure alters the distribution of EPSC amplitudes in an age- and NMDAR-dependent manner. EPSC activity exhibited a bimodal distribution. A-C, The first mode consisted of TTX-insensitive, small EPSCs (< 100 pA). The number of small EPSCs observed during two 2 minute windows, one before and one 15 min after glutamate perfusion, were binned as a function of amplitude (in pA) and averaged across cells. Changes in distribution were assessed by exponential regression (bold lines). Insets, The second mode consisted of TTX-sensitive (see Fig. 4) compound EPSCs (>300 pA). Mean number of compound EPSCs before (hatched columns) and after (gray columns) glutamate exposure are illustrated in the inset of each panel. A: In younger neurons (n = 25), the number and amplitude of small EPSCs increased after glutamate application, and there was a non-significant trend for compound EPSCs to increase (inset). B: In older neurons (n = 14), the number of events in the lowest amplitude bins (<40 pA) increased...
substantially at 15 min after glutamate exposure, whereas compound EPSCs were nearly
eliminated (inset). C: Post-insult application of MK-801 to older neurons (n = 9),
produced a remarkable stabilizing effect. Both the amplitude/frequency histogram and
the number of compound EPSCs (inset) were unchanged after glutamate exposure (see
also Fig. 2C and 3C). *Difference from pre-glutamate baseline, p < 0.05. See text for
analyses of # events.
Figure 6. The PCE and extended neuronal depolarization occur within the same neurons and exhibit strong quantitative and temporal relationships. Simultaneous $[Ca^{2+}]_i$ imaging (with Fura-2) and electrophysiological recording in individual younger ($n = 5$, A and C) and older ($n = 7$, B and D) neurons before, during, and after 5-min exposure to 20 μM glutamate (Glu). Note that, for illustration purposes, recording electrodes have been subtracted from images. A and B: Representative pseudocolor Fura-2 ratiometric images taken immediately before (Pre-Glu), during the first minute of glutamate exposure (1 min Glu), and 15 min after glutamate application (15 min Post Glu). C and D: Time course plots of $[Ca^{2+}]_i$ measurements (Top) and continuous $V_m$ measurements in current-clamp (bottom) within the same neurons. Similar to the results in Figs 1 and 2, the younger neuron was able to recover its $[Ca^{2+}]_i$ and $V_m$ to near basal levels, whereas the older neuron was not. E: Correlation analysis across all younger and older cells between the
post-insult change in $[\text{Ca}^{2+}]_i$ ($\Delta [\text{Ca}^{2+}]_i$) and the simultaneously-recorded post-insult depolarization, indicated a close quantitative relationship between $V_m$ and $[\text{Ca}^{2+}]_i$ in the post-insult period (measured at 15 min post Glu). F, Recovery kinetics for $[\text{Ca}^{2+}]_i$ and $V_m$ immediately after washout of glutamate. Only data from cells in which either the $V_m$ or the $[\text{Ca}^{2+}]_i$ returned to near pre-glutamate levels were included (all younger neurons and one older neuron). In all cases, $\text{Ca}^{2+}$ recovery followed the repolarization of the $V_m$. 
Figure 7. Active repolarization of the membrane immediately after glutamate exposure restores [Ca\textsuperscript{2+}] to pre-insult levels. A and B: [Ca\textsuperscript{2+}] imaging (with Fura-2) and electrophysiological recording in an individual older neuron studied by hybrid current-clamp/voltage-clamp. Note that, for illustration purposes, the recording electrode has been subtracted from images. The V_m was recorded in current-clamp mode before and during glutamate application (20 \textmu M, 5 min), then voltage-clamped to -80 mV immediately after glutamate application. A: Representative pseudocolor Fura-2 ratiometric images taken immediately before (Pre-Glu), during the first minute of glutamate exposure (1 min Glu), and 15 min after glutamate application (15 min Post Glu). B: Time course plots of [Ca\textsuperscript{2+}] measurements. (top) and continuous V_m and current measurements (bottom) in the neuron illustrated in A. Plot symbols and traces in B that correspond to current clamp conditions are in red, whereas those following the switch to...
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