Anoxia-Induced NF-kB-Dependent Upregulation of NCX1 Contributes to Ca\(^{2+}\) Refilling Into Endoplasmic Reticulum in Cortical Neurons

Rossana Sirabella, PhD, MD; Agnese Secondo, PhD; Anna Pannaccione, PhD; Antonella Scorziello, PhD, MD; Valeria Valsecchi, PhD; Annagrazia Adornetto, PhD; Leonilda Bilo, MD; Gianfranco Di Renzo, MD; Lucio Annunziato, MD

**Background and Purpose**—The 3 gene products of the Na\(^+/Ca^{2+}\) exchanger (NCX), viz, NCX1, NCX2, and NCX3, may play a pivotal role in the pathophysiology of brain ischemia. The aim of this study was to investigate the transductional and posttranslational mechanisms involved in the expression of these isoforms during oxygen and glucose deprivation and their role in endoplasmic reticulum Ca\(^{2+}\) refilling in cortical neurons.

**Methods**—NCX1, NCX2, and NCX3 transcript and protein expression was evaluated in primary cortical neurons by reverse transcriptase–polymerase chain reaction and Western blot. NCX currents (I\(_{\text{NCX}}\)) and cytosolic Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)\(_i\)]) were monitored by means of patch-clamp in whole-cell configuration and Fura-2AM single-cell video imaging, respectively.

**Results**—Exposure of cortical neurons to 3 hours of oxygen and glucose deprivation yielded dissimilar effects on the 3 isoforms. First, it induced an upregulation in NCX1 transcript and protein expression. This change was exerted at the transcriptional level because the inhibition of nuclear factor kappa B translocation by small interfering RNA against p65 and SN-50 prevented oxygen and glucose deprivation-induced NCX1 upregulation. Second, it elicited a downregulation of NCX3 protein expression. This change, unlike NCX1, was exerted at the posttranscriptional level because it was prevented by the proteasome inhibitor MG-132. Finally, we found that it significantly increased I\(_{\text{NCX}}\) both in the forward and reverse modes of operation and promoted an increase in ER Ca\(^{2+}\) accumulation. Interestingly, such accumulation was prevented by the silencing of NCX1 and the NCX inhibitor CB-DMB that triggered caspase-12 activation.

**Conclusions**—These results suggest that nuclear factor kappa B-dependent NCX1 upregulation may play a fundamental role in Ca\(^{2+}\) refilling in the endoplasmic reticulum, thus helping neurons to prevent endoplasmic reticulum stress during oxygen and glucose deprivation. (Stroke. 2009;40:922-929.)

**Key Words:** [Ca\(^{2+}\)], homeostasis ■ ER stress ■ Na\(^+\)-Ca\(^{2+}\) exchanger ■ neuroprotection ■ OGD

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The Na\(^+/Ca^{2+}\) exchanger (NCX),\(^1,2\) a 9-transmembrane protein able to couple the efflux/ influx of Ca\(^{2+}\) to the influx/exflux of Na\(^+\) ions by operating in a bidirectional way,\(^2\) is the major regulator of Na\(^+\) and Ca\(^{2+}\) homeostasis. In the brain, unlike other tissues, this exchanger is present in 3 different gene products, named NCX1, NCX2, and NCX3,\(^2,5\) with a distinct distribution pattern in different brain regions.\(^6\)

Under physiological conditions, its primary role is to extrude Ca\(^{2+}\) through a forward mode of operation in response to a depolarization or to an increase in intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)\(_i\)]) coupled to receptor stimulation.\(^2,7\) However, during hypoxic conditions, NCX is also able to operate in the reverse mode, thus extruding Na\(^+\) ions while promoting Ca\(^{2+}\) influx.\(^8\)

A new emerging concept is that NCX1, NCX2, and NCX3 may exert different roles during in vitro\(^9\) and in vivo\(^9\) anoxic conditions. Indeed, in cells singly and stably transfected with NCX3, this isoform contributes more significantly to the maintenance of [Ca\(^{2+}\)\(_i\)] homeostasis during experimental conditions mimicking ischemia, thereby preventing mitochondrial Δψ collapse and cell death.\(^9\) In addition, in in vivo experiments, the selective knocking down of NCX1 and NCX3, but not of NCX2, by antisense oligodeoxynucleotide strategy,\(^10\) or the disruption of the ncx3 gene, renders the brain more susceptible to the ischemic insult.\(^8\) In addition, our most recent experiments in vivo, entailing the induction of permanent middle cerebral artery occlusion in rats, have demonstrated that NCX1 mRNA is upregulated in the peri-infarct area.\(^11\) This event seems to play a
crucial role in counteracting the dysregulation of \([Ca^{2+}]_{i}\) homeostasis. It is also equally important to consider that under anoxic conditions and cerebral ischemia, the progressive dysregulation of \([Ca^{2+}]_{i}\) homeostasis can lead to endoplasmic reticulum (ER) dysfunction. In particular, the disruption of ER \([Ca^{2+}]_{i}\) homeostasis plays an important role in the induction of ER stress because the depletion of \([Ca^{2+}]_{i}\) ER and ATP can disrupt proper peptide folding and trigger ER stress. However, the cellular mechanisms underlying \([Ca^{2+}]_{i}\) ER dysregulation after ischemia and reperfusion are not defined. On the other hand, it has recently been demonstrated that during normoxic conditions, a functional relationship exists between NCX working in the reverse mode and \([Ca^{2+}]_{i}\) refilling into the ER. Consequently, in the present study, we explored the possibility that during anoxic conditions, NCX can play a role in ER \([Ca^{2+}]_{i}\) refilling. To this aim, in cortical neurons exposed to oxygen and glucose deprivation (OGD) followed by reoxygenation, the expression of NCX1, NCX2, and NCX3 transcripts and proteins and the putative transcriptional and posttranslational factors involved in their modulation were evaluated. In addition, to study the specific role played by each NCX isoform in the modulation of \([Ca^{2+}]_{i}\) storage during OGD, NCX currents were detected by electrophysiology in whole-cell configuration and cytosolic \([Ca^{2+}]_{i}\) concentrations were monitored by Fura-2AM single-cell video imaging. Finally, because ER depletion and caspase-12 activation have been considered ER stress markers, we examined whether changes in NCX expression and activity during anoxia could interfere with these 2 mechanisms, thus resulting in a protective effect.

**Materials and Methods**

**Primary Cortical Neurons**

Cortical neurons were prepared from brains of 16-day-old Wistar rat embryos (Charles River) and used at 7 to 10 days in vitro. Briefly, dissection and dissociation were performed in the Department of Neuroscience lab in \([Ca^{2+}]/Mg^{2+}\)-free phosphate-buffered saline containing glucose (30 mmol/L). Tissues were incubated with papain for 10 minutes at 37°C and dissociated by trituration in Earl’s balanced salt solution containing DNAse (0.16 U/mL), bovine serum albumin (10 mg/mL), and ovomucoid (10 mg/mL). Cells, plated both in plastic Petri dishes (Falcon; Becton-Dickinson) and on glass coverslips precoated with poly-D-lysine (20 mg/mL), were grown in MEM/F12 containing glucose, 5% of deactivated fetal bovine serum, and 5% horse serum, glutamine (2 mmol/L), penicillin (50 U/mL), and streptomycin (50 \(\mu\)g/mL; Invitrogen).

**Combined Oxygen and Glucose Deprivation**

In cortical neurons, OGD insult was reproduced in vitro by exposing cells to a medium containing (in mmol/L) 116 NaCl, 5.4 KCl, 0.8 MgSO4, 26.2 NaHCO3, 1 NaH2PO4, 1.8 CaCl2, 0.01 glycine, and 0.001 wt/vol phenol red. Hypoxic conditions were maintained using a hypoxia chamber (Billups Rothemberg Inc; temperature 37°C, atmosphere 5% CO2, and 95% N2).

**Small Interfering RNA Against p65 and NCX1**

Double-stranded small interfering (si) RNAs corresponding to different sequences of rat p65 gene (NM_019268) were designed by Qiagen (Qiagen-Xeragon). The following gene-specific sequences were used successfully: siRNA-p65 sense 5'-GAAGCAUUAACUUCCCUGA-3' and antisense 3'-'UCAGGGAAGUUAAUGCUUC-5'. Hypoxic conditions were maintained using a hypoxia chamber (Billups Rothemberg Inc; temperature 37°C, atmosphere 5% CO2, and 95% N2).
for 60 minutes before use. Cell transfection was carried out as follows: siRNA-p65 (20 nM) was condensed with RNAiFect Transfection Reagent (Qiagen) for 15 minutes at room temperature according to the manufacturer’s instructions. The transfection complex was diluted in culture medium without serum and added directly to the neurons at 7 days in vitro. Then, it was replaced with fresh medium 3 hours later.

The mammalian expression vector, pSUPER.retro.puro (Oligo-Engine), was used to express siRNA against NCX1 (siRNA-NCX1; NM_019268) in cortical neurons. To prepare siRNA-NCX1, a 60-base oligonucleotide and another oligonucleotide with the complementary sequence were annealed and inserted into pSUPER.retro.puro as previously reported.17 A mismatch sequence cloned in the same vector was used as an experimental control. After 72 hours’ plating, cortical neurons were transfected with siRNA-NCX1 or siRNA mismatch in OPTIMEM by means of lipofectamine2000 (Invitrogen). After 5 hours, it was replaced with fresh medium.

Western Blot Analysis
Cortical neurons were collected by scraping in ice-cold lysis buffer containing protease inhibitor cocktail II (Roche Diagnostic) as previously reported.10 Proteins (30 μg) were separated on 8% (for NCX and nuclear factor kappa B [NF-kB] p65) or 12% (for caspase-12) sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare). Membranes were blocked with 5% nonfat dry milk in 0.1% Tween20 (2 mmol/L Tris-HCl, 50mmol/L NaCl, pH 7.5) for 2 hours at room temperature and subsequently incubated overnight at 4°C in the blocking buffer with 1:1000 antibody for NCX1 (polyclonal rabbit antibody; Swant), 1:1000 antibody for NCX2 (polyclonal rabbit antibody; Alpha Diagnostic), 1:1500 antibody for NCX3 (polyclonal rabbit antibody; Philipson’s Laboratory), 1:1000 antibody for caspase-12 (polyclonal rabbit antibody; Santa Cruz Biotechnology), 1:1000 antibody for NF-kb p65 (polyclonal rabbit antibody; Santa Cruz Biotechnology), and 1:1000 antibody for β-actin (monoclonal mouse antibody; Sigma). Nuclear extracts were performed in lysis buffer containing (10 mmol/L TRIS-HCl pH 7.4, 0.32 mol/L sucrose, 20 mmol/L MgCl2, 0.1 mmol/L EDTA, 0.1% Triton X100, 10 mmol/L DTT, 1 mmol/L Na3VO4, 10 mmol/L NaF, and protease inhibitor cocktail). Immunoreactive bands were detected with the ECL reagent (GE Healthcare). The optical density of the bands was determined by the Chemi-Doc Imaging System (Bio-Rad).

Reverse Transcriptase–Polymerase Chain Reaction Analysis
Total RNA was extracted from cortical neurons with Trizol following the instructions of the supplier (Invitrogen). Total RNA was treated with ribonuclease-free deoxyribonuclease I for 15 minutes at room temperature. The first-strand cDNA was synthesized with 5 μg of the total RNA and 500 ng of random primers using the SuperScript first-strand synthesized system for reverse transcriptase–polymerase chain reaction (Invitrogen). The samples were treated as previously reported.17 The pairs of oligonucleotide used were 5′-ACCACCAAGACTACGTGCG-3′ and 5′-TTGGAAGCTGGTCTGTCTCC-3′ for NCX1; 5′-GGGTGTGGCGATGCTCA-3′ and 5′-GACCTCGAGGCGACAGTTC-3′ for NCX2; and 5′-CTTGGAAGGCGATGGACCC-3′ and 5′-GTTTAGGGTGTTACCCAAATA-3′ for NCX3. Hypoxanthine-
guanine–phosphoribosyl-transferase mRNA expression was used as an internal standard, and the primers used were 5'-CCTGTCTGGA-TTACATTAAAAGCCTG-3' and 5'-CCTGAAGTACTCATTATG-TCAGG-3'.

**[Ca**2+]i Measurement**

[Ca**2+]i was measured by single-cell computer-assisted video imaging in cortical neurons loaded with 6 μmol/L Fura-2 acetoxyethyl ester (Fura-2AM; EMD Biosciences; 30 minutes, room temperature). At the end of the loading period, the coverslips were placed into a perfusion chamber (Medical Systems) mounted onto a Zeiss Axiovert 200 microscope. The experiments were performed as previously reported.

**Electrophysiology**

I_{SCX} was recorded from primary cortical neurons by the patch-clamp technique in whole-cell configuration as previously described. Currents were filtered at 5 kHz and digitized using a Digidata 1322A interface (Molecular Devices). Data were acquired and analyzed using the pClamp software (Version 9.0; Molecular Devices). I_{SCX} was recorded starting from a holding potential of −60 mV up to a short-step depolarization at +60 mV (60 ms). Then, a descending voltage ramp from +60 mV to −120 mV was applied. The current recorded in the descending portion of the ramp was used to plot the current voltage (I-V) relation curve. The magnitudes of I_{SCX} were measured at the end of +60 mV (reverse mode) and at the end of −120 mV (forward mode), respectively. The Ni**2+**-insensitive components were subtracted from total currents to isolate I_{SCX}. The I_{SCX} were normalized for membrane capacitance as previously reported.

**Determination of Neuronal Death**

Cell death was evaluated by means of fluorescein diacetate (36 μmol/L) and propidium iodide (7 μmol/L) measuring the ratio between dead and living cells as previously described.

**Drugs and Chemicals**

SN-50 and SN-50M were purchased from EMD Biosciences. ω-conotoxin was purchased from Alomone Labs. MG-132, thapsigargin, ω-agatoxin, nimodipine, and all the reagents were purchased from Sigma.

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical analysis was performed with analysis of variance followed by Newman-Keuls test. Statistical significance was accepted at the 95% confidence level (P<0.05).

**Results**

Expression of NCX1, NCX2, and NCX3 Transcripts and Proteins in Cortical Neurons Exposed to Oxygen and Glucose Deprivation Followed by Reoxygenation and Transcriptional and Posttranscriptional Mechanisms Involved

Immunoblot analysis showed that cortical neurons express NCX1, NCX2, and NCX3 proteins (Supplemental Figure I, available online at http://stroke.ahajournals.org). In addition, in these neurons, the increase in NCX activity in the reverse mode of operation, elicited by extracellular Na**+** removal and measured by single-cell Fura-2-AM-detected [Ca**2+]i increase, was prevented by the NCX pan-inhibitor CB-DMB (3 μmol/L), which was exerted exclusively on NCX.

The exposure of cortical neurons to 3 hours OGD induced an increase in NCX1 transcript and protein expression (Figure 1A–D). When the OGD was followed by 24 hours' reoxygenation, NCX1 transcript and protein expression returned to control values (Figure 1A–D). Interestingly, NCX1 protein level returned to the baseline already after 1 hour of reoxygenation.
reoxygenation (Supplemental Figure II). The inhibition of NF-κB active complex translocation into the nucleus with the cell-permeable peptide SN-50 (5 μmol/L) prevented the upregulation of NCX1 protein expression induced by OGD in cortical neurons (Figure 1E–F), whereas it did not interfere with its basal expression (data not shown). By contrast, the inactive homolog of SN-50, SN-50M (5 μmol/L), failed to inhibit OGD-induced upregulation of NCX1 protein expression (Figure 1E–F). To corroborate these data, 20 nM synthetic siRNAs directed against specific sequences of p65, that translocated into the nucleus after 30 minutes’ OGD (Figure 2A–B), was introduced into cortical neurons at 7 days in vitro to inhibit the expression of this NF-κB subunit (Figure 2C–D). siRNA-p65 prevented the upregulation of NCX1 protein expression induced by OGD in cortical neurons (Figure 2E–F). This suggested an involvement of the transcriptional factor NF-κB in inducing NCX1 upregulation in cortical neurons exposed to OGD.

By contrast, NCX2 transcript and protein expression did not change after OGD or after OGD plus 24 hours of reoxygenation (data not shown). Unlike NCX1, the exposure of neurons to OGD caused a downregulation of NCX3 protein that was not associated with a reduction of its respective transcript, suggesting that the effect of anoxia on NCX3 expression was exerted at the posttranslational level (Figure 3A–D). The inhibition of the proteasomal system with the inhibitor MG-132 (40 μmol/L) prevented NCX3 downregulation induced by OGD in cortical neurons, whereas it did not interfere with its basal expression (Figure 3E–F).

**Effect of Oxygen and Glucose Deprivation or Oxygen and Glucose Deprivation Followed by Reoxygenation on I\(_{\text{NCX}}\) Recorded by the Patch-Clamp Technique in the Reverse and Forward Modes and on [Ca\(^{2+}\)]\(_i\) in Cortical Neurons**

In cortical neurons exposed to 1 and 3 hours of OGD, I\(_{\text{NCX}}\) recorded by patch-clamp in both the reverse and the forward modes of operation, were significantly higher than those detected under normoxic conditions (Figure 4A–B). Fura-2AM detection in single cortical neurons revealed that after 1 hour OGD, [Ca\(^{2+}\)]\(_i\) markedly increased. After 3 hours of OGD or OGD plus reoxygenation, [Ca\(^{2+}\)]\(_i\) levels were significantly lower than those detected after 1 hour of OGD, reaching basal levels thereafter (Figure 4C).

**Effect of NCX1 Activity on Ca\(^{2+}\) Refilling Into Endoplasmic Reticulum and on Caspase-12 Activation in Cortical Neurons Exposed to Oxygen and Glucose Deprivation and Oxygen and Glucose Deprivation Followed by Reoxygenation**

After 3 hours OGD, the SERCA inhibitor thapsigargin induced a release of Ca\(^{2+}\) from ER stores higher than that
observed in normoxic conditions, demonstrating that during OGD, a larger Ca\textsuperscript{2+} accumulation occurs in ER (Figure 5A–B). This larger ER accumulation, however, was prevented when the NCX inhibitor CB-DMB (3 μmol/L) was preincubated during OGD (Figure 5A–B), thus revealing the important contribution of NCX—whose reverse mode of operation is markedly increased during OGD (Figure 4A–B)—in the ER-refilling process. More relevantly and in agreement with the pharmacological data, the silencing of NCX1, by reducing basal expression of this exchanger (Figure 6A–B) and its activity (Figure 6C–D), prevented OGD-induced ER-Ca\textsuperscript{2+} accumulation (Figure 6E). Interestingly, when such a refilling process was prevented by the plasma membrane NCX blockade or by NCX1 knocking-down, an activation of caspase-12, a specific marker of ER stress, occurred together with an increased neuronal vulnerability to OGD. Altogether these data suggested the protective role played by NCX when it works in the reverse mode. In fact, the increase in free Ca\textsuperscript{2+} concentration, indeed by NCX, within the ER appears to be a protective key factor in that it determines the synthesis and processing of proteins within this organelle, a crucial early self-protective mechanism against ER stress. Intriguingly, it is also well known that Ca\textsuperscript{2+} accumulation and NF-kB translocation into the nucleus constitute relevant self-protective mechanisms against ER stress. Remarkably, whereas the transcriptional factor NF-kB

**Discussion**

The results of this study indicated that in primary cortical neurons, transcript and protein expression of the 3 isoforms, NCX1, NCX2, and NCX3, respond differently to anoxic injury. In particular, 3 hours of OGD induced an NF-kB-dependent upregulation of NCX1 and a proteasomal-dependent NCX3 downregulation, leaving, however, NCX2 unaffected. These changes in NCX isoform expression during OGD were accompanied by increases in I\textsubscript{NCX}, both in the reverse and forward modes of operation, and by cytosolic Ca\textsuperscript{2+} levels comparable to those found under normoxic conditions. Consistent with an elevation of NCX activity and in accordance with normal cytosolic [Ca\textsuperscript{2+}], we found that during OGD, an increased refilling of Ca\textsuperscript{2+} into ER occurred. This augmented refilling was prevented by NCX inhibition and by NCX1 knocking-down, thus suggesting that this plasma membrane antiporter is crucial for the Ca\textsuperscript{2+} refilling process. Interestingly, when such a refilling process was prevented by the plasma membrane NCX blockade or by NCX1 knocking-down, an activation of caspase-12, a specific marker of ER stress, occurred together with an increased neuronal vulnerability to OGD.
was responsible for NCX1 upregulation in cortical neurons exposed to OGD, the inhibition of its translocation into the nucleus prevented NCX1 overexpression.

It should be underlined that in our previous studies, we observed an increase in reactive oxygen species production in cortical neurons exposed to OGD. Moreover, this increase has been reported to directly activate the transcription factor NF-κB, thus inducing NCX1 upregulation. Unlike NCX1, the other brain-specific isoform NCX3 displayed a downregulation during OGD that was not exerted at the transcriptional level but was rather proteasomal-dependent. Particularly, evidence that proteasome inhibition did not affect basal NCX3 expression suggests that this system is specifically activated by OGD. Interestingly, the proteasomal system appears to be involved in the early phase of ER stress as an upstream signal able to induce caspase and calpain activation. In addition to the effect of the proteasomal system, the NCX3 downregulation could also be ascribed to the activation of calpains involved in glutamate-induced excitotoxicity in cerebellar granule cells. However, our current findings demonstrated that the inhibition of the proteasomal system completely prevented OGD-induced NCX3 downregulation, thereby suggesting that under OGD, this degradation pathway is the only operative system.

In agreement with the data showing that there was an upregulation of NCX1 expression, we found that the total $I_{NCX}$ recorded in the reverse and forward modes of operation were higher than those in controls at 1 and 3 hours after OGD. However, the re-exposure of cortical neurons to 24 hours of reoxygenation significantly reduced $I_{NCX}$ in the reverse mode. Noticeably, the enhancement of $I_{NCX}$ began just 1 hour after OGD, a time when no increases in NCX1 protein expression were detected. This evidence thus suggested that this $I_{NCX}$ increase was most likely due to an OGD-induced functional modulation rather than to protein overexpression. This assumption was further confirmed by cytosolic Ca2⁺ variations observed during OGD. The increase in [Ca2⁺], after 1 hour of hypoxia was probably due to the increased activity of NCX in the reverse mode during the same time period, whereas its return to control levels after 3 hours of OGD was probably the result of NCX-dependent Ca2⁺ refilling into ER. In fact, this refilling was blocked by CB-DMB and by siRNA against NCX1. In agreement with these results, in anoxic astrocytes and in Ca2⁺ oscillating muscle cells, NCX blockade prevents ER Ca2⁺ refilling.

Figure 6. Effect of si-RNA against NCX1 on $I_{NCX}$ Ca²⁺ refilling into ER, caspase-12 activation, and cell death in cortical neurons exposed to OGD. A–B, Effect of si-RNA-NCX1 on NCX1 protein expression and its densitometric analysis, respectively. *P<0.05 versus CTL. C, $I_{NCX}$ superimposed traces recorded from normoxic cortical neurons (CTL), normoxic plus siRNA-NCX1, normoxic plus mismatch sequence, after 3 hours OGD, after 3 hours OGD plus siRNA-NCX1, and after 3 hours OGD plus mismatch. D, Quantification of $I_{NCX}$ under the experimental conditions reported in (C; n=15 cells in 3 independent experimental sessions). *P<0.05 versus their respective CTLs. E, Quantification of thapsigargin-induced [Ca²⁺] release under the experimental conditions reported in (C; n=50 cells in 3 independent experimental sessions). *P<0.05 versus CTL. F, Caspase-12 activation in control conditions, after 3 hours OGD, after OGD plus si-RNA-NCX1, and after OGD plus mismatch sequence (n=5). G, Cell death detected in the previously mentioned conditions in cortical neurons and represented as percentage of the ratio between PIpositive cell values and PI fluoresceinpositive cell values. *P<0.05 versus all.
NCX1’s protective role against OGD insult has also been highlighted in our recent study. Indeed, we have demonstrated that in Akt-positive mutants, NCX1 constitutes a prosurvival target because its upregulation contributes to the neuronal survival during chemical hypoxia.17 In addition, when in vivo experiments, we selectively knocked down this isoform with oligodeoxynucleotides,10 we further demonstrated that an exacerbation of ischemic damage occurs. In addition, Boscia et al11 have demonstrated that in the brain regions surrounding the core and containing surviving neurons, NCX1 mRNA is upregulated after stroke, whereas in the ischemic core, a region characterized by an extended cell death, this transcript is greatly reduced.

Overall, the results of the present study indicate that NF-kB-dependent NCX1 upregulation, occurring during OGD, may play a fundamental role in ER Ca2+ refilling and that NCX activation during OGD may help to prevent ER stress and cell death. These results might be of clinical relevance because the increase of NCX1 activity in the ischemic core may help to refill the ER Ca2+ stores and to prevent ER stress during anoxic conditions, thus contributing to cell rescue, although drugs selectively activating NCX1 are not yet available. Unfortunately, the in vitro experiments of our work could not at this time be reproduced in vivo because it is not technically feasible to mimic in vivo an experimental condition in which Ca2+ refilling in the endoplasmic reticulum is monitored in living neurons and, at the same time, NCX1 is knocked down.

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**Disclosures**

None.

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