Coupling diverse routes of calcium entry to mitochondrial dysfunction and glutamate excitotoxicity

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Overactivation of NMDA receptors (NMDARs) is a critical early step in glutamate-evoked excitotoxic injury of CNS neurons. Distinct NMDAR-coupled pathways specified by, for example, receptor location or subunit composition seem to govern glutamate-induced excitotoxic death, but there is much uncertainty concerning the underlying mechanisms of pathway selection. Here we ask whether, and if so how, route-specific vulnerability is coupled to Ca^{2+} overload and mitochondrial dysfunction, which is also a known, central component of exitotoxic injury. In cultured hippocampal neurons, overactivation of only extrasynaptic NMDARs resulted in Ca^{2+} entry strong enough to promote Ca^{2+} overload, which subsequently leads to mitochondrial dysfunction and cell death. Receptor composition per se appears not to be a primary factor for specifying signal coupling, as NR2B inhibition abolished Ca^{2+} loading and was protective only in predominantly NR2B-expressing young neurons. In older neurons expressing comparable levels of NR2A- and NR2B-containing NMDARs, amelioration of Ca^{2+} overload required the inhibition of extrasynaptic receptors containing both NR2 subunits. Prosurvival synaptic stimuli also evoked Ca^{2+} entry through both N2A- and NR2B-containing NMDARs, but, in contrast to excitotoxic activation of extrasynaptic NMDARs, produced only low-amplitude cytoplasmic Ca^{2+} spikes and modest, nondamaging mitochondrial Ca^{2+} accumulation. The results—showing that the various routes of excitotoxic Ca^{2+} entry converge on a common pathway involving Ca^{2+} overload-induced mitochondrial dysfunction—reconcile and unify many aspects of the ''route-specific'' and ''calcium load-dependent'' views of exitotoxic injury.

extrasynaptic receptors | hippocampal neurons | mitochondria | NMDA receptor | synaptic activation

Overactivation of NMDA receptors (NMDARs) plays a key role in glutamate-dependent excitotoxic injury of CNS neurons (1). Several lines of evidence indicate that toxicity depends on the activation of specific NMDA-dependent sources of Ca^{2+} entry (reviewed in refs. 2 and 3). For example, intense synaptic stimulation has been shown to promote survival in hippocampal neurons, whereas overactivation of extrasynaptic NMDARs was linked to excitotoxic death (4), implying that NMDAR location plays an important role in specifying survival vs. death pathways. Beyond location, the subunit composition of NMDARs might be important. Thus, it has been suggested that excitotoxicity is triggered by the selective activation of NMDARs containing the NR2B subunit (5, 6).

An alternative hypothesis considers that toxicity depends on the magnitude of the Ca^{2+} load imposed on the cell by Ca^{2+} entry (7–9). In this view, excessive Ca^{2+} entry is responsible for triggering the well-established pathway linking Ca^{2+} overload-dependent mitochondrial dysfunction to exitotoxic injury (10–12). There is presently little evidence to relate excitotoxicity mechanisms depending on generalized Ca^{2+} overload to source-specific mechanisms mentioned above. Cheng et al. (8) showed a correlation between NR2B expression, cytosolic Ca^{2+} elevations, and susceptibility to excitotoxicity in cortical neurons.

Concerning location-specific signaling, Hardingham and colleagues (4) showed that toxic activation of extrasynaptic NMDARs also depends on mitochondrial dysfunction, which implies a role for Ca^{2+} overload but, on the negative side, both toxic and prosurvival stimuli were reported to evoke similar cytosolic Ca^{2+} elevations, arguing against any necessary dependence on Ca^{2+} loading (4). Given the limited and conflicting information available, the relationship, if any, between route specificity and Ca^{2+} overload remains open to question.

In this study, we find that in cultured hippocampal neurons at different developmental stages there are multiple pathways for glutamate-stimulated Ca^{2+} entry, but toxic stimuli are uniformly characterized by an obligatory link to massive mitochondrial Ca^{2+} loading through NMDARs that are primarily extrasynaptic. The findings support the unifying hypothesis that the effects of factors such as NMDAR location or subunit composition on death signaling can be accounted for by a common, convergent impact on intracellular Ca^{2+} levels and mitochondrial injury.

Results

Mechanism of Excitotoxic Injury in Young Hippocampal Neurons: Ca^{2+} Overload via One Dominant Route. In relatively young CNS pyramidal neurons, excitotoxic injury is mainly mediated by NR2B-containing NMDARs (6, 13, 14). NR2B is the dominant NR2 subunit early in development (5, 13, 15, 16). In our hippocampal cultures at 15 days in vitro, where the NR2B/NR2A subunit expression ratio is ≈1.6 (Fig. 1*A*), the ifenprodil-like noncompetitive NR2B-selective antagonists Co 101244 and Ro 25–6981 were strongly neuroprotective. Though either glutamate or NMDA killed 40%–60% of cells in control cultures, both antagonists improved the survival rate by >80% (Fig. 1*B*). In contrast, the NR2A-selective blocker Zn^{2+} (500 nM) (15, 17) was only weakly effective (Fig. 1*B*) and nontoxic (data not shown), whereas CNQX and nimodipine, blockers of AMPA receptors (AMPARs) and L-type voltage-gated Ca^{2+} channels (VGCCs), respectively, were ineffective (data not shown).

NR2B-Containing NMDARs Mediate Excitotoxic Cytosolic Ca^{2+} Elevations. Because elevated intracellular Ca^{2+} plays a critical role in excitotoxicity (18), we first asked whether blockade of NR2B-containing NMDARs is protective because these receptors are the dominant route of Ca^{2+} entry in younger neurons. Indeed, Co 101244 reduced glutamate- or NMDA-induced cytosolic Ca^{2+}

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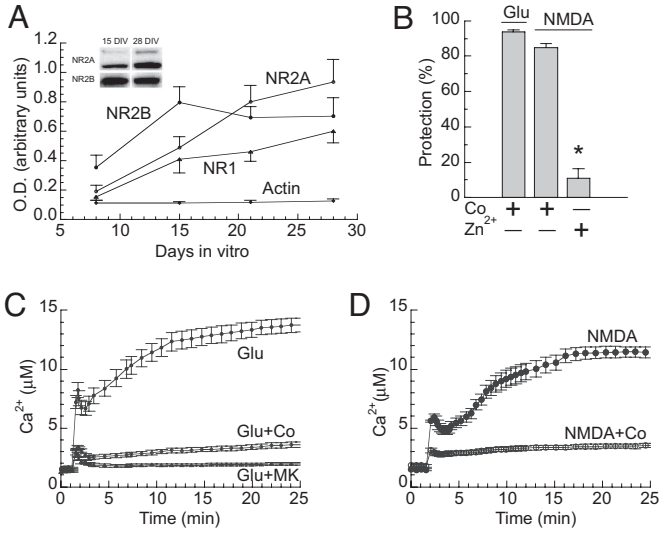

Fig. 1. Excitotoxic Ca^{2+} elevations and cell death in young hippocampal neurons are mediated by NR2B-containing NMDARs. (*A*) Relative immunoreactivity on Western blots of NR1, NR2A, and NR2B NMDAR subunits, plus actin loading control, in hippocampal neurons as a function of age in culture. Points are average of 7 experiments ± SEM. NR1 expression increases monotonically throughout this developmental period, whereas NR2B expression is maximal by ≈15 days in vitro DIV. NR2A expression increases continuously after ≈14 (DIV). The NR2B/NR2A ratio is ≈1.6 at 15 DIV and ≈0.7 at 28 DIV. (*Inset*) Representative bands at 15 and 28 days. (*B*) Protection afforded by the NR2B antagonist Co 101244 (5 μM) or the NR2A blocker Zn^{2+} (500 nM) in young neurons (14–17 DIV) was assayed 24 h after exposure to glutamate (500 μM, 30 min) or NMDA (100 μM, 30 min). Zn^{2+} is much less protective than Co 101244 (*$P < 0.01$). (*C*) Time courses of glutamate-induced free Ca^{2+} elevations (fura-2FF, mean ± SEM) without ($n = 35$) or with Co 101244 ($n = 31$) or MK-801 ($n = 24$); cells from 3 independent platings. Co 101244 reduced Ca^{2+} elevations almost as effectively as MK-801 (10 μM) in 84% of cells. (*D*) NR2B inhibition ($n = 32$) is comparably effective against NMDA exposure ($n = 27$) in 72% of cells.

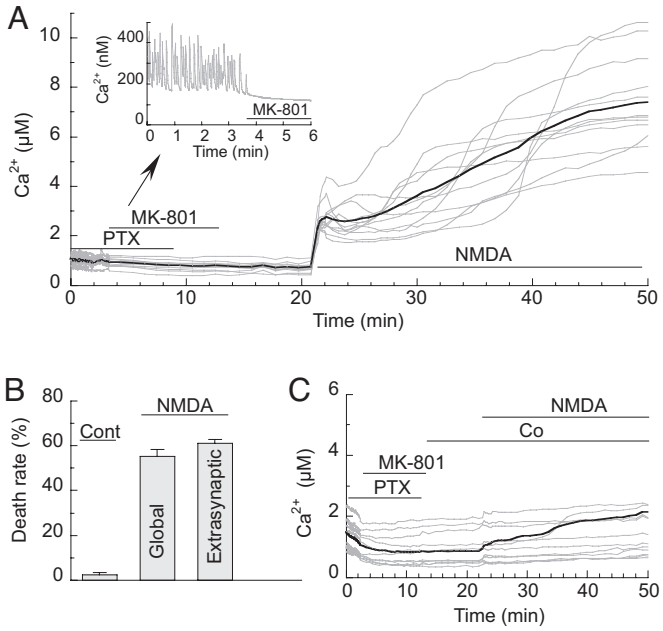

Fig. 2. Activation of extrasynaptic NMDARs induces large amplitude Ca^{2+} elevations. (*A*) Single-cell Ca^{2+} transients (fura-2FF) in young neurons triggered by PTX (100 μM) activation of synaptic NMDARs, followed by MK-801 (10 μM) to block open (i.e., synaptic) NMDARs. With this low-affinity Ca^{2+} probe, submicromolar synaptically induced somatic transients are barely visible in the 0- to 3-min portion of the trace. Application of NMDA after washout activates only previously unactivated extrasynaptic NMDARs, leading to large Ca^{2+} elevations. (*Inset*) Experiment parallel to the first 7 min of the main trace but using high-affinity fura-2 shows that low-amplitude synaptically induced Ca^{2+} elevations (average of 9 cells) are blocked by MK-801; they are also abolished by TTX pretreatment (Fig. S3*B*). Synaptic Ca^{2+} spikes evoked by bicuculline are comparable to those for PTX (Fig. S3*A*). (*B*) Death rate after bath NMDA exposure (100 μM, 30 min; global) was not different from that evoked by activation of only extrasynaptic NMDARs (extrasynaptic). (*C*) Extrasynaptic Ca^{2+} elevations are mainly eliminated in the presence of Co 101244. Average traces are shown in bold.

elevations >80% (Fig. 1 *C* and *D* and Fig. S1 *A* and *B*), which is comparable to that achieved by the NMDAR antagonist MK-801 (Fig. 1*C*). Two other NR2B-selective inhibitors, ifenprodil and Ro 25–6981, also strongly reduced peak Ca^{2+} levels (Fig. S1 *C* and *D*). Inhibiting other routes of Ca^{2+} entry (e.g., NR2A-containing NMDARs, AMPARs, or VGCCs) had no significant effect on glutamate-induced Ca^{2+} elevations (Fig. S1*E*). These findings suggest that in younger neurons the excessive Ca^{2+} entry and cytoplasmic Ca^{2+} elevations that precede excitotoxic cell death are predominantly mediated by NR2B-containing NMDARs.

Extrasynaptic NMDARs Mediate Excitotoxic Ca^{2+} Loading. There is evidence that only extrasynaptic NMDARs are linked to mitochondrial dysfunction (3), but it remains unclear how differently disposed NMDARs might be differently coupled to survival or death pathways. To ask whether the degree of Ca^{2+} loading is a major factor, we quantified Ca^{2+} elevations specifically mediated by extrasynaptic NMDARs, taking advantage of a protocol that selectively activates only these receptors (4, 6). Synaptic NMDARs were first activated by applying the $GABA_A$ antagonists bicuculline or picrotoxin (PTX) to enhance spontaneous synaptic activity, then blocked by the open channel blocker MK-801 (Fig. 2*A* *Inset*). Following washout, bath application of NMDA activates the remaining, extrasynaptic NMDARs. Selective activation of extrasynaptic NMDARs resulted in a death rate similar to global activation (Fig. 2*B*) and induced Ca^{2+} elevations of similar amplitude (compare Figs. 1*D* and 2*A*). Co 101244 abolished these large NMDA-induced Ca^{2+} elevations (Fig. 2*C*). These findings suggest that in young cells, extrasynaptic NR2B-containing NMDARs represent a selective route to excitotoxic injury, which is mediated by cytosolic Ca^{2+} overload.

Inhibition of Extrasynaptic NMDARs Reduces Excitotoxic Mitochondrial Ca^{2+} Accumulation, Damage, and Dysfunction. To evaluate the impact of NMDA-evoked, NR2B-mediated Ca^{2+} loading on mitochondrial function we used electron probe x-ray microanalysis (EPMA) to measure stimulus-induced increases in total intracellular Ca concentrations. NMDAR overstimulation led to large increases in the concentration of total cytoplasmic Ca, paralleled by the accumulation of large amounts of Ca in individual mitochondria (Fig. 3*A* and Table S1). Consistent with the inhibition of free Ca^{2+} elevations (Fig. 1 *C* and *D*), Co 101244 or Ro 25–6981 dramatically reduced cytoplasmic total Ca loading and abolished mitochondrial Ca accumulation (Fig. 3*A* and Table S1).

Excitotoxic injury is preceded by fast depolarization of the mitochondrial membrane potential (MMP), which may be followed by a partial recovery (9, 19). To estimate MMP depolarization we used the fluorescent probe tetramethylrhodamine methyl ester (TMRM), following published protocols that allow one to take account of the effects of plasma membrane (PM) depolarization (20). NMDA exposure resulted in a rapid loss of TMRM fluorescence (Fig. 3*C* and Fig. S2*A*); some combination of PM plus MMP depolarization underlies this loss of fluorescence. Contributions from PM depolarization can be independently estimated using changes in fluorescence from the PM-sensitive probe PMPI as input to the computational model of Nicholls (20) (Fig. S2*C*), which here reveals strong (≈60 mV) PM depolarization and a ≈30 mV loss of

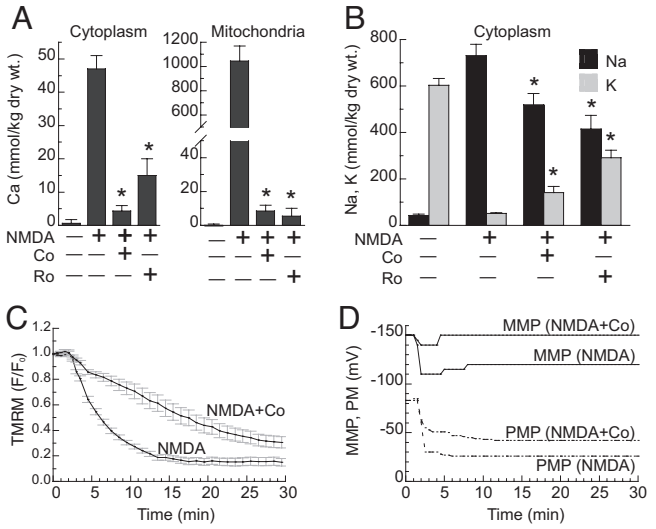

Fig. 3. NR2B-containing NMDARs mediate mitochondrial Ca^{2+} overload and dysfunction in young hippocampal neurons. (*A*) Concentrations of total Ca in cytoplasm and mitochondria of neurons immediately after exposure to NMDA (100 μM, 30 min) in the presence or absence of Co 101244 (5 μM) or Ro 25–6981 (0.5 μM). Both antagonists strongly reduce NMDA-induced Ca accumulation in both compartments. (*B*) Cytoplasmic concentrations of Na and K under the same conditions. Both inhibitors only slightly affected NMDA-induced changes in Na and K. Data are mean ± SEM, as measured by EPMA; asterisks indicate significantly different ($P < 0.01$) from NMDA alone. (*C*) Averaged TMRM fluorescence traces ± SEM from individual cells from 3 independent platings in the presence of NMDA with ($n = 20$) or without Co 101244 ($n = 26$). NR2B inhibition slightly reduced NMDA-induced loss of TMRM fluorescence. (*D*) Deconstruction of the TMRM fluorescence into MMP (solid lines) and plasma membrane potential (PMP, dashed lines) components reveals that NR2B blockade essentially prevents MMP depolarization, while reducing PM depolarization by ≈40%.

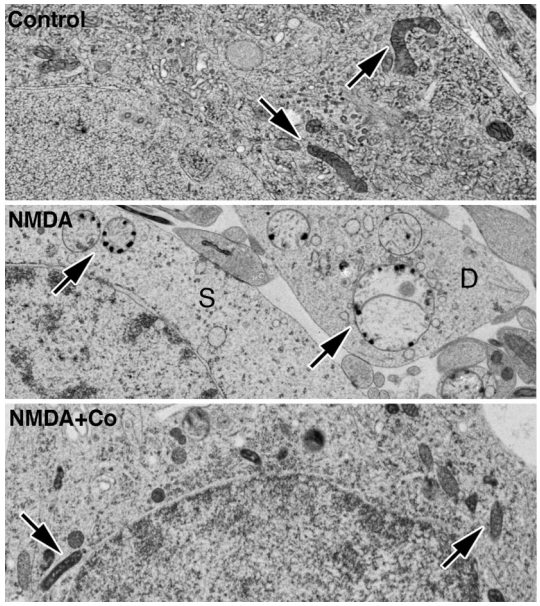

Fig. 4. NR2B inhibition prevents NMDA-induced mitochondrial structural changes in young neurons. Electron micrographs of high-pressure frozen, freeze-substituted preparations of representative hippocampal neurons under control conditions, immediately after exposure to NMDA (100 μM, 30 min) and after NMDA exposure in the presence of Co 101244 (5 μM). Mitochondria (arrows) in somata and dendrites (*Middle*, S and D, respectively) of NMDA-treated neurons, but not glia, contain many swollen mitochondria with multiple electron-dense, Ca-rich inclusions that are diagnostic for strong Ca accumulation. NR2B blockade completely prevented NMDA-induced mitochondrial swelling and precipitate formation, so that in the presence of Co 101244 mitochondrial structure is similar to that in control. (Scale bar, 1 μm.)

the MMP (Fig. 3*D*), consistent with previous findings in cerebellar neurons (20). Co 101244 delayed and attenuated the NMDA-induced loss of TMRM fluorescence, which now mainly recovered within 60 min (Fig. 3*C* and Fig. S2*B*). Corrections for PM depolarization revealed that NR2B inhibition completely abolished MMP depolarization (Fig. 3*D*), an effect that reflects maintenance of the MMP due to limited mitochondrial Ca^{2+} accumulation. NMDA-dependent PM depolarization is a consequence of the redistribution of Na^+ and K^+ (Fig. 3*B*). Though inhibition of NR2B-containing NMDARs strongly reduced NMDA-induced Ca^{2+} entry, it did not prevent reversal of the Na^+/K^+ gradient (Fig. 3*B*). The reason for persistent Na^+/K^+ reversal and PM depolarization under conditions of NR2B inhibition is unknown.

In concert with the functional effects of NMDAR overactivation, toxic NMDA exposure also induced dramatic structural changes, including massive mitochondrial swelling and the formation of calcium-rich precipitates within mitochondrial matrices (Fig. 4). The fraction of damaged mitochondria, characterized by increased volume and low matrix density, was 48 ± 3% ($n = 52$), as estimated from EM images of cells cryofixed 30 min after NMDA exposure. NR2B inhibition completely prevented these structural changes to mitochondria (Fig. 4 *Bottom*).

Prosurvival Synaptic NMDAR Stimulation Evokes Minimal Ca^{2+} Loading. $GABA_A$ inhibition results in persistent, TTX-sensitive cytosolic Ca^{2+} spikes (Fig. 2*A Inset* and Fig. S3 *A* and *B*). Synaptically evoked Ca^{2+} entry was not toxic; indeed, it appears to promote survival pathways, as judged by a significant increase in nuclear phospho-CREB staining and a decrease in NMDA-induced cell death (unpublished observations and ref. 4). In our hands, various protocols of synaptic activation produce Ca^{2+} elevations on the order of 250–500 nM (Fig. 2*A Inset* and Fig. S3 *B* and *C*), which is consistent with previous reports for cultured neurons (21, 22), as well as with our own estimates of Ca^{2+} elevations induced by high-frequency field stimulation (Fig. S3*C*). It is also in agreement with the magnitude of transients induced by strong physiological stimuli in intact preparations (23, 24), but, importantly, quite low relative to the ≈10 μM Ca^{2+} elevations induced in the same cells by global or extrasynaptic NMDAR activation (Figs. 1 *C* and *D* and 2*A*, respectively).

The size of the Ca^{2+} loads associated with synaptically driven Ca^{2+} transients was measured using EPMA. Synaptically induced Ca^{2+} entry led to modestly higher cytoplasmic total Ca in both dendrites and soma relative to control, but the increase was much lower than that induced by NMDA or glutamate (Fig. 5*A* and Table S1). Synaptic NMDAR activation also resulted in detectable Ca accumulation in mitochondria, but the relatively modest levels achieved in both somatic and dendritic mitochondria, <6 mmol/kg dry weight, are more than 2 orders of magnitude smaller than the ≈1,000 mmol/kg Ca loads induced by NMDA or glutamate (Fig. 5*A* and Table S1). Some dendritic mitochondria, presumably those exposed to the largest Ca^{2+} transients, had higher Ca levels and contained high-Ca inclusions (Fig. 5*D Inset*). Nonetheless, there were no synaptically driven structural changes in any mitochondria, including those containing inclusions (Fig. 5*D*). Synaptic activation induced relatively small changes in the basal concentrations of Na and K, which contrasts with the large changes induced by excitotoxic stimulation (Table S1). Consistent with physiologically low Ca elevations, no significant changes in TMRM fluorescence were seen following synaptic activation (Fig. 5*B*).

Because NR2B-containing NMDARs constitute a significant fraction of synaptic NMDARs at this stage of development (25, 26), we examined the role of this receptor subtype in synaptically induced Ca^{2+} elevations. Co 101244 reduced the Ca^{2+} spike

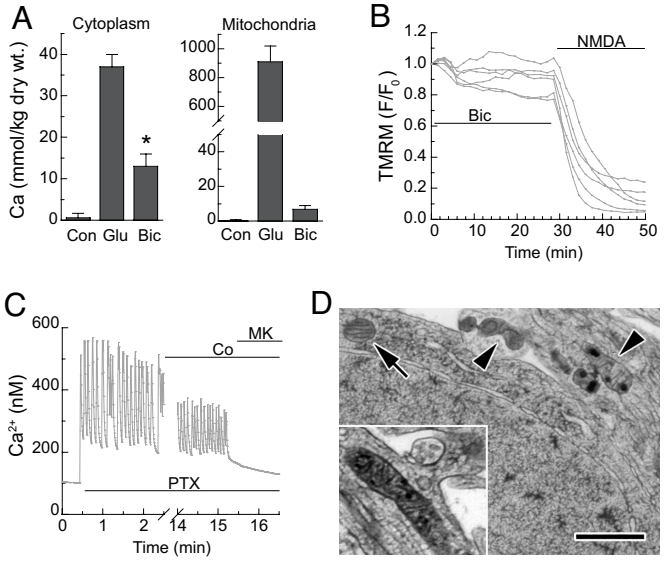

Fig. 5. Activation of synaptic NMDARs induces physiological levels of Ca^{2+} accumulation and no structural or functional damage. (*A*) Concentrations of total Ca in cytoplasm and mitochondria in young hippocampal neurons 30 min after synaptic activation (50 μM bicuculline plus 2.5 mM 4-AP) are much lower than Ca levels induced by glutamate bath application (500 μM, 30 min). (*B*) Synaptic activation induces negligible decrease in TMRM fluorescence compared with the strong loss induced by NMDA (100 μM). (*C*) Amplitude of Ca^{2+} spikes induced by synaptic activation (100 μM PTX; average of 13 cells) is reduced by Co 101244 (5 μM). (*D*) Representative electron micrograph of high-pressure frozen, freeze-substituted neuronal cell body and surrounding neuropil after 30 min bicuculline exposure. Somatic (arrows) and dendritic (arrowheads) mitochondria appear normal, although some dendritic mitochondria contain evident Ca^{2+} inclusions, as illustrated in the rightmost arrowhead and by a mitochondrion at the base of a dendritic spine (*Inset*). (Scale bar, 1 μm.)

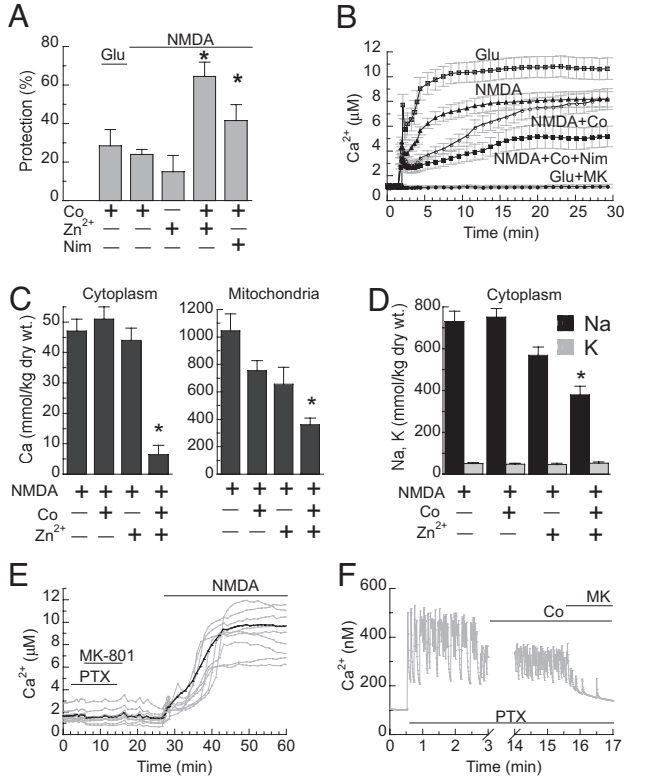

Fig. 6. Both NR2A- and NR2B-containing NMDARs mediate excitotoxic Ca^{2+} accumulation in older neurons. (*A*) Protection against glutamate (500 μM, 30 min) or NMDA (100 μM, 30 min) in older neurons (21–28 DIV) is enhanced by simultaneous blockade of both NR2A- and NR2B-containing NMDARs with Co 101244 (5 μM) and Zn^{2+} (500 nM), respectively. Co 101244 plus nimodipine (10 μM) also enhanced protection. (*B*) Averaged traces of evoked free Ca^{2+} elevations (fura-2FF) induced by glutamate ($n = 19$), glutamate plus MK-801 ($n = 22$), NMDA ($n = 22$), NMDA plus Co 101244 ($n = 25$), and NMDA plus Co 101244 and nimodipine ($n = 17$). Inhibiting NR2B alone delayed but did not reduce NMDA-induced Ca^{2+} elevation, but simultaneous inhibition of L-type VGCCs did. Glutamate-induced Ca^{2+} elevations were blocked by MK-801. (*C*) Concentrations of total cytoplasmic and mitochondrial Ca after exposure to NMDA (100 μM, 30 min), NMDA with NR2B inhibited (Co), NMDA with NR2A inhibited (Zn^{2+}), and NMDA with both subtypes inhibited. Ca accumulation in both compartments is significantly reduced only in the presence of both NR2 antagonists. (*D*) Elevated NMDA-induced cytosolic Na concentrations are attenuated by inhibition of both NR2 subtypes, but the Na/K ratio remains reversed. Asterisks indicate data significantly different ($P < 0.05$) from Co 101244 alone (*A*) or from only NMDA exposure (*C* and *D*). (*E*) The magnitude of single-cell extrasynaptic Ca^{2+} elevations (fura-2FF), evoked as described in the legend to Fig. 2, is comparable to that seen in young cells (Fig. 2*A*). (*F*) Amplitude of synaptically induced Ca^{2+} spikes (fura-2, 100 μM PTX plus 10 μM CNQX, $n = 10$) is relatively small. Transients are reduced ≈50% by Co 101244 (5 μM) and completely abolished by MK-801 (10 μM). The oscillation frequency is faster than in young cells, both before (≈0.35 vs. ≈0.17 Hz) and after (≈0.50 vs. ≈0.20 Hz) NR2B inhibition.

amplitude by ≈60% (Fig. 5*C*), suggesting that in young cells both NR2B- and NR2A-containing NMDARs contribute to synaptically induced Ca^{2+} entry. Nonetheless, synaptic activation of these receptors was not toxic, which implies, as confirmed in the text following, that receptor subtype identity is not the determining factor for specifying signal coupling.

Mechanisms of Excitotoxic Injury in Mature Hippocampal Neurons: Ca^{2+} Overload Mediated by Multiple Routes of Calcium Entry. Several labs have reported (5, 13, 15, 16), as we have found here, that as pyramidal neurons mature the NR2B/NR2A ratio is reduced, primarily due to enhanced expression of NR2A (Fig. 1*A*). There are also data indicating that in older neurons, activation of either NR2B- or NR2A-containing NMDARs triggers excitotoxicity (14). Similarly, we found that protection against NMDA was enhanced by simultaneously blocking NR2B and NR2A (Fig. 6*A*), which indicates that activation of either NR2 subunit-containing NMDARs can mediate excitotoxicity in older neurons.

NR2B and NR2A Inhibition Is Required to Strongly Reduce Excitotoxic Ca^{2+} Elevations. To determine whether in older cells, as in younger cells, the size of Ca^{2+} loads still parallels neuroprotection, free and total Ca elevations were measured by fluorescence microscopy and EPMA, respectively. Free Ca^{2+} elevations induced by glutamate or NMDA were similar to those in younger cells (compare Fig. 6*B* with Fig. 1 *C* and *D*), and completely abolished by MK-801 (Fig. 6*B*). However, NMDA-induced Ca^{2+} elevations were, in contrast to younger cells, relatively insensitive to NR2B inhibition (compare Fig. 6*B* with Fig. 1 *C* and *D*), which suggests that Ca^{2+} entry through NR2B-containing receptors is no longer the only significant source of toxic Ca^{2+} and therefore implicates NR2A-containing NMDARs. Because fura-2FF responds to Zn^{2+} at concentrations necessary to block NR2A-containing NMDARs, we evaluated the effect of NR2A blockade by measuring total Ca accumulation with EPMA. NMDA-stimulated increases in both cytoplasmic and mitochondrial Ca were similar to younger cells (Fig. 6*C* vs. Fig. 3*A*; Table S1), but, in contrast to younger cells, inhibiting NR2B alone was without effect. To achieve effective reduction of excessive NMDA-induced Ca accumulation, blockade of both NR2A- and NR2B-containing receptors was necessary (Fig. 6*C* and Table S1), which parallels the data on neuroprotection. These observations imply that both NR2 subtypes are routes for excitotoxic Ca^{2+} entry in older neurons.

Voltage-Gated Ca^{2+} Entry Can Contribute to Toxic NMDA-Induced Cytosolic Ca^{2+} Elevations. Even though simultaneous inhibition of NR2A and NR2B is reasonably protective in older cells, this combination is not as effective as MK-801 or NR2B inhibition alone in younger cells, implying that there may well be additional routes of Ca^{2+} loading that can contribute to excitotoxicity. Brewer et al. (16) have recently reported that blocking L-type VGCCs is neuroprotective in mature, but not young, hippocampal neurons. Here we find that coapplication of the L-channel blocker nimodipine together with Co 101244 enhanced protection (Fig. 6*A*). Nimodipine plus Co 101244 also attenuated NMDA-induced cytosolic Ca^{2+} elevations (Fig. 6*B*). Although these observations suggest that VGCCs can contribute to excitotoxic Ca^{2+} overload, they do not distinguish between various mechanisms that might activate VGCCs. EPMA showed that none of the pharmacological agents used, except MK-801, eliminate NMDA-induced PM depolarization (as judged by reversal of cytoplasmic Na^+/K^+ ratios (Fig. 6*D* and Table S1), which suggests that activation of NMDARs containing either NR2 subunit may lead to synergistic VGCC Ca^{2+} entry.

In Older Cells, Activation of Extrasynaptic NMDARs Induces Large Toxic Ca^{2+} Elevations. The level of NMDA-induced Ca^{2+} elevations via extrasynaptic NMDARs in older cells was similar to that in young cells (Fig. 6*E*), but NR2B inhibition was much less effective, blocking only ≈40% of extrasynaptic Ca^{2+} entry (data not shown). This implies that a substantial fraction of extrasynaptic Ca^{2+} influx is mediated by NR2A-containing NMDARs. Also similar to younger cells, synaptically induced Ca^{2+} elevations were much smaller than those induced by activation of extrasynaptic NMDARs (Fig. 6 *E* and *F*). NR2B inhibition significantly reduced the amplitude of synaptically induced Ca^{2+} oscillations, indicating that NR2B-containing NMDARs remain an important component of synaptic receptors (Fig. 6*F*). However, the frequency of Ca^{2+} oscillations increased ≈2× in older cells, and even more with NR2B inhibition (Fig. 6*F* vs. Fig. 5*C*), probably reflecting a greater proportion of synaptic NR2A-containing NMDARs, which are known to have faster decay times (27). Taken together, these observations indicate that extrasynaptic NR2A-containing NMDARs represent an additional route of excitotoxic Ca^{2+} entry that becomes important later in development.

Discussion

NMDA receptors play important and diverse roles in CNS function, ranging from the regulation of synaptic plasticity and neuronal growth and survival to the initiation of cell death. It is thought that distinct NMDAR-mediated routes specified by receptor location or subunit composition are responsible for the induction of survival vs. death pathways (4, 6), but the mechanisms underlying pathway selection remain uncertain. Two popular ideas, referred to as the "source specificity" and "calcium load" hypotheses, consider that excitotoxic Ca^{2+} signaling is specified either by the location and/or composition of signaling NMDARs, or by the relative strength of Ca^{2+} signals—that is, by the size of NMDAR-mediated Ca^{2+} loads (28). In this study, we provide experimental support for the Ca^{2+} load hypothesis, presenting the first data to quantify the large differences in Ca^{2+} loading between prodeath and prosurvival signals. The findings explain why only certain routes of Ca^{2+} entry are toxic, and serve to rationalize and reconcile the literature showing dependencies on NMDAR location and subunit composition.

Concerning receptor location in death vs. survival decisions, findings show that extrasynaptic NMDARs alone account for the large majority of Ca^{2+} accumulation and cell death under conditions of agonist bath application. Thus, these receptors are sufficient to induce large, toxic Ca^{2+} elevations on the order of 10 μM, strong enough to trigger mitochondrial overload and dysfunction. Ca^{2+} increases of this magnitude are consistent with previous reports that Ca^{2+} elevations induced by toxic NMDAR activation, when measured with low-affinity fluorescent Ca^{2+} probes, are much larger than those evoked by alternative strong stimuli (7–9, 29). In contrast, synaptic stimuli are not toxic because they evoke Ca^{2+} accumulation that is too small to overload mitochondria. Quantitative estimates indicate that GABA antagonist-induced spontaneous synaptic activation evokes NMDAR-dependent Ca^{2+} increases on the order of several hundred nanomolar, which are ≈100-fold less than elevations induced by agonist bath application. The large difference between the strength of synaptic and bath agonist stimulation is confirmed by the dramatic differences in mitochondrial Ca accumulation, as well as changes in Na and K concentrations, determined by EPMA.

Our measurements contradict the findings of a previous study, which found no differences between synaptic and extrasynaptic Ca^{2+} elevations when evoked by essentially the same protocols used here (4). The discrepancy is likely explained by the saturation of the high-affinity Ca^{2+} probe used in that study (Fig. S3 *A* and *B*). Although the modest Ca^{2+} accumulation evoked by synaptic activation probably reflects the regulated, controlled nature of transmitter release in intact synapses, present findings do not rule out the possibility that synaptic NMDARs are capable of mediating Ca^{2+} overload under conditions of extremely high glutamate exposure (e.g., as might be encountered in ischemia). This would be consistent with the observation that selective perturbation of synaptic NMDARs protects against oxygen/glucose deprivation but not against NMDA or glutamate (30), and might rationalize the influence of postsynaptic density modifications on excitotoxic vulnerability (31, 32).

The developmental maturation of NMDARs is accompanied by a decrease in the NR2B/NR2A expression ratio (13–16), which provides an opportunity to test the effect of NMDAR subunit composition on the choice of Ca^{2+}-linked transduction pathways. Inhibition of NR2B-containing NMDARs eliminated glutamate-evoked Ca^{2+} overload only in young neurons, mainly because their NMDARs are predominantly NR2B containing. In older cells it was necessary to inhibit both NR2A- and NR2B-containing receptors to reduce Ca^{2+} accumulation to nontoxic levels and achieve satisfactory protection. This indicates that toxic Ca^{2+} loading can be mediated by receptors of either subtype. Moreover, both NR2B- and NR2A-containing NMDARs contribute to synaptically induced Ca^{2+} entry, which is prosurvival, not toxic. Taken together, this implies that subunit composition alone is not sufficient to specify signal coupling.

Although NMDARs are the primary route of Ca^{2+} entry regardless of developmental stage, in mature neurons nimodipine in combination with Co 101244 effectively reduces NMDA-evoked Ca^{2+} elevations while enhancing neuroprotection. This observation indicates that Ca^{2+} entry through VGCCs acts synergistically with NMDARs to increase the cumulative Ca^{2+} load, reinforcing the point that the amount of Ca^{2+} accumulated is more important than the route of entry.

The large cytosolic Ca^{2+} elevations and mitochondrial Ca^{2+} overload that characterize excitotoxic conditions are generally paralleled by MMP loss and mitochondrial swelling, both implying mitochondrial dysfunction. In neurons, the exact mechanisms are not completely clear, but MMP loss reduces ATP production (10, 11), whereas irreversible swelling is a characteristic of MPT (33). It appears that mitochondrial Ca^{2+} accumulation may also be responsible for ROS production and activation of poly(ADP-ribose) polymerase-1 (PARP-1) (34, 35). Strong NMDAR activation is also generally associated with significant Na^+ loading, which has the potential to promote ATP depletion as a result of increased ATP demand by Na/K-ATPase (36). However, our experiments reveal conditions under which cells have elevated Na^+ but low Ca^{2+}, yet there is no MMP loss and no cell death. This implies that Ca^{2+}, not Na^+, plays the pivotal role in mitochondrial dysfunction. Earlier studies have identified other circumstances where Na^+ loading per

se did not affect MMP or ATP levels (37). However, under conditions of continuous, prolonged exposure to low concentrations of NMDA, ATP depletion following from elevated Na^+ is clearly a major factor in delayed Ca^{2+} deregulation (38).

To summarize, this study provides evidence that the dichotomous nature of NMDAR-mediated Ca^{2+} signaling can be understood in the context of the strength of the Ca^{2+} signal. The effects of other factors, such as receptor composition and location, can be rationalized within the framework of excessive mitochondrial Ca^{2+} loading. It seems likely that understanding the mechanisms of excitotoxic death will inform therapeutic approaches to stroke, as well as to other neurodegenerative disorders that may present mechanistically similar disease pathways.

Materials and Methods

Cell Culture, Stimulation Protocols, and Survival Assays. Primary cultures of rat hippocampal neurons were prepared in accordance with the National Institute of Neurological Disorders and Stroke, National Institutes of Health Animal Care and Use Committee Protocol, as previously described (9). To induce excitotoxicity, cultures were exposed to 500 μM glutamate or 100 μM NMDA in a Mg^{2+}-free Hepes buffered salt solution for 30 min at room temperature. Selective synaptic activation was accomplished according to published protocols (4, 6).

Fluorescence Microscopy. Two Ca^{2+}-sensitive ratiometric fluorescent dyes, fura-2 (K_D = 0.14 μM; Invitrogen) and fura-2FF (K_D = 25 μM; Tef Labs), were used to estimate concentrations of intracellular free Ca^{2+}, as described in *SI Materials and Methods*. The membrane-permeant cationic dye tetramethylrodamine methyl ester (TMRM; Invitrogen) was used to assay MMP changes. PM potential was estimated using the fluorescent plasma membrane potential indicator (PMPI, as described [20]).

Western Blot Analysis. Hippocampal cultures were harvested by scraping plates incubating in Nonidet P-40 cell lysis buffer (Invitrogen), 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture (Complete Mini; Roche). Dispersed lysates were electrophoresed on NuPAGE 4–12% Bis Tris Gels (Invitrogen). Blots were incubated overnight at 4 °C with appropriate antibodies (see *SI Materials and Methods*). For semiquantitative analysis, O.D. scans recorded with a BioRad GS800 densitometer were analyzed with Quantity One software (BioRad).

Electron Microscopy and EPMA. For conventional electron microscopy, cultures grown in gold specimen ''hats'' were high-pressure frozen immediately after appropriate reagent exposure using a Baltec HPM010 high-pressure freezing machine (Boeckeler Instruments) and freeze-substituted by means of a Leica AFS freeze-substitution device, both as previously described (9). For EPMA, cultures grown on plastic coverslips were plunge-frozen in liquid ethane at appropriate time points, processed, and analyzed as described (9).

Statistics. Statistical comparisons were carried out (*InStat* software; GraphPad) by one-way ANOVA with post hoc Dunnett multiple comparisons test (for normally distributed data), or by nonparametric Kruskal-Wallis rank ANOVA with post hoc Dunn's test (for non-normally distributed data).

Further details are provided in *SI Text*.

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1. Choi DW (1992) Excitotoxic cell death. *J Neurobiol* 23:1261–1276.
2. Arundine M, Tymianski M (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell Calcium* 34:325–337.
3. Hardingham GE, Bading H (2003) The Yin and Yang of NMDA receptor signalling. *Trends Neurosci* 26:81–89.
4. Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci* 5:405–414.
5. Zhou M, Baudry M (2006) Developmental changes in NMDA neurotoxicity reflect developmental changes in subunit composition of NMDA receptors. *J Neurosci* 26:2956–2963.
6. Liu Y, et al. (2007) NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. *J Neurosci* 27:2846–2857.
7. Carriedo SG, Yin HZ, Sensi SL, Weiss JH (1998) Rapid Ca^{2+} entry through Ca^{2+}-permeable AMPA/Kainate channels triggers marked intracellular Ca^{2+} rises and consequent oxygen radical production. *J Neurosci* 18:7727–7738.
8. Cheng C, Fass DM, Reynolds IJ (1999) Emergence of excitotoxicity in cultured forebrain neurons coincides with larger glutamate-stimulated $[Ca^{2+}]_i$ increases and NMDA receptor mRNA levels. *Brain Res* 849:97–108.
9. Pivovarova NB, et al. (2004) Excitotoxic calcium overload in a subpopulation of mitochondria triggers delayed death in hippocampal neurons. *J Neurosci* 24:5611–5622.
10. Nicholls DG (2004) Mitochondrial dysfunction and glutamate excitotoxicity studied in primary neuronal cultures. *Curr Mol Med* 4:149–177.
11. Duchen MR (2004) Mitochondria in health and disease: Perspectives on a new mitochondrial biology. *Mol Aspects Med* 25:365–451.
12. Starkov AA, Chinopoulos C, Fiskum G (2004) Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury. *Cell Calcium* 36:257–264.
13. Sinor JD, et al. (2000) NMDA and glutamate evoke excitotoxicity at distinct cellular locations in rat cortical neurons in vitro. *J Neurosci* 20:8831–8837.
14. von Engelhardt J, et al. (2007) Excitotoxicity in vitro by NR2A- and NR2B-containing NMDA receptors. *Neuropharmacology* 53:10–17.
15. O'Donnell LA, et al. (2006) Human immunodeficiency virus (HIV)-induced neurotoxicity: Roles for the NMDA receptor subtypes. *J Neurosci* 26:981–990.
16. Brewer LD, et al. (2007) Increased vulnerability of hippocampal neurons with age in culture: Temporal association with increases in NMDA receptor current, NR2A subunit expression and recruitment of L-type calcium channels. *Brain Res* 1151:20–31.
17. Rachline J, Perin-Dureau F, Le Goff A, Neyton J, Paoletti P (2005) The micromolar zinc-binding domain on the NMDA receptor subunit NR2B. *J Neurosci* 25:308–317.
18. Choi DW (1994) Calcium and excitotoxic neuronal injury. *Ann NY Acad Sci* 747:162–171.
19. Pivovarova NB, et al. (2008) Reduced calcium-dependent mitochondrial damage underlies the reduced vulnerability of excitotoxicity-tolerant hippocampal neurons. *J Neurochem* 104:1686–1699.
20. Nicholls DG (2006) Simultaneous monitoring of ionophore- and inhibitor-mediated plasma and mitochondrial membrane potential changes in cultured neurons. *J Biol Chem* 281:14864–14874.
21. McLeod JR, Jr, Shen M, Kim DJ, Thayer SA (1998) Neurotoxicity mediated by aberrant patterns of synaptic activity between rat hippocampal neurons in culture. *J Neurophysiol* 80:2688–2698.
22. Young KW, Bampton ET, Pinon L, Bano D, Nicotera P (2008) Mitochondrial Ca^{2+} signalling in hippocampal neurons. *Cell Calcium* 43:296–306.
23. Cormier RJ, Greenwood AC, Connor JA (2001) Bidirectional synaptic plasticity correlated with the magnitude of dendritic calcium transients above a threshold. *J Neurophysiol* 85:399–406.
24. Pivovarova NB, Pozzo-Miller LD, Hongpaisan J, Andrews SB (2002) Correlated calcium uptake and release by mitochondria and endoplasmic reticulum of CA3 hippocampal dendrites after afferent synaptic stimulation. *J Neurosci* 22:10653–10661.
25. Thomas CG, Miller AJ, Westbrook GL (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. *J Neurophysiol* 95:1727–1734.
26. Harris AZ, Pettit DL (2007) Extrasynaptic and synaptic NMDA receptors form stable and uniform pools in rat hippocampal slices. *J Physiol* 584:509–519.
27. Cull-Candy SG (September 28, 2007) NMDA receptors. *Encyclopedia of Life Sciences*, 10.1002/9780470015902.a0000254.pub2.
28. Wittmann M, Bengston CP, Bading H (2004) in *Pharmacology of Cerebral Ischemia*, eds Krieglestein J, Klumpp S (Medpharm Scientific Publishers, Stuttgart), pp 253–266.
29. Hyrc K, Handran SD, Rothman SM, Goldberg MP (1997) Ionized intracellular calcium concentration predicts excitotoxic neuronal death: Observations with low-affinity fluorescent calcium indicators. *J Neurosci* 17:6669–6677.
30. Sattler R, Xiong Z, Lu WY, MacDonald JF, Tymianski M (2000) Distinct roles of synaptic and extrasynaptic NMDA receptors in excitotoxicity. *J Neurosci* 20:22–33.
31. Aarts M, et al. (2002) Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. *Science* 298:846–850.
32. Soriano FX, et al. (2008) Specific targeting of pro-death NMDA receptor signals with differing reliance on the NR2B PDZ ligand. *J Neurosci* 28:10696–10710.
33. Bernardi P, et al. (2006) The mitochondrial permeability transition from in vitro artifact to disease target. *FEBS J* 273:2077–2099.
34. Duan Y, Gross RA, Sheu SS (2007) Ca^{2+}-dependent generation of mitochondrial reactive oxygen species serves as a signal for poly(ADP-ribose) polymerase-1 activation during glutamate excitotoxicity. *J Physiol* 585:741–758.
35. Abramov AY, Duchen MR (2008) Mechanisms underlying the loss of mitochondrial membrane potential in glutamate excitotoxicity. *Biochim Biophys Acta* 1777:953–964.
36. Nicholls DG, Johnson-Cadwell L, Vesce S, Jekabsons M, Yadava N (2007) Bioenergetics of mitochondria in cultured neurons and their role in glutamate excitotoxicity. *J Neurosci Res* 85:3206–3212.
37. Chinopoulos C, Tretter L, Rozsa A, Adam-Vizi V (2000) Exacerbated responses to oxidative stress by an Na^+ load in isolated nerve terminals: The role of ATP depletion and rise of $[Ca^{2+}]_i$. *J Neurosci* 20:2094–2103.
38. Vander Jagt TA, Connor JA, Shuttleworth CW (2008) Localized loss of Ca^{2+} homeostasis in neuronal dendrites is a downstream consequence of metabolic compromise during extended NMDA exposures. *J Neurosci* 28:5029–5039.