Mitochondrial Dysfunction and Dendritic Beading during Neuronal Toxicity

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Mitochondrial dysfunction (depolarization and structural collapse), cytosolic ATP depletion, and neuritic beading are early hallmarks of neuronal toxicity induced in a variety of pathological conditions. We show that, following global exposure to glutamate, mitochondrial changes are spatially and temporally coincident with dendritic bead formation. During oxygen-glucose deprivation, mitochondrial depolarization precedes mitochondrial collapse, which in turn is followed by dendritic beading. These events travel as a wave of activity from distal dendrites toward the neuronal cell body. Despite the spatiotemporal relationship between dysfunctional mitochondria and dendrites, mitochondrial dysfunction increases neuronal vulnerability to these morphological changes during normal physiological activity. Our findings support a mechanism whereby, during glutamate excitotoxicity, Ca$^{2+}$ influx leads to mitochondrial depolarization, whereas Na$^+$ influx leads to an unsustainable increase in ATP demand (Na$^+$, K$^+$-ATPase activity). This leads to a drop in ATP levels, an accumulation of intracellular Na$^+$ ions, and the subsequent influx of water, leading to microtubule depolymerization, mitochondrial collapse, and dendritic beading. Following the removal of a glutamate challenge, dendritic recovery is dependent upon the integrity of the mitochondrial membrane potential, but not on a resumption of ATP synthesis or Na$^+$, K$^+$-ATPase activity. Thus, dendritic recovery is not a passive reversal of the events that induce dendritic beading. These findings suggest that the degree of calcium influx and mitochondrial depolarization inflicted by a neurotoxic challenge, determines the ability of the neuron to recover its normal morphology.

Mitochondrial dysfunction, dendritic spine remodeling, and the formation of neuritic beads are early hallmarks of neuronal toxicity induced in a variety of pathological conditions (1–6). In particular, in Alzheimer disease, the existence of extracellular amyloid plaques and intracellular neurofibrillary tangles have been associated with neuritic beading, spine loss, neurite breakage, and shaft atrophy (7–10).

An understanding of the importance of mitochondrial dysfunction in neurological diseases continues to grow. Mitochondria are highly dynamic organelles with their trafficking and elongated morphology being regulated by neuronal (11, 12) and mitochondrial (13) activity. It appears that mitochondrial transport, morphology, and function are affected by physiological processes such as synaptic transmission and pathological processes such as excitotoxicity.

Despite the fact that mitochondrial dysfunction and dendritic beading are both initiated by glutamate excitotoxicity, these events are believed to be independent due to their distinct requirements for Ca$^{2+}$ (1–4, 6, 14) versus Na$^+$ (2, 3) influx. Moreover, dendritic beading occurs much more rapidly than Ca$^{2+}$-dependent excitotoxic cell death (2, 3).

Although Ca$^{2+}$-induced excitotoxicity and Na$^+$-induced dendritic beading appear to be distinct, both processes are activated simultaneously by excitotoxic stimuli. Indeed, NMDA receptor agonists are permeable to both ions and are implicated in both processes (5, 6), yet the spatial and temporal relationship between these events have not been examined.

The role of mitochondria in excitotoxicity is well established (15), but how they relate to dendritic beading is unknown. Interestingly, previous studies have observed the existence of “hollow” beads that were devoid of cytosol (4, 14, 16). Indeed, the existence of “membraneous structures” within some beads has been observed in situ. These inclusions were attributed to the presence of autophagic vesicles or secondary lysosomes (7). Mitochondrial changes in morphology have been reported to occur following exposure to glutamate (11). Therefore, it is possible that the structures observed within beads might, in fact, be dysfunctional mitochondria, with altered morphology.

To investigate the temporal and spatial regulation, and ionic dependence, of these events, we performed time-lapse microscopy on hippocampal neurons during an excitotoxic challenge. Using this approach, we have monitored changes in mitochondrial morphology and function, and correlated this to the formation of dendritic beads. We find that these events are related temporally and spatially, with the majority of beads containing a dysfunctional mitochondrion. Furthermore, the morphological recovery of neurons appears to depend on the degree of mitochondrial damage during the insult. However, mitochondrial changes appear to be secondary to a perturbation in neuronal microtubules as a result of cytoplasmic water influx.

**EXPERIMENTAL PROCEDURES**

Materials—pECFP-C1 and pDsRed2-Mito vectors were purchased from Clontech (BD Biosciences). The construct encoding firefly (Photinus pyralis) luciferase targeted to the cyto-
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plasm (PcDNA3lucLL/V) was kindly donated by Dr. Giovanni Manfredi, Cornell University, New York (17). Rhodamine 123, JC-1, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP),2 2,4-dinitrophenol, oligomycin (a mixture of oligomycins A, B, and C), L-glutamic acid, and L-glycine were purchased from Sigma. Ouabain, CGP 3157, diazoxide, 5-hydroxydcanolic acid, MK-801, and CNQX were purchased from Tocris (UK). D-Luciferin was purchased from Pierce.

Neuronal Cell Culture—Primary cultures of rat hippocampal neurons were prepared as follows. Hippocampi from 1–3-day-old Sprague-Dawley rats of both sexes were dissected and cut into small pieces with a scalpel. Tissue was suspended in 5 ml of filter-sterilized buffer comprising (mM) 116 NaCl, 5.4 KCl, 26 NaHCO3, 1.3 NaH2PO4, 2 MgSO4, 2 CaCl2, 0.5 EDTA, and 25 D(+)-glucose, pH 7.4) supplemented with 1.5 mg/ml papain and incubated at 37 °C for 20 min. Tissue was then transferred to 2 ml of buffer (supplemented with 10 mg/ml ovomucoid and 10 mg/ml bovine serum albumin) and triturated with a series of flame-polished glass Pasteur pipettes of decreasing tip diameter. The cell suspension was centrifuged at 1,200 × g for 2 min and the pellet resuspended in culture medium consisting of Neurobasal-A Medium (Invitrogen) supplemented with 2% (v/v) B-27 (Invitrogen) and 2 mM L-glutamine at a density of 5 × 104 cells/ml. Cells were plated at a density of 3.8 × 104 cells/cm2 onto sterile coverslips or 8.3 × 104 cells/cm2 onto white 96-well plates pretreated with poly-D-lysine (15 μg/ml in borate buffer; pH 8). Cultures were incubated in culture medium and maintained in a humidified atmosphere at 37 °C in 5% CO2. After 5 days, cytosine β-D-arabinofuranoside (final concentration 10 μM) was added to inhibit glial cell proliferation. Experiments were performed after 9–13 days (no differences due to age were observed) in vitro at room temperature in HEPES-buffered saline comprising (mM) 135 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, and 25 D(+)-gluconate (pH 7.4), supplemented with 0.5 μM tetrodotoxin. For ion replacement solutions, NaCl or CaCl2 were replaced with equimolar N-methyl-D-glucamine or MnCl2, respectively. Hypertonic buffer was achieved by the addition of 200 mM sucrose. A rapid shift into hypotonic buffer was achieved by a 2-fold dilution of HEPS-buffered saline into HEPES buffer lacking NaCl, to achieve a 67 mM final concentration of NaCl.

Neuronal Transfection—Hippocampal cultures were transfected using the calcium phosphate precipitate technique as previously described (18). Briefly, DNA/Ca2+-phosphate precipitate was prepared by diluting (per coverslip) 2 μl of 1 mg/ml plasmid DNA in 20.5 μl of filter-sterilized TE (10 mM Tris-Cl, 1 mM EDTA, pH 7.3) followed by addition of 2.5 μl of filter-sterilized HEPES-buffered CaCl2 (2.5 mM CaCl2, 10 mM HEPES, pH 7.2). The resulting solution was added dropwise to 25 μl of filter-sterilized 2× HEPES-buffered saline (273.8 mM NaCl, 9.9 mM KCl, 1.4 mM Na2HPO4, 42.0 mM HEPES, 11.1 mM D(+)-glucose, pH 7.2) and vortexed (3 s). The precipitate solution was incubated in the dark at room temperature and briefly vortexed every 2 min for a total of 30 min. The resulting precipitate was dispersed over a neuron-seeded coverslip in one well of a 24-well cell culture plate containing 250 μl of culture medium supplemented with 2 mM kynurenic acid. Cells were incubated at 37 °C in 5% CO2 for 3 h to allow for settling and endocytosis of the precipitate. Treated coverslips were then washed in 1 ml of acidic wash medium (culture medium equilibrated to 10% CO2 and supplemented with 2 mM kynurenic acid) for 20 min at 37 °C in 5% CO2 before being returned to the original culture medium. Cells grown in 96-well plates were transfected as above using one-fourth of the stated volumes per well. Experiments were performed 24–48 h post-transfection.

Epifluorescent Microscopy— Cultures cotransfected with pECFP-C1 and pDsRed2-Mito were loaded with rhodamine 123 (1 μg/ml in HEPES-buffered saline) for 15 min in the dark at room temperature. Quantification of mitochondrial polarization was achieved using ratiometric JC-1 imaging (1 μg/ml, 15 min at 37 °C in the dark). Cells were subsequently washed, sealed into a non-perfusion chamber, and imaged using a Hamamatsu ORCA-ER camera and a 40× PL Fluotar or 100× PL APO objective lens on a Leica DM-IRB inverted microscope using OpenLab 5.0.1 software (Improvement, UK). Images were collected as follows: CFP (excitation 430 ± 25, emission 470 ± 30), DsRed (excitation 527 ± 23, emission 630 ± 60), rhodamine 123 (excitation 500 ± 20, emission 535 ± 30), and JC-1 (excitation 492 ± 18, emission 630 ± 60 [polarized]/535 ± 30 [depolarized]) presented as excitation 630/emission 535 multiplied by 100. For quantitative analysis, areas of interest containing dendritic beads or mitochondria were selected and the skeletal length/diameter and/or fluorescence intensities measured for each time point using Velocity 3.7 software (Improvement, UK). Values were normalized to a starting value of 100 and mean ± S.E. values calculated accordingly.

Cytoskeletal Detection—Neurons were fixed (3% paraformaldehyde in PBS, 15 min) and permeabilized (0.1% Triton X-100 in PBS, 10 min). Actin staining was achieved using Alexa 488-conjugated phalloidin (Sigma, 50 μg/ml). MAP2 distribution was probed using 2 μg/ml monoclonal anti-MAP2 antibodies (Sigma) and goat anti-mouse Alexa 488 (Molecular Probes, 1 μg/ml).

In Situ ATP Assay— Cultures transfected with pC3lucLL/V were incubated in HEPES-buffered saline supplemented with 1 mM D-luciferin plus appropriate drugs where necessary. Bioluminescence was monitored using a LB96V microplate luminometer using WinGlow software (Berthold Technologies). Luminescence values were normalized to a starting value of 100 and the mean ± S.E. values of the percentage difference from vehicle control calculated accordingly.

Oxygen-Glucose Deprivation— Cultures cotransfected with pECFP-C1 and pDsRed2-Mito were loaded with rhodamine 123 and imaged as described above. Cells were sealed into a non-perfusion chamber and incubated in artificial cerebrospinal fluid comprising (mM) 124 NaCl, 3 KCl, 26 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 10 D(+)-glucose, supplemented with 0.5 μM tetrodotoxin and bubbled with 95:5% O2:CO2. Oxygen-glucose deprivation was achieved by replacing standard artificial cerebrospinal fluid with an identical artificial cer-

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2 The abbreviations used are: FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; CFP, cyan fluorescent protein; G/G, glutamate/glycine; OGD, oxygen-glucose deprivation; MAP, mitogen-activated protein; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DsRed, discosoma red fluorescent protein.
Mitochondrial depolarization was monitored using rhodamine with mitochondrially targeted DsRed and cytoplasmic CFP. Chronically and exhibit spatial overlap in response to glutamate avoidance secondary glutamate release following the generation of vesicles (in rhodamine 123) (Fig. 1B). During an excitotoxic challenge with glutamate (100 μM) was included in all experiments to avoid secondary glutamate release following the generation of action potentials.

Mitochondrial Dysfunction and Neuritic Beading Occur Synchronously and Exhibit Spatial Overlap in Response to Glutamate Excitotoxicity—To examine changes in mitochondrial morphology and depolarization, simultaneously with dendritic structural changes, hippocampal neurons were transfected with mitochondrially targeted DsRed and cytoplasmic CFP. Mitochondrial depolarization was monitored using rhodamine 123. Tetrodotoxin (0.5 μM) was included in all experiments to avoid secondary glutamate release following the generation of action potentials. During an excitotoxic challenge with glutamate (100 μM) and glycine (10 μM)(G/G), four events occur simultaneously; 1) mitochondria depolarize immediately (as evidenced by a transient increase in rhodamine 123 fluorescence) (Fig. 1). 2) Mitochondrial morphology collapses. Dynamically, this appears as if mitochondria are stretched out on an elastic support that was severed at several points simultaneously. Thus, mitochondria appear to collapse into short, rounded structures with a reduced length and an increased girth. 3) Beads form along dendrites as focal swellings with reduced volume present between beads (supplemental materials Movie 1). To analyze the temporal sequence of events, quantitative measurements for each parameter were determined using Volocity (Improvisation) software. From this analysis, it can be seen that mitochondrial collapse (monitored as reduced length), mitochondrial depolarization, and dendritic beading (monitored as increased width) are initiated immediately and simultaneously (Fig. 1B).

Within the window of time used (6 s between image collection), all dendritic mitochondria appeared to depolarize simultaneously, suggesting that differential rates of mitochondrial dysfunction do not determine the site of beading. 4) Dendritic beading has been proposed to correlate with a drop in cytoplasmic [ATP] (5, 19), although the earliest time points examined were 1 (5) or 6 h (19). Using a cytoplasmic luciferase reporter construct (17) to monitor cytoplasmic [ATP], we observed a rapid depletion of ATP within the time scale (15 min) of dendritic beading (Fig. 1C) (n > 50).

G/G induced beading and mitochondrial dysfunction are blocked by antagonists of glutamate receptors (10 μM MK801 plus 20 μM CNQX). Furthermore, G/G treatment in the presence of either MK801 or CNQX, or glutamate receptor activation by either NMDA/glycine (100 μM/10 μM), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (100 μM), or kynurenic acid (100 μM), revealed that all the ionotropic glutamate receptors are capable of inducing dendritic bead formation in hippocampal neurons (supplementary materials Fig. 1).

An apparent overlap of dysfunctional (depolarized and morphologically collapsed) mitochondria and dendritic beads is evident (Fig. 1A and supplemental Movie 1). However, dysfunctional mitochondria are not exclusively localized to beads, so it is likely that some mitochondria may associate with beads by chance. To determine the existence of mitochondria within beads as predicted by chance, we estimated the average mitochondrial density to be 0.082 ± 0.003 (mean ± S.E.: 82 dendrites, 6 neurons) mitochondria/μm of dendrite and the average bead length to be 4.42 ± 0.08 μm (mean ± S.E.: 541 beads, 10 neurons). Thus, 36.3 ± 1.3% of beads would be expected to contain a dysfunctional mitochondrial by chance. Quantification of the observed overlap reveals that 92.3 ± 1.1% (mean ± S.E.: 372 beads, 13 neurons) of dendritic beads are associated with dysfunctional mitochondria. This is likely to be an underestimate, as unlabeled, or out of focus mitochondria are not included in the analysis. Thus, dysfunctional mitochondria appear to be preferentially associated with sites of dendritic beading following G/G treatment. Sequence of Events during Oxygen-Glucose Deprivation—Glutamate has been shown to mediate neuronal injury during ischemia. Using oxygen-glucose deprivation (OGD) as an in vitro model for ischemia, glutamate release (20), mitochondrial depolarization, ATP depletion (21), and dendritic beading (22) have been observed. OGD is able to trigger neuronal injury by localized synaptic NMDA receptor activation (23, 24). Therefore, we investigated whether these events exhibited the same spatial and temporal relationship during OGD, as observed during global G/G treatment. To avoid secondary effects due to the generation of action potentials, 0.5 μM tetrodotoxin was included.

As expected, mitochondrial depolarization, mitochondrial collapse, and dendritic beading occurred in response to OGD (Fig. 2; supplemental Movie 2). Again, almost all beads (91.1 ± 1.8% (mean ± S.E.: 256 beads, 8 neurons) contained a dysfunctional (depolarized and collapsed) mitochondrion. Although the spatial correlation between mitochondrial collapse and beading during OGD is identical to that of G/G treatment, the temporal profile was different. The time required for the initi-
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FIGURE 2. Temporal profile of dendritic changes during oxygen-glucose deprivation. Hippocampal neurons (DIV 10–14) were transfected with cDNAs encoding Mito-DsRed and CFP. Neurons were pre-loaded with rhodamine 123 and transferred to artificial cerebrospinal fluid (+0.5 μM tetrodotoxin). Prior to treatment (left panel) neurons exhibit no dendritic beading. Following OGD dendritic beading is evident (right panel). The temporal course for mitochondrial depolarization (filled circles), mitochondrial collapse (filled triangles), and dendritic beading (filled squares) is depicted as a function of distance from the cell soma (y axis) and time (x axis). For further information, see supplementary Movie 2.

The analysis of these events varied between experiments from ~30 min to 6 h of OGD (n = 10). This most likely represents the time required for individual neuronal responses to be induced rather than experimental deviation. This is evident in the variability of individual neurons to undergo mitochondrial depolarization within an individual experiment (rhodamine 123 labeling, supplementary Movie 2). However, once initiated, the events followed a similar temporal order with mitochondrial depolarization (set at t = 0) preceding mitochondrial collapse and the initiation of beading (t = 7–26 and 61–74 min, respectively).

Particularly striking is the observation that mitochondrial depolarization, within an individual neuron, occurs sequentially, and rapidly, traveling from distal dendrites toward the cell body (Fig. 2, supplementary Movie 2). Similarly, both mitochondrial collapse and dendritic beading occur sequentially from distal dendrites toward the cell body. To illustrate this, an analysis of these events from a single neuron at individual beading sites relative to the distance from the cell body reveals both the temporal relationship between mitochondrial depolarization, mitochondrial collapse, and dendritic beading (Fig. 2) and illustrates how these events migrate toward the cell body (n = 5).

Role of Mitochondrial Depolarization and ATP Deposition in Dendritic Beading—Given the localization of dysfunctional mitochondria to sites of dendritic beads, combined with the observation that mitochondrial depolarization precedes the other events during OGD, we postulated that mitochondrial depolarization might be responsible for bead formation. To address this, mitochondria were depolarized by the addition of a protonophore (FCCP, 1 μM). To avoid any unforeseen secondary effect due to glutamate receptor activation, glutamate receptor antagonists (10 μM MK801, 20 μM CNQX) and tetrodotoxin (0.5 μM) were included in these experiments. Given the putative relationship between ATP and dendritic beading (5, 19) (Fig. 1C), we investigated whether beading would occur following mitochondrial depolarization and subsequent ATP depletion. In neurons treated with FCCP, mitochondria depolarized as expected (not shown), but no mitochondrial collapse or dendritic beading was observed (Fig. 3A), despite the rapid drop in cytoplasmic [ATP] (Fig. 3B). Thus, neither mitochondrial depolarization, nor a drop in intracellular [ATP], is sufficient to induce dendritic beading.

However, when mitochondria depolarize in response to G/G, this occurs in the presence of significant sodium and calcium influx. Therefore, we investigated whether both events (mitochondrial depolarization and ion influx) were required to induce dendritic beading. Ion influx was initiated by plasma membrane depolarization using 50 mM KCl and mitochondrial depolarization was induced by oligomycin/FCCP treatment. Oligomycin was included to prevent ATP consumption by mitochondria. Secondary effects due to glutamate release were prevented by the inclusion of tetrodotoxin, MK801, and CNQX. As expected, neither treatment alone induced dendritic beading. However, when combined, extensive dendritic beading is evident (Fig. 4) (n > 5). Thus, dendritic beading may be induced by a combination of mitochondrial depolarization (or the subsequent failure of ATP synthesis) and ion (sodium and/or calcium) influx. Thus, mitochondrial dysfunction renders neurons vulnerable to normally innocuous ionic challenges.

Role of Na+ and Ca2+ Influx in Mitochondrial Depolarization, Collapse, and Dendritic Beading—Glutamate-induced neurotoxicity involves Ca2+ influx (6) and mitochondrial dysfunction (25), whereas dendritic beading has been reported to
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FIGURE 4. Mitochondrial depolarization increases neuronal vulnerability to intracellular ion influx. Hippocampal neurons were transfected with cDNAs encoding CFP. Neurons were treated (30 min) with KCl (50 mM), oligomycin/FCCP (1 μg/ml and 1 μM, respectively), or a combination of KCl and oligomycin/FCCP, as indicated, and imaged to detect dendritic bead formation. Secondary glutamate release was prevented using 0.5 μM tetrodotoxin and the glutamate receptor antagonists, 10 μM MK801, 20 μM CNQX. Scale bar = 30 μM (n = 3).

FIGURE 5. Ionic dependence of dendritic beading and mitochondrial collapse. Hippocampal neurons were transfected with cDNAs encoding MitosRed (MR) and CFP to detect mitochondrial collapse and dendritic beading, respectively. A, Ca2+ (CaCl2) was replaced with equimolar MnCl2, and neurons imaged for dendritic beading (CFP) and mitochondrial collapse (MR) prior (Pre-G/G), or following (20′/G/G) 20 min treatment with 100 μM glutamate, 10 μM glycine. Following this, the medium was replaced with normal HEPES-buffered solution containing Ca2+ and the glutamate/glycine insult continued. B, Na+ (NaCl) was replaced with equimolar N-methyl-D-glucamine and neurons imaged for dendritic beading (CFP) and mitochondrial collapse (MR) prior (Pre-G/G), or following (20′/G/G) 100 mM glutamate, 10 mM glycine treatment. Following this, the medium was replaced with normal HEPES-buffered solution containing Na+ and the glutamate/glycine insult continued. Scale bar = 30 μM (n = 3).

Occur through an independent mechanism(s) that requires Na+ influx (2, 14, 26, 27). To confirm the same dependence exists in hippocampal neurons and to dissect their temporal and spatial contribution to these morphological changes, we performed ion replacement experiments. Na+ or Ca2+ was replaced with equimolar N-methyl-D-glucamine or MnCl2, respectively. Upon the replacement of Ca2+ and exposure to G/G, we observed that dendritic beading and mitochondrial collapse occurred as normal and no further changes in these parameters were evident upon the re-introduction of Ca2+ (Fig. 5A). In keeping with previous observations (2, 14, 26, 27), the removal of Na+ prevented dendritic beading in response to G/G treatment. However, partial mitochondrial collapse occurred (Fig. 5B). Upon the re-introduction of Na+, dendritic beading and full mitochondrial collapse occurred (Fig. 5B).

To determine the ionic dependence for mitochondrial depolarization, identical experiments were performed using rhodamine 123-loaded hippocampal neurons. In the absence of Na+, mitochondrial depolarization appeared to be enhanced (Fig. 6A). In contrast, in the absence of Ca2+, no mitochondrial depolarization occurred until Ca2+ was re-introduced. Importantly, in the absence of Ca2+, polarized mitochondria existed with a collapsed phenotype within dendritic beads, providing firm evidence that mitochondrial depolarization is not a prerequisite for these morphological changes (Fig. 6B). Although mitochondria were still able to depolarize in the absence of Na+, cytoplasmic ATP depletion was prevented. In contrast, the depletion of ATP still occurred in the absence of Ca2+, despite the failure of mitochondria to depolarize (Fig. 6C). Together, these findings suggest that the influx of Na+, not mitochondrial depolarization, appears to be required for cytoplasmic ATP depletion during G/G exposure.

Role of Na+,K+-ATPase in ATP Depletion—The major route for Na+ efflux from neurons is the ATP-dependent plasma membrane Na+,K+-ATPase. Indeed, it has been reported that Na+ efflux by this exchanger may be prevented in metabolically compromised neurons with low [ATP] (26), leading to an accumulation of intracellular Na+. Such a situation is likely to exist during G/G exposure (large Na+ influx and Ca2+-induced mitochondrial depolarization), leading to a high consumption of ATP to maintain Na+ homeostasis. Alternatively, mitochondria might consume ATP in an attempt to maintain their membrane potential. To address these possibilities directly, we investigated the effects of the inhibition of mitochondrial F0F1-ATPase (1 μg/ml oligomycin) or the plasma membrane Na+,K+-ATPase (500 μM ouabain) activity on cytoplasmic ATP levels during G/G exposure. Hippocampal neurons incubated with G/G in the presence of oligomycin revealed that ATP depletion still occurred (Fig. 7). In contrast, incubation...
with ouabain abolished ATP depletion during G/G treatment (Fig. 7). However, G/G + ouabain did not prevent mitochondrial depolarization, collapse, or dendritic beading (not shown) supporting the conclusion that ATP depletion is insufficient to induce these events as sodium influx is required. These findings identify that the plasma membrane Na$^+$,K$^+$-ATPase is responsible for consuming ATP during G/G exposure. This is consistent with the fact that, although depolarized mitochondria can consume ATP (Fig. 3), their depolarization is not a prerequisite for dendritic beading (Fig. 5, Ca$^{2+}$).

Role of Water Influx and Cytoskeletal Disruption in Dendritic Bead Formation—It has been reported previously that sodium influx leads to water influx and so to dendritic beading (2). In support of this, we observed that preventing water influx by the incubation of neurons in hypertonic (200 mM sucrose) medium during G/G exposure prevented both dendritic beading and mitochondrial collapse (Fig. 8A). Thus, it is not Na$^+$ influx that leads to the observed morphological changes in hippocampal dendrites, but subsequent water influx. To explore if water influx is sufficient to induce beading, we shifted hippocampal neurons from isotonic (135 mM NaCl) HEPES medium into hypotonic (67 mM NaCl) conditions. Dendritic beading formed within seconds following this treatment (Fig. 8B). Again, these beads frequently exhibited a hollow region, due to the coincident collapse of mitochondrial structure.

The accumulation of disrupted cytoskeleton within beads has been reported (5), therefore, we investigated cytoskeletal distribution prior to, and following, G/G exposure. Actin staining (phalloidin) exhibits a predominantly clustered distribution that was unaltered following G/G treatment. At sites of dendritic beading, peripheral actin staining was evident around the
beads (Fig. 8C). In contrast, MAP2 staining, a marker for microtubules, is clearly disrupted following G/G treatment, forming small aggregates. In keeping with a role for water influx, hypotonic extracellular medium also leads to microtubule disruption within seconds, as determined by immediate fixation and immunofluorescence using MAP2 antibodies (not shown).

Role of Mitochondria in Dendritic Recovery—Given that mitochondria are present in dendritic beads, they are ideally located to participate in the recovery of dendritic morphology upon the removal of an excitotoxic insult. It has been reported that dendritic beads resolve much more quickly if Ca\(^{2+}\) influx is prevented (2). Given our findings that mitochondria do not depolarize in the absence of Ca\(^{2+}\) (Fig. 6), it is possible that mitochondrial function may play a role in the recovery process.

To explore this possibility, we examined the recovery of dendritic and mitochondrial morphology following G/G exposure. Once the G/G insult was terminated (after beading and mitochondrial collapse had occurred) by the replacement of G/G with glutamate receptor antagonists, 10 μM MK801, 20 μM CNQX, dendritic beads resolve within 1 h. However, mitochondrial collapse does not resolve within the period of bead recovery (Fig. 9A). Likewise, no recovery of cytoplasmic [ATP] is observed (Fig. 9B). Thus, dendritic morphological recovery does not require the recovery of ATP synthesis to re-initiate the plasma membrane Na\(_{\text{K}}\)-ATPase activity and resume Na\(^{+}\) efflux. Obtaining information on mitochondrial repolarization using rhodamine 123 is hampered due to the dissipation of the mitochondrially released dye (supplemental Movie 2) and the lack of quantitative information provided. Using JC-1, we determined that no recovery of mitochondrial membrane potential occurred within the time scale of dendritic recovery (Fig. 9C).

Given that Ca\(^{2+}\) influx leads to mitochondrial depolarization (Figs. 1, 6, and 9C), combined with the detrimental effect of Ca\(^{2+}\) influx on the recovery of dendritic morphology (2), it is possible that mitochondrial membrane potential may influence the recovery process. Although mitochondrial membrane potential does not recover during the period of bead resolution (Fig. 9C), it is possible that mitochondria do not fully depolarize during a glutamate challenge. This is supported by the observation that glutamate induces only a ~68% depolarization, as determined by the subsequent incubation with 1 μM FCCP (Fig. 9D).

We determined to investigate, directly, the effects of mitochondrial membrane potential, mitochondrial ATP production, and Na\(^{+}\),K\(^{+}\)-ATPase activity in the recovery of the dendritic morphology. In keeping with the failure to restore intracellular ATP levels during dendritic recovery, blocking

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**FIGURE 8.** Water influx is both necessary and sufficient for dendritic bead formation and mitochondrial collapse. Hippocampal neurons were transfected with cDNAs encoding CFP and/or Mito-Red. A, neurons were imaged prior to (Pre G/G) and following (Post G/G) exposure to 100 μM glutamate, 10 μM glycine (G/G) for 20 min in the presence of 200 mM sucrose to prevent extracellular water influx. Application of G/G did not induce dendritic beading or mitochondrial structural collapse (right panels). B, neurons were incubated in normal (135 mM NaCl) HEPES buffer (isotonic), followed by replacement in low sodium (68 mM NaCl) HEPES buffer (hypotonic) to induce water influx into neurons. Neurons were imaged for the formation of dendritic beads (CFP) or mitochondrial collapse (Mito-Red). Images are representative of at least 3 independent experiments. C, neurons were imaged prior to (UT) and following (G/G) exposure to 100 μM glutamate, 10 μM glycine for 20 min and stained for actin (phalloidin) or MAP2. Scale bar, 50 μm.
mitochondrial ATP production (1 μg/ml oligomycin) or the inhibition of Na⁺,K⁺-ATPase activity (500 μM ouabain) did not prevent dendritic recovery (Fig. 9E). These results suggest that the resolution of the morphological changes within dendrites is not achieved by a simple reversion of events (Na⁺,K⁺-ATPase activity and ATP production) that induce beading and mitochondrial dysfunction. In contrast, dendritic beads do not recover if mitochondria are completely depolarized by FCCP (Fig. 9E). To ensure that FCCP treatment does not reduce ATP levels beyond that encountered during G/G treatment, oligomycin/FCCP treatment was also tested during the recovery period. Again, dendritic recovery was prevented (not shown).

To address a possible role for plasma membrane depolarization, induced by a nonspecific action of FCCP (28), we examined another mitochondrial uncoupling agent, 2,4-dinitrophenol (1 mM 2,4-dinitrophenol), which does not depolarize the plasma membrane (supplementary Fig. S2). In keeping with a specific role for mitochondrial repolarization, dendritic recovery was prevented (not shown). Moreover, a direct depolarization and dendritic beads, mitochondrial depolarization and the consequential drop in intracellular [ATP] is not sufficient to induce beading.

Dendritic cytoplasm between beads appears to be drawn (or squeezed) into the forming beads (supplementary Movie 3). However, the observation that hypertonic medium prevented beading, whereas hypotonic medium induced beading (Fig. 8) supports the previous conclusions (2, 14, 27) that extracellular water influx is a critical element of the beading process.

In contrast to the simultaneous events occurring during global G/G exposure, when neurons are exposed to OGD conditions a distinct temporal profile is evident, with mitochondrial depolarization preceding mitochondrial collapse, followed by a significant lag (~1 h) before dendritic beading occurs. Interestingly, a transient appearance of filopodial structures is evident prior to beading during OGD (supplementary Movie 2) but not G/G exposure (supplementary Movie 1). It is possible that filopodial outgrowth may reflect the preferential activation of synap-
tic glutamate receptors during OGD as opposed to synaptic and extrasynaptic receptors during G/G exposure (23).

Interestingly, during OGD, this train of events migrates from distal dendrites toward the cell body (supplementary Movie 2). This may be initiated by the formation of discrete foci of glutamate release during OGD, or some intrinsic mechanism governed by “trigger factors,” leading to its initiation at distal sites, independent of the site of insult (16). It seems likely that the spreading of events observed in our study represents anoxic depolarization/spreading depression, which is observed during focal ischemia in brain slices, in which a transient increase in light transmittance (attributed to cell swelling) is followed by a decrease in light transmittance (attributed to dendritic beading) (29).

As glutamate excitotoxicity is clearly Ca\(^{2+}\) dependent (1, 6, 25), whereas dendritic beading is dependent upon Na\(^{+}\) influx (2, 4, 14, 27), we investigated the ionic dependence of mitochondrial function and dendritic beading simultaneously. Influx of Na\(^{+}\) is required for dendritic beading, a drop in intracellular [ATP], and the complete morphological collapse of mitochondria, but not mitochondrial depolarization. In contrast, Ca\(^{2+}\) is only required for mitochondrial depolarization. The observation that mitochondrial depolarization induced by G/G, in the absence of Na\(^{+}\), does not lead to ATP depletion supports the conclusion that mitochondrial depolarization by G/G is only partial and is not responsible for the initial depletion of intracellular ATP. Indeed, it has been reported previously that G/G exposure does not necessarily lead to complete mitochondrial depolarization and ATP consumption (30).

That mitochondrial depolarization, when combined with an innocuous ion influx, is sufficient to induce beading, supports a role for mitochondria in providing ATP to drive normal physiological Na\(^{+}\) efflux. Nevertheless, mitochondrial depolarization is neither sufficient, nor necessary, for beading to occur. Thus, normal mitochondria are unable to cope with the increased demand for ATP under these conditions of Na\(^{+}\) influx. In situations where mitochondrial function is perturbed (excessive Ca\(^{2+}\) influx), neurons may become vulnerable to normally innocuous Na\(^{+}\) influx.

The most likely scenario to explain this phenomena is that water influx perturbs the microtubule cytoskeleton, leading to the concomitant collapse of mitochondrial structure. It is unclear why beads form, rather than generalized swelling of dendrites. It is possible that localized increases in calcium influx (2) may induce localized mitochondrial depolarization, decreases in [ATP] and Na\(^{+}\) efflux. Alternatively, these events may be driven by cytoskeletal depolymerization and/or degradation (30). Such an hypothesis would be consistent with the observation of cytoskeletal components within dendritic beads (2, 5, 31). Although a contribution to dendritic beading of Na\(^{+}\)-dependent protease activity on the cytoskeleton has been reported (19), this was not within the time scale reported here. Previous evidence for cytoskeletal disruption being causative in dendritic beading is difficult to interpret given the long term (6 h) treatment required (19). However, in this study we have shown that water influx induces rapid (within seconds) microtubule (MAP2) and mitochondrial collapse and dendritic beading.

Although our results with OGD indicate that mitochondrial collapse precedes bead formation, mitochondrial and microtubule changes are likely to occur concomitantly. Therefore, the possibility that these collapsed intracellular elements lead to a physical blockage of dendrites and so bead formation (rather than generalized swelling) are not supported by the sequence of events during OGD. Thus, the question of how water influx is limited to bead structures remains unresolved.

Interestingly, the maintenance of residual mitochondrial membrane potential is essential for dendritic recovery to occur. However, neither mitochondrial ATP production, nor Na\(^{+}\), K\(^{+}\)-ATPase dependent efflux of Na\(^{+}\) is required for dendritic recovery. Thus, dendritic recovery does not occur by a passive reversal of the events that induce dendritic beading. The degree of mitochondrial depolarization is dependent on the degree of insult and the amount of Ca\(^{2+}\) influx/mitochondrial sequestration experienced (6). In support of this, Ca\(^{2+}\) is elevated in dendritic beads and beads recover much more quickly in the absence of extracellular Ca\(^{2+}\) (2), conditions in which we have shown that mitochondria do not depolarize. In addition, the inclusion of FCCP during a glutamate exposure (but not present during the subsequent recovery), resulted in a faster recovery rate of mitochondrial structure in cortical neurons (11). Thus, mitochondrial recovery may be related to their level of calcium uptake.

In summary, our findings provide evidence that the very early responses to excitotoxicity (dendritic morphological changes and mitochondrial dysfunction) are connected temporally, spatially, and functionally. Interestingly, dendritic recovery does not occur by a passive reversal of conditions that induce beading. These results are consistent with recovery from dendritic beading occurring as a result of regulatory volume decrease and cytoskeletal remodeling (32). Indeed, Ca\(^{2+}\)-dependent impairment of regulatory volume decrease, by glutamate exposure, has been reported recently. It seems likely that the ability for dendritic recovery is reflected in the degree of mitochondrial depolarization experienced during an excitotoxic insult. Therefore, these two apparently independent events appear to converge at the level of mitochondrial dysfunction.

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REFERENCES
1. Tymianski, M., Charlton, M. P., Carlen, P. L., and Tator, C. H. (1993) J. Neurosci. 13, 2085–2104
2. Hasbani, M. J., Hyrc, K. L., Faddis, B. T., Romano, C., and Goldberg, M. P. (1998) Exp. Neurol. 154, 241–258
3. Swann, J. W., Al-Noori, S., Jiang, M., and Lee, C. L. (2000) Hippocampus 10, 617–625
4. Ikegaya, Y., Kim, J. A., Baba, M., Iwatsubo, T., Nishiyama, N., and Matsuki, N. (2001) J. Cell Sci. 114, 4083–4093
5. Takeuchi, H., Mizuno, T., Zhang, G., Wang, J., Kawanokuchi, J., Kuno, R., and Suzumura, A. (2005) J. Biol. Chem. 280, 10444–10454
6. Ward, M. W., Kushnareva, Y., Greenwood, S., and Connolly, C. N. (2005) J. Neurochem. 92, 1081–1090
7. Hall, G. F., Chu, B., Lee, G., and Yao, J. (2000) J. Cell Sci. 113, 1373–1387
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8. Tsai, J., Grutzendler, J., Duff, K., and Gan, W. B. (2004) Nat. Neurosci. 7, 1181–1183
9. Brendza, R. P., Bacskai, B. J., Cirrito, J. R., Simmons, K. A., Skoch, J. M., Klunk, W. E., Mathis, C. A., Bales, K. R., Paul, S. M., Hyman, B. T., and Holtzman, D. M. (2005) J. Clin. Investig. 115, 428–433
10. Spires, T. L., Meyer-Luehmann, M., Stern, E. A., McLean, P. J., Skoch, J., Nguyen, P. T., Bacskai, B. J., and Hyman, B. T. (2005) J. Neurosci. 25, 7278–7287
11. Rintoul, G. L., Filiano, A. J., Brocard, J. B., Kress, G. J., and Reynolds, I. J. (2003) J. Neurosci. 23, 7881–7888
12. Li, Z., Okamoto, K., Hayashi, Y., and Sheng, M. (2004) Cell 119, 873–887
13. Rintoul, G. L., Bennett, V. J., Papaconstandinou, N. A., and Reynolds, I. J. (2006) J. Neurochem. 97, 800–806
14. Al-Noori, S., and Swann, J. W. (2000) Neuroscience 101, 337–348
15. Nicholls, D. G. (2005) Cell Calcium 38, 311–317
16. Oliva, A. A., Jr., Lam, T. T., and Swann, J. W. (2002) J. Neurosci. 22, 8052–8062
17. Manfredi, G., Yang, L., Gajewski, C. D., and Mattiazzi, M. (2002) Methods 26, 317–326
18. Jiang, M., Deng, L., and Chen, G. (2004) Gene Ther. 11, 1303–1311
19. Ikegami, K., Kato, S., and Koike, T. (2004) Brain Res. 1030, 81–93
20. Fujimoto, S., Katsuaki, H., Kume, T., Kaneko, S., and Akaike, A. (2004) Neurosci. Res. 50, 179–187
21. Almeida, A., Delgado-Esteban, M., Bolanos, J. P., and Medina, J. M. (2002) J. Neurochem. 81, 207–217
22. Zhang, S., Boyd, J., Delaney, K., and Murphy, T. H. (2005) J. Neurosci. 25, 5333–5338
23. Sattler, R., Xiong, Z., Lu, W. Y., MacDonald, J. F., and Tymianski, M. (2000) J. Neurosci. 20, 22–33
24. Hasbani, M. J., Schlief, M. L., Fisher, D. A., and Goldberg, M. P. (2001) J. Neurosci. 21, 2393–2403
25. Sattler, R., and Tymianski, M. (2001) Mol. Neurobiol. 24, 107–129
26. Chinopoulos, C., Trettter, L., Rozsa, A., and Adam-Vizi, V. (2000) J. Neurosci. 20, 2094–2103
27. Beck, I., Lenart, B., Kintner, D. B., and Sun, D. (2003) J. Neurosci. 23, 5061–5068
28. Nicholls, D. G. (2006) J. Biol. Chem. 281, 14864–14874
29. Joshi, I., and Andrew, R. D. (2001) J. Neurophysiol. 85, 414–424
30. Nicholls, D. G., Budd, S. L., Castilho, R. F., and Ward, M. W. (1999) Ann. N. Y. Acad. Sci. 893, 1–12
31. Faddis, B. T., Hasbani, M. J., and Goldberg, M. P. (1997) J. Neurosci. 17, 951–959
32. Loo, L. S., and McNamara, J. O. (2006) J. Neurosci. 26, 10177–10187