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Cleavage of the Plasma Membrane Na^+/Ca^{2+} Exchanger in Excitotoxicity

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Summary

In brain ischemia, gating of postsynaptic glutamate receptors and other membrane channels triggers intracellular Ca^{2+} overload and cell death. In excitotoxic settings, the initial Ca^{2+} influx through glutamate receptors is followed by a second uncontrolled Ca^{2+} increase that leads to neuronal demise. Here we report that the major plasma membrane Ca^{2+} extruding system, the Na^+/Ca^{2+} exchanger (NCX), is cleaved during brain ischemia and in neurons undergoing excitotoxicity. Inhibition of Ca^{2+}-activated proteases (calpains) by overexpressing their endogenous inhibitor protein, calpastatin or the expression of an NCX isoform not cleaved by calpains, prevented Ca^{2+} overload and rescued neurons from excitotoxic death. Conversely, down-regulation of NCX by siRNA compromised neuronal Ca^{2+} handling, transforming the Ca^{2+} transient elicited by non-excitotoxic glutamate concentrations into a lethal Ca^{2+}overload. Thus, proteolytic inactivation of NCX-driven neuronal Ca^{2+} extrusion is responsible for the delayed excitotoxic Ca^{2+} deregulation and neuronal death.

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Introduction

Excitotoxicity (Olney, 1969) plays a central role in neuronal demise during brain ischemia. Prolonged overstimulation of the glutamate N-methyl-D-aspartate (NMDA) receptor subtype leads to Ca^{2+} and Na^+ overload in postsynaptic neurons (Choi, 1988; Lipton and Rosenberg, 1994; Rothman and Olney, 1995). In cultured neurons, and also in stroke models, the initial Ca^{2+} influx triggered by glutamate through NMDA-Rs is followed by a delayed massive Ca^{2+} accumulation, which invariably results in cell death (Budd and Nicholls, 1996; Rothman et al., 1987). The amplitude of the initial Ca^{2+} increase mediated by glutamate is within the range of nonlethal stimulations, whereas the delayed intracellular Ca^{2+} rise is sustained and apparently beyond rectification, suggesting that additional mechanisms are involved to terminally deregulate cellular Ca^{2+} handling. The mechanisms responsible for the delayed Ca^{2+} increase that precedes neuronal demise remain unclear.

Ca^{2+} inflow through voltage-dependent or independent channels can lead to neuronal Ca^{2+} overload under excitotoxic (Fryer et al., 1999), anoxic (Aarts et al., 2003), or ischemic (Xiong et al., 2004) conditions. In addition, mitochondria Ca^{2+} accumulation and its subsequent release may play an important role in maintaining a Ca^{2+} overload (Schinder et al. 1996). It is also well established that mitochondrial dysfunction is involved in excitotoxic demise (Ankarcrona et al., 1995; Reynolds, 1999; Ward et al., 2000).

Nevertheless, the combination of increased Ca^{2+} influx into neurons and mitochondrial Ca^{2+} release may not fully account for the irreversible buildup of intracellular Ca^{2+} after excitotoxic stimulation. Conceivably, the delayed increase in cellular Ca^{2+} should be rectified by the mechanisms operating cellular Ca^{2+} extrusion. In neurons, Ca^{2+} extrusion is operated by the plasma membrane Ca^{2+} pump (PMCA) and by Na^+/Ca^{2+} exchangers (NCX). PMCA has high Ca^{2+} affinity but low transport capacity, whereas NCX has a low affinity, but a higher capacity to transport Ca^{2+} (Carafoli et al., 2001). Inhibition of Ca^{2+} efflux from cells is sufficient to cause a sustained intracellular Ca^{2+} elevation and the demise of nonneuronal cells by activating Ca^{2+}-dependent hydrolytic enzymes including members of the calpain protease family (Nicotera et al., 1986).

Calpains (Mellgren et al., 1989; Murachi et al., 1987) modulate a variety of physiological processes (Robles et al., 2003) but can also become important mediators of cell death (Neumar et al., 2003). Ample evidence docu-

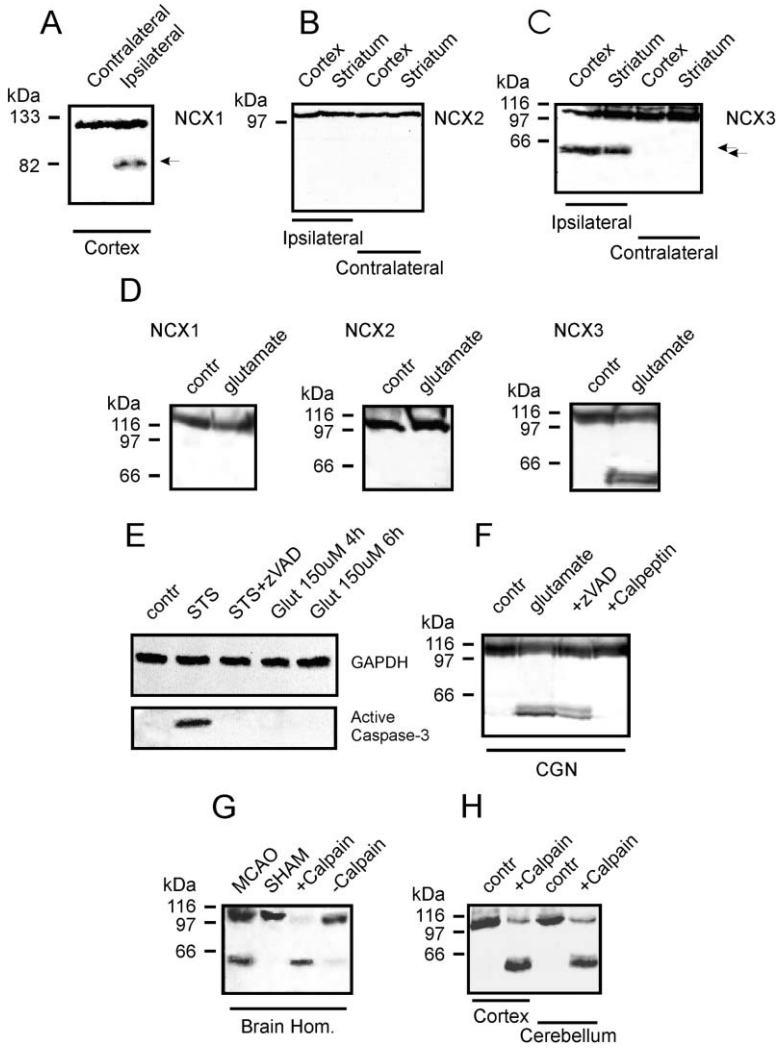

Figure 1. Cleavage of NCX Isoforms

Focal brain ischemia was induced in neonatal P7 Wistar rats, and brains were removed, homogenized, and treated as described in the Experimental Procedures. Extracts were loaded on SDS-PAGE: the blots were incubated with antibodies for NCX1 (A), NCX2 (B), and NCX3 (C). NCX3 was cleaved in two fragments of about 58–60 kDa, whereas NCX1 was only partially degraded and NCX2 was not cleaved.

(D) Cerebellar granule neurons (grown in BME 25 mM KCl, in the presence or absence of 100 nM FK506) were incubated for 2.5 hr with 100 μM glutamate. Cells were harvested, the membrane fraction was loaded on SDS-PAGE, and the nitrocellulose filters were immunoblotted with the antibodies specific for the three NCX isoforms. Only NCX3 degradation was apparent, again generating a doublet of about 58–60 kDa.

(E) CGNs were treated either with 1 μM STS (in presence or absence of 100 μM Z-VAD-fmk) for 18 hr or with glutamate (150 μM for 4–6 hr). Cell extracts were separated on SDS-PAGE and immunoblotted with antibody against the active caspase-3 (GAPDH as loading control).

(F) CGNs were preincubated with 100 μM z-VAD-fmk or 5 μM calpeptin and subsequently exposed to 100 μM glutamate for 2.5 hr. NCX breakdown products were detected in extracts separated on SDS-PAGE and analyzed by Western blotting.

(G and H) Whole-brain homogenates (G), cortex, or cerebellar homogenates (30 μg protein) (H) were incubated at 37°C for 15 min with 0.5 μg calpain m or μ (shown are the experiments with μ calpain); the same amounts of brain homogenate from Sham-treated and MCAO were loaded for comparison.

ments the activation of calpains in brain ischemia and excitotoxic neuronal degeneration (Lankiewicz et al., 2000; Leist et al., 1998; Siman and Noszek, 1988). However, the nature of the calpain substrates during ischemic injury is largely unknown as are the mechanisms by which they mediate neuronal demise.

Here, we present evidence that the plasma membrane Na^+/Ca^{2+} exchanger (NCX) (Philipson and Nicoll, 2000) is cleaved in brain ischemia and in cultured cerebellar granule neurons (CGNs) exposed to glutamate. In particular, we show that proteolysis of NCX isoform 3 (Nicoll et al., 1996) by calpains plays a prominent role in the delayed, irreversible excitotoxic Ca^{2+} elevation leading to neuronal demise.

Results

Calpains Cleave NCXs During Brain Ischemia and in Neurons Exposed to Excitotoxins

Brain ischemia was induced by middle cerebral artery occlusion (MCAO), and samples were collected from cortical and striatal regions after 24 hr. The exchanger isoforms NCX1 and NCX3 were cleaved in the cortex and NXC3 also in the striatum, whereas NCX2 was not degraded (Figures 1A–1C). NCX3 was cleaved to a doublet of approximately 58–60 kDa, whereas NCX1 was degraded to an 82 kDa fragment. Excitotoxic stimulation of neuronal cultures (i.e., treatment of cultured CGNs with glutamate) did not cause degradation of NCX1, whereas NCX3 was processed to two products of about 58–60 kDa (Figure 1D). To visualize NCX2 in cultured CGNs, it was necessary to block its rapid downregulation by a calcineurin-mediated process (Li et al., 2000) using the calcineurin inhibitor FK506. Under these conditions, NCX2 did not undergo proteolytic cleavage (Figure 1D). In contrast, NCX3 was cleaved as in control cultures (data not shown).

We then decided to characterize the cleavage of NCX isoform 3. CGN can undergo both necrosis and apoptosis (Bonfoco et al., 1995; Ankarcrona et al., 1995). We selected conditions that would cause mostly nonapoptotic cell death. To test whether caspases were at all active in these conditions, we examined processing of caspase-3 to the p17-20 fragment, which forms an essential part of the activated enzyme. Figure 1E shows that caspase-3 was processed when CGNs were exposed to staurosporine (STS) but not when stimulated by glutamate.

The broad-spectrum caspase inhibitor, z-VAD-fmk, moderately reduced NCX3 cleavage in glutamate-treated

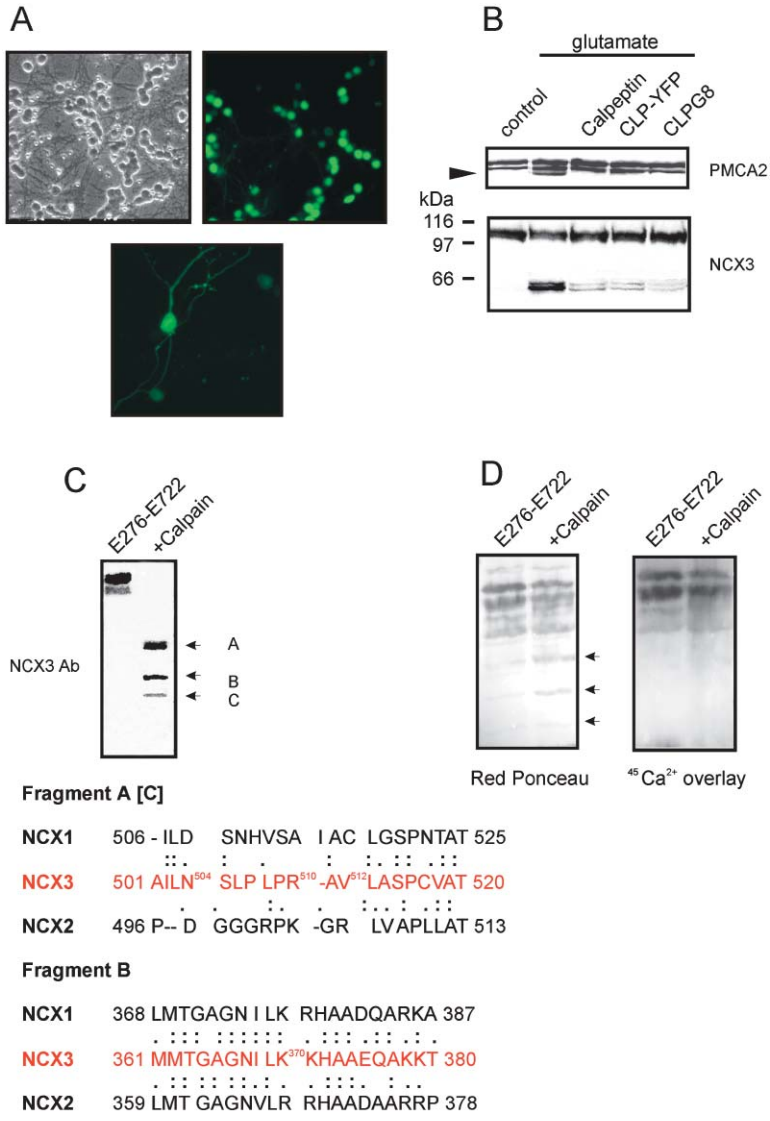

Figure 2. NCX3 Degradation by Calpains

(A and B) Overexpression of calpastatin prevents NCX3 degradation.

(A) The chimera CLP-YFP was transduced in CGNs as described in the Experimental Procedures. At the seventh DIV, between 80% and 90% cells were positive for YFP fluorescence. The chimera localized in the soma and in the projections.

(B) Wild-type and transduced CGNs were treated with glutamate as in Figure1. Neurons were collected, and membrane proteins were separated on 7.5% SDS-PAGE and immunoblotted with the NCX3 antibody. Both the chimera and full-length calpastatin (CLPG8) were able to prevent the PMCA2 and the NCX3 degradation.

(C and D) The calpain cleavage site on NCX3.

(C) The peptide corresponding to the intracellular loop of NCX3 was purified and incubated with calpains as described in Experimental Procedures. The degradation products were N-terminally sequenced and the cleavage site(s) of calpains was determined. As reported in the inset, calpain cleaved NCX 3 at positions 504, 510, 512 and at position 370. The corresponding regions of the other two NCX isoforms were aligned for comparison.

(D) Red-Ponceau staining (left panel) of *E. coli* lysate containing the fusion protein E276-E722 and the relative $^{45}Ca^{2+}$ overlay on the lysate (right panel) are presented. The fragments produced by calpains did not bind Ca^{2+}. About ten times more protein than that used in the blot was loaded onto this gel.

CGNs, in line with the observation that methyl ketone protease inhibitors of caspases can also partially inhibit other protease families (Waterhouse et al., 1998) including calpains (data not shown). In contrast, calpeptin, which inhibits calpains with high selectivity (Lee et al., 2000), fully prevented NCX3 fragmentation (Figure 1F). The pattern of NCX3 degradation after cerebral ischemia was then compared to that obtained in whole-brain, cortical, and cerebellar homogenates incubated with recombinant calpains (Figures 1G and 1H). In all cases, NCX3 was processed to products of the same size as found after MCAO.

Calpastatin Overexpression Inhibits NCX3 Cleavage

With the exception of the latest generation of synthetic caspase inhibitors (Han et al., 2002; Hotchkiss et al., 2000; Schwab et al., 2002), many protease inhibitors have overlapping specificities. Therefore, we decided to selectively neutralize calpains by modulating the expression of their natural protein inhibitor calpastatin. A chimera between the calpastatin domain, 1–251, which contains the calpain inhibitory sequence TIPPKYR (Emori et al., 1988), and the yellow fluorescent protein (EYFP) was generated and cloned into a lentiviral transfer vector to transduce CGNs. The chimera localized to both the cell soma and the neurites of CGNs (Figure 2A). As controls, we utilized cells overexpressing either full-length calpastatin (CLPG8) or GFP alone. The overexpression of either calpastatin-EYFP (CLP-YFP) or full-length calpastatin in CGNs prevented the cleavage of NCX3 (Figure 2B) and also the cleavage of another calpain substrate chosen as control, the PMCA isoform 2 (Guerini et al., 2003). Additional experiments to confirm the selectivity of CLP-YFP for calpains are shown in Supplemental Figure S1 at http://www.cell.com/cgi/content/full/120/2/275/DC1.

The Calpain Cleavage Site on NCX3

The structural features of substrate recognition by calpains are not yet fully understood, and sequence specificity is but one requirement. Based on our previous

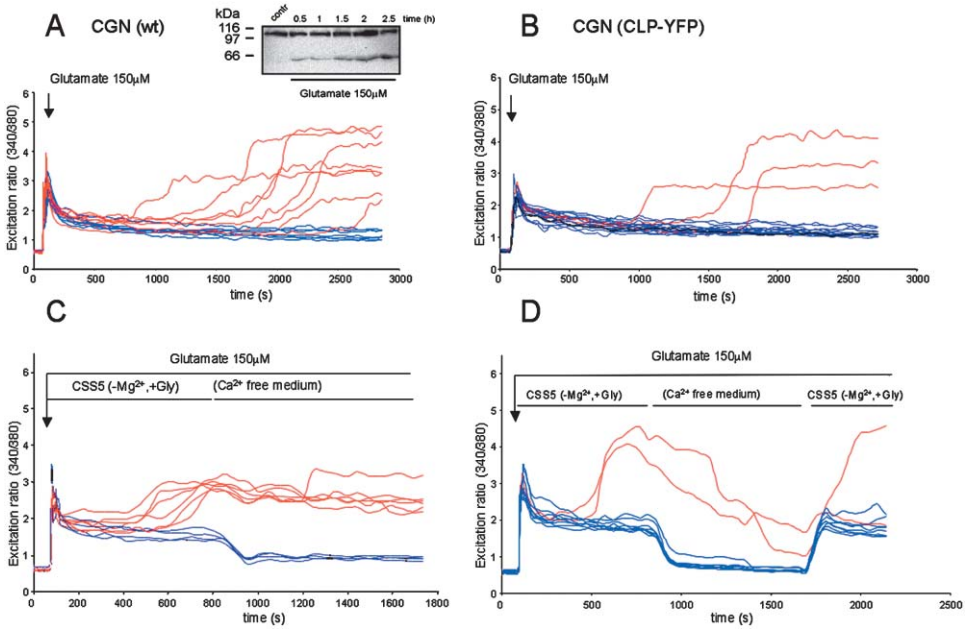

Figure 3. Calpastatin Prevents the Ca^{2+} Deregulation Induced by Glutamate

CGNs loaded with 2 μM Fura-2-AM were perfused with CSS5 buffer, supplemented with 10 μM glycine (Gly) and without $MgCl_2$. At 50 s the cells were exposed to 150 μM glutamate, which was then present for the entire duration of the experiment. Frames were collected every 45 s. The Ca^{2+} response of 15 representative cells is shown: approximately 50% of wild-type neurons underwent a delayed Ca^{2+} elevation (shown by the red traces) (A), whereas the secondary Ca^{2+} deregulation did not take place in most neurons expressing the chimera (B). The magnitude and timing of the initial Ca^{2+} spike caused by glutamate in the two neuronal populations was comparable (nine separate experiments; 450 cells). CGNs overexpressing GFP alone showed similar responses as wild-type neurons (data not shown).

(C and D) Granule neurons (control, [C]; overexpressing CLP-YFP [D]) were stimulated with 150 μM glutamate in CSS5 (without $MgCl_2$ and supplemented with 10 μM glycine). After approximately 750 s, the cells were perfused with a Ca^{2+}-free buffer (in the continued presence of glutamate). Where indicated, cells were then returned to the Ca^{2+}-containing buffer. The insert shows the time course of NCX3 degradation in cells exposed to 150 μM glutamate.

experiments, it was unlikely that calpain cleavage occurred within the two transmembrane regions of NCX3 that flank the large cytosolic loop, as this would have resulted in a very bulky fragment. Since the antibody we used was directed against the large intracellular loop and the size of the fragment was around 58–60 kDa (compatible with a cleavage within this region), we decided to express this loop in *E. coli* and to subject it to in vitro proteolysis with recombinant calpains. The cleavage products were analyzed by N-terminal sequencing. Cleavage of the cytoplasmic loop yielded three products (Figure 2C). Fragment A (top) was a doublet containing equal amounts of the sequences SLPLP/AVLAS/LASP, which correspond to region 501-AILN^{504}-SLPLPR^{510}-AV^{512}-LASPCVAT-520 in NCX3. Fragment B contained a KHAAEQA, sequence, which corresponds to the sequence 361-MMTGAGNILK^{370}-KHAAEQAKKT-380 on the whole NCX3 protein. Fragment C was a shortened version of fragment A as it contained the same SLPL sequence, which is only found in the 501–520 region of NCX3. Of these regions, the sequence AILN^{504}-SLPLPR^{510}-AV^{512}-LASPCVAT-520 has high homology with the recently identified recognition sequence for calpain cleavage (Tompa et al., 2004). A cleavage in this position is consistent with the generation of a 56 kDa fragment similar to the 58–60 kDa NCX3 fragments generated in vivo and in vitro by calpains. As shown in Figure 2C (bottom), there was no significant homology between NCX1, NCX2, and NCX3 in this region. Interestingly, this calpain cleavage site is in close proximity to the high-affinity Ca^{2+} binding regulatory domain, which is essential to modulate NCX transport activity (Levitsky et al., 1994; Matsuoka et al., 1995). In order to test whether cleavage of NCX3 would affect the Ca^{2+} regulatory domain, we performed a $^{45}Ca^{2+}$ overlay experiment on lysates from *E. coli* expressing the exchanger loop (fusion protein E276-E722). The experiment showed that the fragments generated by calpains did not bind Ca^{2+} (Figure 2D).

Notably, fragment B of NCX3 contained a putative calpain recognition sequence and was cleaved at position K^{370}. In this region, the three NCX isoforms share similar sequences. Because neither NCX1 nor NCX2 were cleaved in CGNs exposed to glutamate, it is most likely that this potential cleavage site is not accessible to calpains in vivo.

Calpastatin Prevents the Secondary Ca^{2+} Overload in Excitoxicity

To test whether the calpains were involved in the delayed Ca^{2+} increase, $[Ca^{2+}]_i$ was measured in wild-type and in calpastatin-overexpressing CGNs exposed to glutamate. Following the addition of glutamate, $[Ca^{2+}]_i$ increased rapidly and recovered only partially in virtually

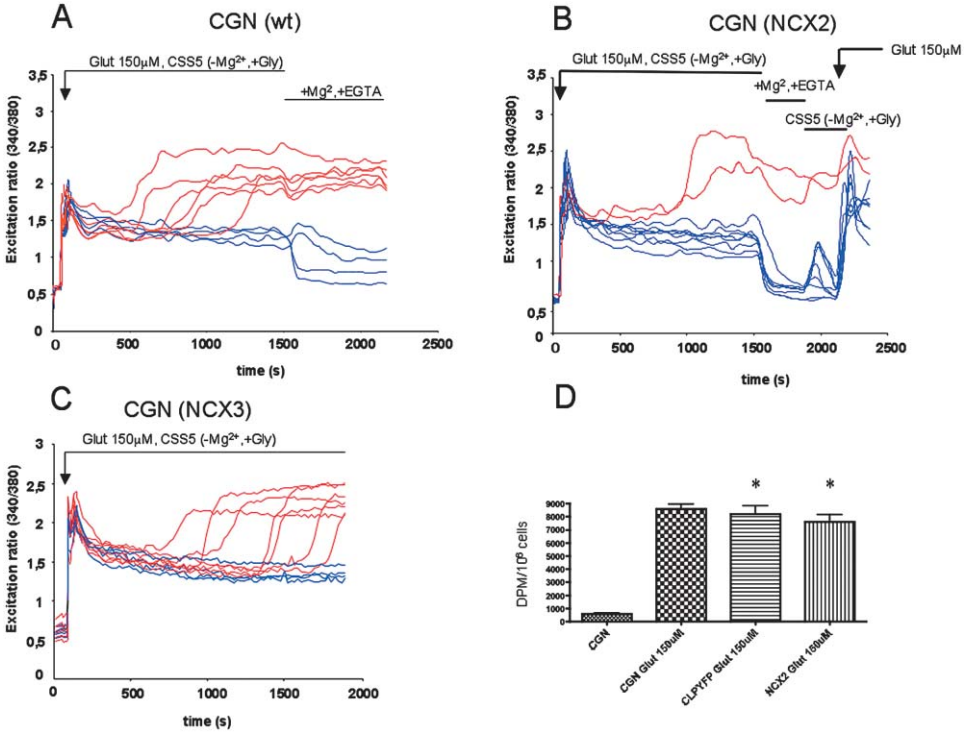

Figure 4. Overexpression of NCX2 Abolishes the Delayed Ca^{2+} Overload

CGNs wild-type (A) and CGNs overexpressing NCX2 (B) and NCX3 (C) were loaded with 2 μM Fura-2-AM. The cells were stimulated with 150 μM of glutamate in CSS5 buffer (supplemented with 10 μM glycine and without $MgCl_2$). Where indicated, the neurons were incubated in a Ca^{2+}-free buffer or in a buffer containing Mg^{2+} (to block Ca^{2+} influx through the NMDA-R). In (D) are shown the amounts of $^{45}Ca^{2+}$ taken up by wild-type or transduced neurons. The results are the mean ± SD from four separate experiments.

all wild-type neurons (traces in Figure 3A show a typical experiment). A secondary rapid Ca^{2+} rise started around 15 min after stimulation (red traces) in the majority of neurons. In CGN overexpressing calpastatin-YFP, the magnitude and duration of the first Ca^{2+} increase was virtually identical to that of wild-type neurons (Figure 3B). However, the number of neurons that underwent secondary Ca^{2+} overload was markedly reduced (neurons with secondary rise were: wild-type = 57% ± 10%, CLP-YFP = 21% ± 3%; n = 8; 400 cells). To establish whether an additional, inward-directed Ca^{2+} influx could account for the delayed $[Ca^{2+}]_i$ increase, wild-type neurons were stimulated with glutamate as indicated above and then bathed in a Ca^{2+}-free medium. Since glutamate was present throughout the entire experiment, neurons were gated to Ca^{2+} at all times. As illustrated in Figure 3C, the removal of Ca^{2+} from the bathing solution did not eliminate the secondary Ca^{2+} overshoot, whereas in the majority of neurons overexpressing CLP-YFP, the secondary Ca^{2+} overshoot did not take place (Figure 3D). Here, $[Ca^{2+}]_i$ spontaneously recovered to near-resting concentrations, and removal of external Ca^{2+} only facilitated the complete restoration of the resting $[Ca^{2+}]_i$. Overall, these findings demonstrate that calpains are involved in the generation of the secondary Ca^{2+} deregulation in excitotoxic conditions by interfering with Ca^{2+} influx/efflux pathways. Notably, NCX3 cleavage was marginally detectable 15 min after glutamate addition (not shown), becoming more evident at 30 min (Figure 3, inset), which coincided with the timing when neurons experienced the secondary sustained Ca^{2+} increase.

Expression of NCX2 Prevents the Delayed Ca^{2+} Overload

If NCX3 proteolysis by calpains was required for the delayed Ca^{2+} overload, we would predict that replacement of NCX3 with the NCX isoform 2, which was not cleaved by calpains in CGNs exposed to glutamate, should rectify the delayed Ca^{2+} increase. CGNs were transduced with NCX2 and then exposed to glutamate. The initial transient Ca^{2+} rise following glutamate addition remained unchanged (arbitrary fluorescence values in wild-type neurons = 1.9 ± 0.3; in NCX2 overexpressing neurons = 2.1 ± 0.2; n = 6; 180 cells); however, the number of neurons that underwent secondary calcium deregulation decreased markedly (compare Figures 4A and 4B). Only 12% ± 6% of NCX2 overexpressing neurons as opposed to 50% ± 3% of wild-type neurons underwent delayed Ca^{2+} elevation. In contrast, overexpression of NCX3 did not protect against the secondary Ca^{2+} overload (Figure 4C). Expression levels for NCX2 and NCX3 were comparable (as determined by semiquantitative RT-PCR). The protection from the second Ca^{2+} overshoot was not due to the inactivation of the NMDA-R, as the NCX2 overexpressing neurons were still able to respond to a second glutamate addition after washout (Figure 4B). Moreover, the overall amounts of Ca^{2+} accumulated in the early stages of glutamate stim-

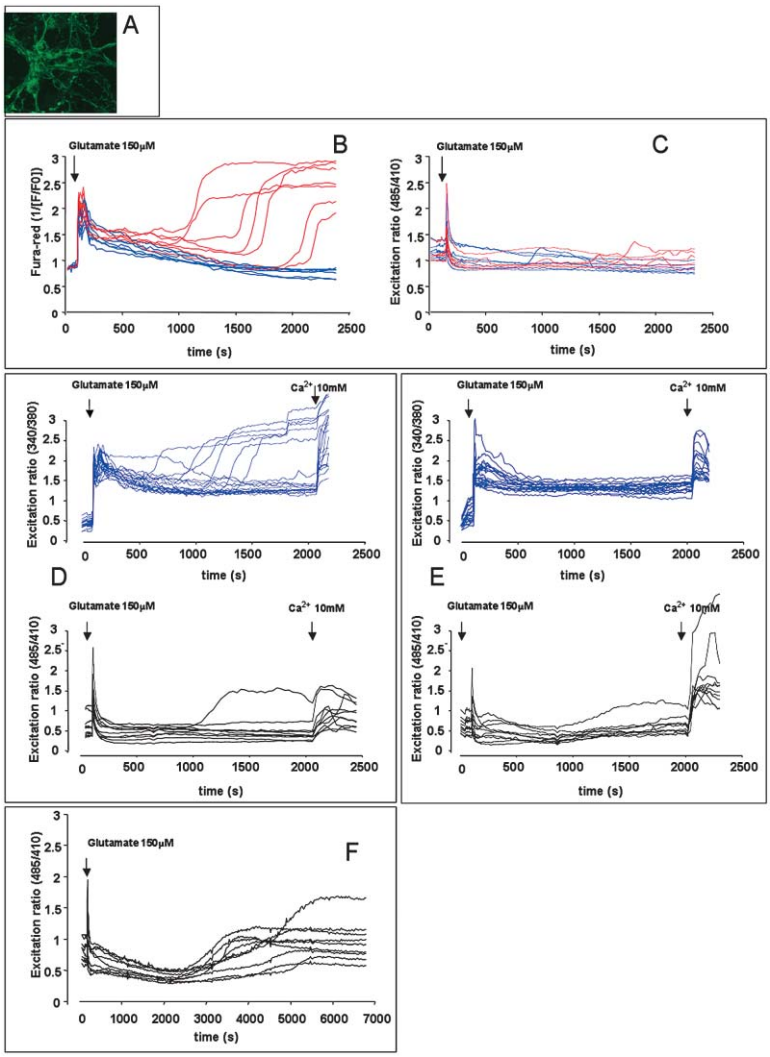

Figure 5. Intramitochondrial Ca^{2+} and Excitotoxic Ca^{2+} Overload

CGNs were transduced with ratio-pericam at 1 DIV and imaged at 7 DIV.

(A) Confocal image of ratio-pericam demonstrates the probe localized to the mitochondrial compartment.

(B and C) Dual imaging of cytosolic Ca^{2+} (with Fura-Red, [B]) and mitochondrial Ca^{2+} (ratio-pericam, [C]). Addition of glutamate caused a rapid increase in both cytosolic and intramitochondria Ca^{2+}. In neurons that underwent secondary Ca^{2+} overload (red traces), no simultaneous changes in mitochondrial Ca^{2+} were observed.

(D and E) Measurement of cytosolic Ca^{2+} (top traces) and mitochondrial Ca^{2+} (lower traces) were performed separately in control cultures (D) or cultures transduced with NCX2 (E). NCX2 expression did not alter the profile of intramitochondrial Ca^{2+} changes, despite preventing the secondary increase in cytosolic Ca^{2+}. Addition of 10 mM $CaCl_2$ to the incubation medium resulted in a further increase in cytosolic and intramitochondrial Ca^{2+}, showing that mitochondria were still capable of accumulating Ca^{2+}.

(F) Longer term recording of intramitochondrial Ca^{2+} shows late mitochondria Ca^{2+} uptake, at time points which coincide with neuronal cell death.

ulation (i.e., before the secondary Ca^{2+} overload) was the same in wild-type and in CLP-YFP or NCX2 transduced neurons, as determined by $^{45}Ca^{2+}$ measurements (Figure 4D).

Secondary Ca^{2+} Overload and Mitochondrial Function

Many studies have shown that the mitochondrial membrane potential inevitably collapses and mitochondria break down at some point during excitotoxicity and brain ischemia. However, it is not known whether the secondary Ca^{2+} overload is triggered by a failure of mitochondria to control their Ca^{2+} levels or whether the secondary Ca^{2+} rise is upstream of mitochondrial damage. We measured Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi$) simultaneously (Supplemental Figures S2A and S2B on the *Cell* website) in individual wild-type neurons. In line with other findings (Budd and Nicholls, 1996) $\Delta\Psi$ was initially retained during the early Ca^{2+} increase but then progressively declined in all neurons. However, although the loss of $\Delta\Psi$ occurred in some neurons synchronously with the secondary $[Ca^{2+}]_i$ increase, in most neurons the two events did not correlate. Also, $\Delta\Psi$ declined with the same kinetics in both wild-type and CLP-YFP-expressing neurons (Supplemental Figures S2C and S2D on the *Cell* website). To investigate directly whether mitochondrial Ca^{2+} discharge could initiate the secondary Ca^{2+} deregulation, CGNs were transduced with the mitochondrial GFP-based Ca^{2+} probe, ratio-pericam (Figure 5A; Nagai et al., 2001). As shown in Figures 5B and 5C, the initial Ca^{2+} increase in the cytosol (as measured by Fura-Red) was mirrored by a simultaneous Ca^{2+} increase in the mitochondria. However, intramitochondrial Ca^{2+} was rapidly rectified back to prestimulation levels despite the continued presence of increased levels of cytosolic Ca^{2+}. Thereafter, while many neurons underwent the secondary Ca^{2+} deregulation, intramitochondrial Ca^{2+} levels remained unchanged (Figures 5B and 5C, red traces). In neurons transduced with either NCX2 (Figure 5E) or full-length calpastatin (data not shown), mitochondrial Ca^{2+} changes were similar to those observed in wild-type neurons (Figure 5D). However, in NCX2- or calpastatin-transduced CGNs, the secondary Ca^{2+} overload did not take place. Together,

these data demonstrate that the secondary Ca^{2+} overload was not caused by sudden discharge of mitochondrial Ca^{2+}. To determine whether mitochondria were still capable of sequestering Ca^{2+} after the sustained secondary Ca^{2+} overload, Ca^{2+} recordings were made at later time points. In such experiments, intramitochondrial Ca^{2+} in individual neurons began to increase around 45 min after glutamate addition, after which neuronal death was commonly observed (Figure 5F).

Calpastatin or NCX2 Overexpression Protects Neurons from Excitotoxic Death

To test whether calpains and NCX cleavage were linked to neuronal death, cell viability was determined in neurons transduced with GFP, CLP-YFP, NCX3, or NCX2. The majority of neurons underwent cell lysis as determined by the loss of calcein staining starting 2.5 hr after glutamate addition (Figure 6A). The caspase inhibitor, Ac-DEVD-CHO (or other caspase inhibitors; data not shown) did not prevent cell death. In contrast, neurons transduced with calpastatin (CLP-YFP) were significantly protected (Figures 6B–6D). Similarly, the MTT test showed that neurons overexpressing NCX2, but not NCX3, were protected from excitotoxic cell death (Figure 6E).

siRNA Suppression of NCX3 Sensitizes CGNs to Ca^{2+} Overload and Excitotoxicity

Finally, to directly examine whether NCX3 inactivation was sufficient to elicit a Ca^{2+} overload upon glutamate stimulation, we used RNA interference (RNAi) to suppress NCX3 expression. Transfection of small interfering duplexes into CGNs was optimized using a labeled, scrambled siRNA. Neurons transfected with these reference siRNAs had a normal morphology and viability. We then used three different hairpins based on a synthetic 21-nucleotide siRNA duplex against NCX3 (N_1–N_3). Each of the three hairpins inhibited NCX3 expression but did not modify expression of NCX1 or other plasma membrane Ca^{2+} transporters such as the PMCA2 and NMDA-R1 (Figure 7A). NCX3 was differentially downregulated by the three hairpins: N_1 was the most effective and fully downregulated NCX3, whereas N_2 and N_3 reduced NCX3 to 40% and 50% of the level measured in control neurons (Figure 7A).

Notably, downregulation of NCX3 increased the rate of spontaneous cell death in CGN cultures at day 8 after plating (see legend to Figure 7B), which suggests that NCX3 expression is a relevant component of the CGN differentiation program. The rate of spontaneous cell death was measured by counting neurons with condensed nuclei and correlated with the extent of NCX3 downregulation (see legend to Figure 7B).

When neurons were stimulated by subexcitotoxic glutamate concentrations (25 μM), control cells recovered near resting calcium levels, and none underwent secondary Ca^{2+} deregulation (Figure 7C). In contrast, in neurons downregulating NCX3, after an initial partial recovery, Ca^{2+} increased irreversibly and remained well above resting levels. In NCX3 downregulated neurons, a further rise in Ca^{2+} occurred within 10 min: in N_1 neurons, the Ca^{2+} deregulation was most rapid and effective (after 800 s, 61% of the N_1 neurons versus 49%

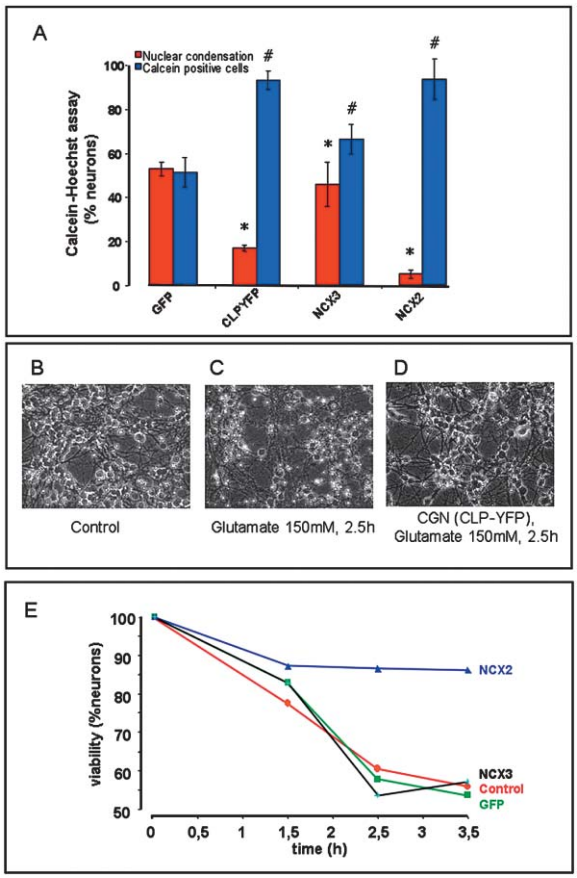

Figure 6. Calpastatin or NCX2 Overexpression Reduced Excitotoxic Neuronal Death

(A) CGNs overexpressing GFP, CLP-YFP chimera, NCX3, and NCX2 were incubated with 150 μM glutamate for 2.5 hr; cell death was determined by staining the neurons with 0.5 μM calcein and 0.5 μg/ml Hoechst-33342. Results are the mean ± SD from 4 separate experiments. Significant difference from control (GFP) at $p < 0.01$ using ANOVA and Bonferroni multiple comparison test (* and #, respectively, for nuclear condensation and calcein staining).

(B–D) Morphology of wild-type CGNs and CGNs overexpressing CLP-YFP and exposed to excitotoxic conditions as indicated.

(E) Wild-type CGNs (circles) and CGNs overexpressing GFP (squares), NCX3 (crosses), or NCX2 (triangles) were incubated with 150 μM glutamate for the time indicated. Viability was measured by the MTT test.

with N_2 and 37% with N_3 had elevated Ca^{2+}), which suggests that the level of NCX3 expression correlates with the ability of CGN to properly rectify intracellular Ca^{2+} elevations. As observed with excitotoxic glutamate concentrations, virtually all neurons with a Ca^{2+} overload then rapidly died (data not shown). Then, we stimulated control neurons and N_1 neurons with excitotoxic glutamate concentrations (150 μM). In the majority of control neurons, the secondary Ca^{2+} deregulation occurred after 15 min (Figure 7B), whereas in most N_1 neurons (70% in the experiment shown in Figure 7D) Ca^{2+} overload was evident 7–8 min after glutamate. Finally, we reasoned that if NCX3 were the most relevant/critical substrate to cause the secondary Ca^{2+} deregulation, CLP-YFP would not modify the secondary Ca^{2+} overload observed in NCX3 downregulated neurons. To this end,

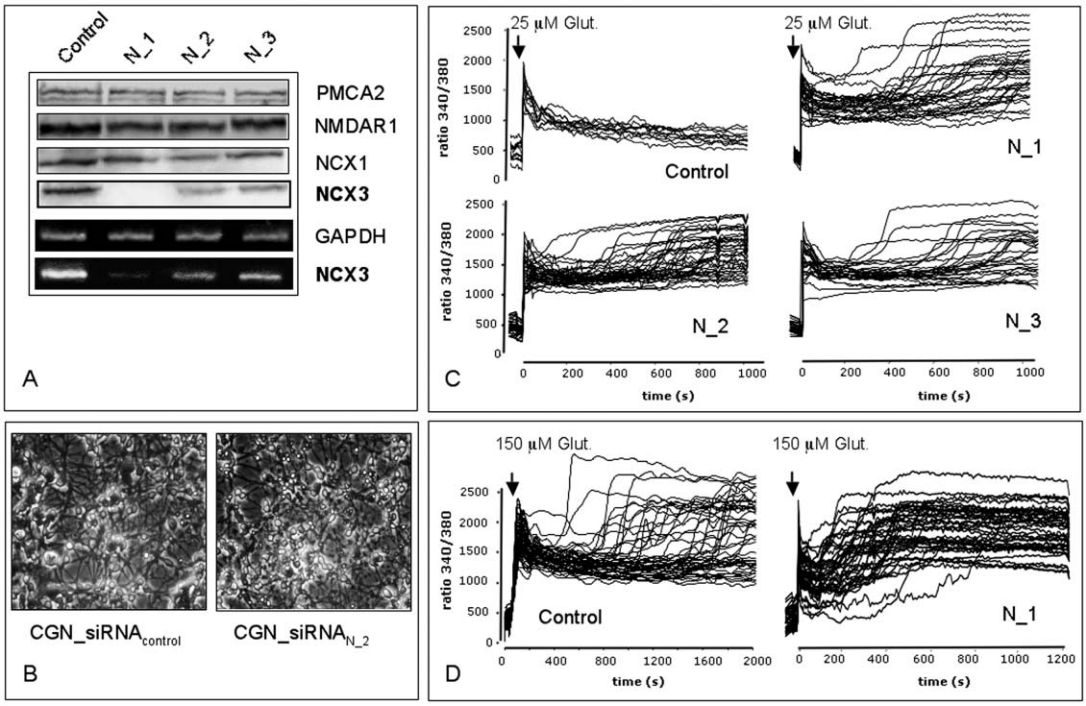

Figure 7. siRNA Inhibition of NCX3 in CGNs

CGNs were transfected either with nonsilencing oligonucleotides (control or siRNA$_{control}$) or with specific siRNA hairpins against the NCX3 (N_1, N_2, N_3). To determine transfection efficiency, we used the Alexa Fluor 488 labeled scrambled duplex. At day 8 when all experiments were performed, 80% of the neurons were positively labeled.

(A) Membrane proteins were loaded on SDS-PAGE and the nitrocellulose filter incubated with the specific antibodies against PMCA2, NMDA-R1, NCX1, and NCX3. As shown in the panel, the three hairpins inhibited NCX3 expression to a different extent: N_1 fully downregulated NCX3, whereas N_2 and N_3, respectively, decreased NCX3 to 40% and 50% of the level found in control neurons (levels were determined by densitometry analysis of the bands on Western blots). The expression of NCX1, PMCA2, or the NMDA-R1 was unchanged. Semiquantitative RT-PCR gave similar results.

(B) Transfection with the different hairpins caused an increase in the spontaneous death rate in CGN at day 8 after plating. The phase contrast images show neurons with condensed, highly refractive nuclei in the cultures transfected with the silencing hairpins (CGN transfected with scrambled hairpins, CGNsiRNA$_{control}$ and transfected with N_2, CGN_siRNA$_{N_2}$). Cell death was quantified by scoring the percentage of neurons with condensed nuclei. It was negligible in control cultures (i.e., $<$2% of the whole population), whereas it correlated with the extent of NCX3 downregulation: in N_1-transfected cultures 31% $\pm$ 5%, whereas in N_2- and N_1-transfected cultures the cell death score was 19% $\pm$ 2% and 12% $\pm$ 3%, respectively (n = 9).

(C) Ca^{2+} imaging of CGNs transfected with nonsilencing duplexes (control) or with each of the three hairpins targeting NCX3 (N_1–N_3). A nonexcitotoxic glutamate concentration (25 μM) evoked a Ca^{2+} elevation in control neurons without the secondary Ca^{2+} overload. Conversely, in siRNA-transfected CGNs, virtually all neurons did not recover resting Ca^{2+} levels and eventually underwent secondary Ca^{2+} deregulation. Approximately 61% of N_1, 49% of N_2, and 37% of N_3 neurons had elevated Ca^{2+} by 7–8 min. The Ca^{2+} responses of ten individual control neurons and 30–50 (for each hairpin) siRNA transfected neurons were examined. The experiments were repeated independently for all the three different hairpins against NCX3 (n = 9).

(D) Excitotoxic glutamate concentrations (150 μM) stimulated a secondary Ca^{2+} deregulation in the majority of control neurons after 15 min. In N_1 cultures, Ca^{2+} overload occurred in the majority of the population after 7–8 min.

we transfected CLP-YFP-transduced neurons with siRNA as described in the Experimental Procedures. Glutamate (150 μM) elicited a Ca^{2+} overload in 70 $\pm$ 5 siRNA neurons, and in 64 $\pm$ 7 CLP-YFP/siRNA neurons (mean $\pm$ SD; n = 3), which confirms that NCX3 is the most relevant calpain substrate for the secondary Ca^{2+} overload.

Discussion

When neurons are exposed to glutamate, the initial Ca^{2+} spike is primarily due to gating of NMDA receptor/ion channels. Plasma membrane hypopolarization and ion influx through voltage-gated channels, release of Ca^{2+} from internal stores, and the Ca^{2+} intake due to the reverse mode of the NCX all contribute to the initial Ca^{2+} increase (for an overview see Lee et al. [1999]). Later, an irreversible ion imbalance, marked by a secondary intracellular Ca^{2+} increase, precedes swelling of the axodendritic network (Bindokas and Miller, 1995) and neuronal lysis (Tymianski et al., 1993).

Our data demonstrate that loss of NCX3 function following its proteolysis is critical for the delayed Ca^{2+} deregulation and excitotoxic demise in CGNs. Since removal of extracellular Ca^{2+} did not reduce the secondary Ca^{2+} overload once it had occurred, we can reasonably assume that opening of inward-gated channels was not sufficient to trigger the secondary Ca^{2+} overshoot. High-capacity Ca^{2+} extrusion is required to relieve substantial Ca^{2+} loads from neurons. At difference with PMCAs that

have high affinity for Ca^{2+} but low capacity, the half-maximal activity of the NCX is around 1–10 μM. The bulk cytoplasmic Ca^{2+} levels measured by low-affinity Ca^{2+} dyes during the secondary overload phase can approach the 10 μM level (Alano et al., 2002 and data not shown) and Ca^{2+} levels in subplasma membrane domains may reach much higher levels than bulk cytosolic Ca^{2+} (Brini et al., 2002). NCXs are therefore perfectly suited to extrude large amounts of Ca^{2+} after a Ca^{2+} load. In line with these findings, suppression of NCX3 by siRNA compromised the ability of CGN to rectify even nonexcitotoxic Ca^{2+} signals. Moreover, siRNA transfected neurons were highly sensitive to excitotoxicity. The finding that in hippocampal pyramidal neurons from NCX2 knockout mice, Ca^{2+} clearance after stimulation with glutamate is markedly delayed, whereas basal or peak values of $[Ca^{2+}]_i$ are approximately the same (Jeon et al., 2003) is consistent with these considerations.

The cleavage of NCX3 occurred in a region that is highly conserved between rodents and humans, which is located close to the high-affinity Ca^{2+} regulatory domain. The high degree of homology in this region is lost in species such as *Drosophila* or *C. elegans* that do not have the Ca^{2+} regulatory domain, suggesting that the cleavage site and the Ca^{2+} regulatory domain are functionally linked. This was supported by the Ca^{2+} overlay experiments.

Other mechanisms invoked to explain the delayed Ca^{2+} deregulation include the loss of mitochondrial Ca^{2+} buffering capacity and the sudden release of mitochondrial Ca^{2+} that would follow mitochondrial permeability transition (Budd and Nicholls, 1996; Duchen, 2000). The experiments using ratio-pericam demonstrated that mitochondria maintained their resting levels of Ca^{2+} for about 45 min after glutamate addition, even in the face of rising cytosolic Ca^{2+} levels. Moreover, these experiments demonstrate that the final mitochondrial Ca^{2+} deregulation and permeability transition are downstream rather than upstream of the secondary Ca^{2+} overload. This is in line with the recent findings that mitochondrial respiration can be retained for a relatively long time in CGN undergoing excitotoxicity (Jekabsons and Nicholls, 2004).

Neurons stimulated by excess glutamate can die by rapid necrosis or succumb to apoptosis depending on the intensity of the insult and the residual cellular energy levels (Ankarcrona et al., 1995; Bonfoco et al., 1995). Whereas the apoptotic mode of cell death has been extensively characterized, much less is known of the biochemical routines involved in early cell lysis (necrosis). The molecular determinants that promote a switch between one and the other death subroutine are only beginning to be understood. Our finding that calpastatin protects neurons from the rapid excitotoxic cell death supports early proposals that the calpain protease family has death-related functions independent from the crosstalk with the caspase family of proteases (Nicotera et al., 1986; Orrenius et al., 2003). Interestingly, apoptosis modulators such as some Bcl-2 family members (Scorrano and Korsmeyer, 2003; Scorrano et al., 2003) or cytochrome c (Boehning et al., 2003) also modulate intracellular Ca^{2+} compartmentalization, while caspases can destabilize Ca^{2+} homeostasis and recruit Ca^{2+} overload into the death program by cleaving and inactivating the plasma membrane Ca^{2+} pump (Schwab et al., 2002). Preliminary findings in our laboratory show that NCXs can also be targeted by caspases. These findings suggest that a pathway, which involves deregulation of Ca^{2+} handling, is invariably activated in dying neurons.

Inhibition of Ca^{2+} extrusion due to NCX proteolysis can explain why the secondary Ca^{2+} increase is sustained and irreversible and unveils a mechanism that may prove to be independent of the source of Ca^{2+} entry, although studies in different models of brain ischemia/anoxia (Aarts et al., 2003; Xiong et al., 2004) are required to corroborate this assumption. Under this hypothesis, treating the sources of Ca^{2+} entry may be effective only in part. More likely, targeting both Ca^{2+} entry routes and the effector proteases may be required to interrupt the lethal loop leading to ischemic brain damage.

Experimental Procedures

Cell Culture

Primary cultures of rat CGNs were grown for 7 days on 6-well plates as described previously (Leist et al., 1997). 293T cells were cultured in IMDM (Iscove's modified Dulbecco medium; SIGMA Life Technologies) supplemented with 10% dialyzed FCS, 2 mM glutamine, 100 units/ml streptomycin, and ampicillin. Excitotoxicity was triggered by addition of glutamate to CGN culture media for the time and concentrations indicated. Where indicated, cells were preincubated for 30 min before treatment with Z-VAD-fmk (100 μM) or calpeptin (5 μM). Cell death was determined by scoring nuclear condensation, plasma membrane integrity, and the conversion of MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrasodiumbromide) to formazan as described (Leist et al., 1998).

Middle Cerebral Artery Occlusion (MCAO)

Experiments were performed on adult Wistar rats (Charles River, United Kingdom). Animals were maintained in a 12 hr light/dark cycle at 22°C, and all experiments performed in accordance with United Kingdom legislation under the 1986 Animals (Scientific Procedures) Act. Focal cerebral ischaemia was induced by occlusion of the right middle cerebral artery (MCAO) using the intraluminal filament technique (Hata et al., 1998). Reduction in cerebral blood flow (approximately 70%) was confirmed by laser doppler flowtometry (Moor Instruments, United Kingdom), and body temperature maintained at 36.5°C–37.5°C. Reperfusion of the tissue was achieved by withdrawing the filament (under brief anesthesia) after 15 min. sham-operated animals received the same surgery as MCAO-treated mice, but the filament was not inserted. Animals were allowed to recover for 24 hr, sacrificed, and their brains immediately removed. Areas of striatum and cortex were dissected from the ischemic and contralateral hemispheres and immediately frozen on dry ice. Samples were homogenized in a Tris-EDTA-EGTA (10:1:1 mM) buffer and prepared for Western blots.

CGN Transduction with Lentiviral Particles

Viral particles were obtained as described (Follenzi and Naldini, 2002). Briefly, 293T cells were transfected with three packaging plasmids plus a vector encoding the protein of interest. After 36 and 60 hr the viral particles were collected and concentrated by ultracentrifugation. The virus concentration was estimated by measuring the amount of p24 protein with a commercial kit (Perkin-Elmer). CGNs were plated for 24 hr before being transduced using 50–100 ng of $p24/10^5$ cells; the medium was changed 8 hr after the addition of the virus, and the cells were allowed to express the protein of interest for at least 96 hr before experiments. The efficiency of viral infection was assessed using viral delivery of GFP or, in the case of CLP-YFP, the YFP itself. Typical efficiency was around 90%.

Immunoblotting

Western blotting was performed as described previously (Schwab et al., 2002) using the isoform polyclonal specific antibodies anti-NCX3 (diluted 1:1000; Thurneysen et al., 2002), anti-NCX2 (1:200), anti-NCX1 (1:400; Li et al., 2000), anti-PMCA2 (1:500; Stauffer et al., 1995), anti-NMDA-R1 (Chemicon; 1:1000), anti-cleaved caspase-3 antibody (Cell Signaling Technology; 1:200). A secondary antibody, conjugated with HRP (Chemicon) was added in a dilution of 1:3000, and immunoreactivity was visualized with ECL development was performed using a Kodak Image Station 440 and the Software Kodak 3.5.

In Vitro Cleavage of NCX3

Membranes solubilized from brain homogenates were resuspended in a buffer containing 50 mM NaCl, 10 mM EGTA, 0.1% Triton, 100 mM HEPES (pH 7.5) (calpain buffer). Subsequently, 0.5 μg of calpain m or μ (Calbiochem) were added to the suspension and the proteolysis performed for 15–30 min, adding $CaCl_2$ at a final concentration of 20 mM. The reaction was stopped by adding 20 μl of sample buffer (8 M urea, 0.5 M DTT, 5 % SDS, 5 mM EDTA, 50 mM Tris/HCl [pH 8], and bromophenol blue). The samples were separated by SDS-PAGE and blotted on nitrocellulose filter. *E. coli* (M15pRep4) was transformed with the plasmid codifying the region from E276 to E722 (kindly provided by Prof. K. Philipson, Departments of Physiology and Medicine and the Cardiovascular Research Laboratory, Los Angeles, California) of the exchanger, and the expression was triggered by adding to the culture 1 mM IPTG when the OD at 600 nm was approximately 0.4. Intracellular bodies were resuspended in PBS (containing a cocktail of proteases), sonicated, and purified by centrifugation. The pellet containing the peptide was resuspended in calpain buffer and incubated with calpains as described above.

$^{45}Ca^{2+}$ Overlay

$^{45}Ca^{2+}$ binding to the fusion protein after transfer to nitrocellulose was measured as described (Branca et al., 1999) with slight modifications. Briefly, the nitrocellulose filter was washed four times for 15–20 min in a solution containing 60 mM KCl, 10 mM imidazole (pH 7.2), and 2 mM $MgCl_2$ and incubated with the same medium supplemented with 2 μCi/ml $^{45}Ca^{2+}$ for 10 min. The nitrocellulose membrane was then rinsed twice for 5 min with 50% ethanol. The filter was air-dried and exposed to X-ray film for two days.

Measurement of $^{45}Ca^{2+}$ Loading

To measure $^{45}Ca^{2+}$ uptake, 1 μCi/ml $^{45}Ca^{2+}$ was added to the CGN in the growing medium for 5 min in presence or absence of 150 μM glutamate. The cells were then rinsed three times with CSS5 buffer, lysed with 0.2% sodium dodecyl sulfate, and counted in a scintillation counter. $^{45}Ca^{2+}$ was normalized to the number of cells used in the experiment.

Intracellular Free Ca^{2+} Concentration ($[Ca^{2+}]_i$) and Mitochondrial Calcium Measurements

$[Ca^{2+}]_i$ changes were measured in individual CGNs by fluorescent microscopy using the Ca^{2+}-indicator Fura-2-AM. Neuronal cells were loaded with 2 μM Fura-2-AM for 30 min at 37°C in CSS-5 medium (120 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 15 mM glucose, and 25 mM HEPES [pH 7.4]) supplemented with 10 μM glycine. The measurements were performed at 37°C using a Nikon microscope with a 40× oil immersion lens. Fura-2-AM was excited at a wavelength of 340 and 380 nm, and emitted fluorescence was collected via a 510 nm band pass filter using a cooled CCD camera.

Mitochondrial calcium measurements were performed using the GFP-based probe, ratio-Pericam, on a Zeiss Axiovert microscope with a 40× oil immersion lens. The probe was alternatively excited at 410 and 485 nm, and the emission was monitored at 535 nm. Cells were incubated in CSS5 (containing 10 μM glycine) and treated with glutamate 150 μM as indicated. Simultaneous measurement of calcium in the cytosol and in the mitochondria was achieved by loading with Fura-Red (2 μM) CGNs transduced with ratio-Pericam. Fura-Red was excited at 485 nm and emission monitored at over 600 nm.

Constructs

See the Supplemental Data on the *Cell* website.

siRNA Reagents and Transfection

A scrambled nonsilencing oligonucleotide conjugated with Alexa Fluor 488 (Qiagen), sequence AAT TCT CCG AAC GTG TCA CGT, was used as control and to quantify the transfection efficiency. siRNA against NCX3 corresponded to coding regions +124–142, +165–183, +227–245 relative to the first nucleotide of the start codon of rat NCX3 (GenBank accession number: U53420). On days 3 and 5 after plating, CGNs were transfected with 60 pmol of synthetic duplex using Lipofectamine 2000 (Invitrogen), following the manufacturer's suggestions, with the only exception being that the medium was not replaced. At day 8 the cells were harvested or used for calcium imaging and microscopy as already described.

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