

The Two Isoforms of the $\text{Na}^+/\text{Ca}^{2+}$ Exchanger, NCX1 and NCX3, Constitute Novel Additional Targets for the Prosurvival Action of Akt/Protein Kinase B Pathway

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ABSTRACT

The proteins NCX1, NCX2, and NCX3 expressed on the plasma membrane of neurons play a crucial role in ionic regulation because they are the major bidirectional system promoting the efflux and influx of Na^+ and Ca^{2+} ions. Here, we demonstrate that NCX1 and NCX3 proteins are novel additional targets for the survival action of the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway. Indeed, the doxycycline-dependent overexpression of constitutively active Akt1 in tetracycline (Tet)-Off PC-12 positive mutants and the exposure of Tet-Off PC-12 wild type to nerve growth factor induced an up-regulation of NCX1 and NCX3 proteins. NCX1 up-regulation induced by Akt1 activation occurred at the transcriptional level because NCX1 mRNA increased, and it was counteracted by cAMP response element-binding protein 1 inhibition through small interfering RNA strategy. In contrast, Akt1-induced NCX3 up-regulation recognized a post-transcriptional mechanism occurring at

the proteasome level because 1) NCX3 transcript did not increase and 2) the proteasome inhibitor *N*-benzyloxycarbonyl (Z)-Leu-Leu-leucinal (MG-132) did not further enhance NCX3 protein levels in Akt1 active mutants as it would be expected if the ubiquitin-proteasome complex was not already blocked by Akt1 pathway. As expected, in PC-12 Tet-Off wild-type cells MG-132 enhanced NCX3 protein levels. This up-regulation produced an increased activity of NCX function. Furthermore, NCX1 and NCX3 up-regulation contributed to the survival action of Akt1 during chemical hypoxia because both the silencing of NCX1 or NCX3 and the pharmacological paninhibition of NCX isoforms reduced the prosurvival property of Akt1. Together, these results indicated that NCX1 and NCX3 represent novel additional molecular targets for the prosurvival action of PI3-K/Akt pathway.

The $\text{Na}^+/\text{Ca}^{2+}$ antiporter, an integral protein belonging to the plasma membrane cation/ Ca^{2+} exchanger superfamily, consists of nine transmembrane segments that can mediate

Ca^{2+} and Na^+ fluxes across the neuronal membrane, depending on the intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) and Na^+ ($[\text{Na}^+]_i$). In particular, NCX can operate either in the "forward mode" by coupling the uphill extrusion of Ca^{2+} with the entrance of Na^+ ions, or in the "reverse mode" by mediating the extrusion of Na^+ to the entrance of Ca^{2+} ions (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000; Annunziato et al., 2004). To date, three *ncx* genes—*ncx1*, *ncx2*, *ncx3*—have been identified and cloned. Whereas NCX1 is ubiquitously expressed, NCX2 and NCX3 are expressed exclusively in the brain and in the skeletal muscle (Lee et al., 1994). Specifically, NCX1, NCX2, and NCX3 are expressed in neurons, astrocytes, oligodendrocytes, and microglia (Quednau et al., 1997; Thurneysen et al., 2002; Nagano et al.,

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ABBREVIATIONS: NCX, $\text{Na}^+/\text{Ca}^{2+}$ exchanger; PKB, protein kinase B; Tet, tetracycline; siRNA, small interfering RNA; FBS, fetal bovine serum; CREB, cAMP response element-binding; PI3-K, phosphatidylinositol 3-kinase; p-, phosphorylated; ECL, enhanced chemiluminescence; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; HA, hemagglutinin; PI, propidium iodide; FDA, fluorescein diacetate; NGF, nerve growth factor; MG-132, *N*-benzyloxycarbonyl (Z)-Leu-Leu-leucinal; tTA, tetracycline-controlled transactivator; EGFP, enhanced green fluorescent protein; GSK, glycogen synthase kinase; PMCA, plasma membrane Ca^{2+} -ATPase; RT-PCR, reverse transcription-polymerase chain reaction; HPRT, hypoxanthine-guanine phosphoribosyl transferase; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate derivative.

2004). During anoxic conditions, owing to the two plasma membrane ATP-dependent pumps— Na^+/K^+ ATPase and Ca^{2+} ATPase—being compromised, NCX assumes a relevant role in controlling the intracellular homeostasis of these two cations. It has recently been proposed that whereas in vitro and in vivo models of anoxia and ischemia, respectively, the pharmacological paninhibition of the NCX gene products, or the more specific antisense knockdown of NCX1 and NCX3 transcripts, can compromise neuronal survival, the activation of the exchanger yields potentially beneficial effects not only on anoxic neurons and glial cells but also in cerebral ischemia (Amoroso et al., 1997, 2000; Annunziato et al., 2004; Pignataro et al., 2004).

Conversely, the Akt/PKB signaling pathway is now largely recognized as one of the most relevant pathways in regulating neuronal survival (Song et al., 2005). Indeed, it has various functions: 1) it regulates glucose metabolism; 2) it inactivates the mitochondrial death pathway (i.e., BAD and caspase 9) and FOXO pathway; 3) it activates CREB phosphorylation, which regulates the expression of genes critical for survival, such as those encoding for cytokines and brain-derived neurotrophic factor; and 4) it activates the antiapoptotic factor nuclear factor- κB through the activation of I κB kinase complex (Fukunaga and Kawano, 2003). In addition, Akt regulates Ca^{2+} homeostasis through the potentiation of L-type voltage-gated calcium channels (Blair et al., 1999), sarco(endo)plasmic Ca^{2+} ATPase (Kim et al., 2003), and intracellular ligand-gated ion channels (Barac et al., 2005), thus enhancing neuronal survival.

In the present study, we examined whether Akt1, an isoform that is ubiquitously and highly expressed in the brain, can, in addition to affecting the other prosurvival cascades, also exert its neuroprotective effect by modulating the expression and activity of NCX1, NCX2, and NCX3 gene products. By means of the Tet-Off strategy, we demonstrate that in positive PC-12 Akt1 mutants, a selective increase of NCX1 and NCX3 isoform expression and activity occurs. The Akt1-induced NCX1 overexpression occurred at the transcriptional level, and it was mediated by CREB activation; by contrast, NCX3 up-regulation seemed to be dependent on Akt inhibition at the level of the proteasome-ubiquitin complex. Furthermore, the selective inhibition of NCX1 and NCX3 by siRNA strategy, and its pharmacological paninhibition, markedly reverted the NCX1 and NCX3 prosurvival action exerted by Akt1.

Materials and Methods

Reagents. Media and sera for cell culture were purchased from Invitrogen (Milan, Italy); antibiotics for cell culture were from Sigma-Aldrich (St. Louis, MO). Tet system-approved fetal bovine serum (FBS) was from BD Biosciences (DBA srl, Milan, Italy). Hygromycin and Geneticin (G418) were from Invitrogen (Inalco, Milan, Italy). Rabbit-polyclonal AKT and p-AKT antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and phosphokinase antibodies were from New England Biolabs (Ipswich, MA). Polyclonal NCX1 antibody was from Swant (Bellinzona, Switzerland), and polyclonal NCX2 and NCX3 antibodies were from Sigma Diagnostics (San Antonio, TX) and Drs. K. D. Philipson and D. A. Nicoll. Western blotting, ECL reagents, and radiochemicals were from GE Healthcare (Milan, Italy). LY294002 was purchased from Calbiochem (San Diego, CA). pTRE-2Hyg expression vector was from Invitrogen. The positive mutant hemagglutinin (HA) antigen-tagged HA-Akt $_{\Delta 4-129}$

(Akt D+) and inactive mutant HA-Akt $_{k179M}$ (Akt D−) plasmids were donated by P. Formisano (University of Naples “Federico II”, Naples, Italy), as described previously (Eves et al., 1998; Trencia et al., 2003). Oligomycin, 2-deoxy-glucose, propidium iodide (PI), fluorescein diacetate (FDA), and all other reagents were from Sigma (Milan, Italy). NGF was purchased from Millipore (Billerica, MA). MG-132 was from Sigma. siRNA-CREB and siRNA-CONTROL were purchased from Dharmacon RNA Technologies (Thema Ricerca, Italy).

Cloning, Cell Culture, and Development of Double-Stable Cell Lines. HA-Akt $_{\Delta 4-129}$ (Akt D+) was obtained by fusing the c-Akt and retroviral Gag protein with 21 additional amino acids derived from the translation of 63 nucleotides of the c-Akt 5', placed in phase between Gag and Akt. The myristoylation site in the Gag sequence targets Akt to the plasma membrane, and it results in high basal kinase activity. By contrast, HA-Akt $_{k179M}$ (Akt D−) was mutated at the ATP binding site, and it results in an inactive kinase. Akt D+ and Akt D− were subcloned into the Bam-EcoRI sites of the pEGFP-N1 vector (Clontech, Mountain View, CA), and then they were cloned into the Nhe1-Not1 sites of pTRE-2Hyg vector.

PC-12 Tet-Off cells were obtained from Clontech. This system is based on the regulatory elements of the tetracycline-resistance operon of *Escherichia coli*, characterized by a tetracycline-controlled transactivator (tTA) and a tTA-dependent promoter. The latter is virtually silent in the presence of tetracycline and doxycycline, but it becomes active in their absence, as indicated in the Tet-Off and Tet-On gene expression systems and cell lines user's manual (Clontech). These cells were grown on plastic dishes in Dulbecco's modified Eagle's medium composed of 10% horse serum, 5% FBS, 100 UI/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin.

The double-stable Tet-Off PC-12 cell lines, expressing Akt D+ and Akt D−, were obtained by transfecting pTRE-2Hyg-Akt1-EGFP vectors into PC-12 Tet-Off cells. Upon reaching 80% confluence, PC-12 Tet-Off cells were transfected with standard protocol by using 10 μg of the two pTRE-2Hyg-Akt1-EGFP vectors and 30 μl of Lipofectamine 2000 (Invitrogen). To select stably transfected cells, selection was carried out by isolating hygromycin-resistant clones incu-

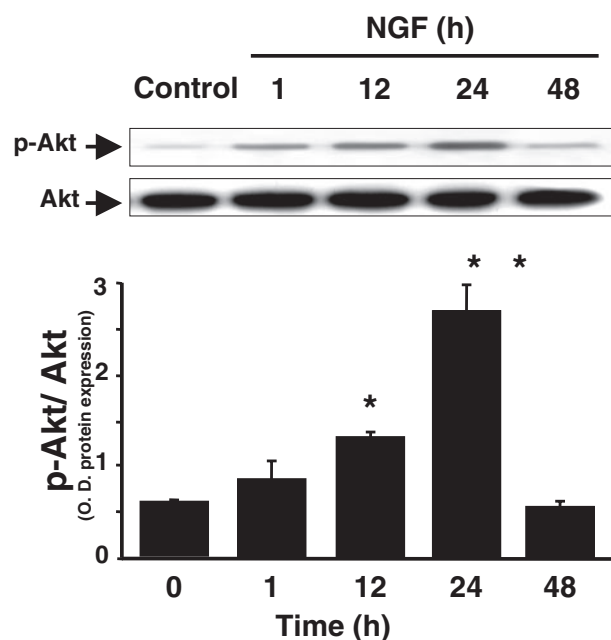


Fig. 1. Time course of the effects of NGF on Akt phosphorylation in PC-12 Tet-Off wild-type cells. The Western blots represent phosphorylated Akt (top) and total-Akt (bottom) in control conditions and in the presence of 100 ng/ml NGF for 1, 12, 24, and 48 h. The bar graph at the bottom shows the quantification of p-Akt normalized to total-Akt and reported as optical density values. *, $p < 0.05$ versus control values, at 1 and 48 h. **, $p < 0.05$ versus all the other experimental groups. Western blots were determined in at least three different experimental sessions.

bated for 2 weeks in a medium containing Dulbecco's modified Eagle's medium, 5% Tet system-approved fetal bovine serum, 10% horse serum, 200 μ g/ml hygromycin, 100 μ g/ml G418, and 10 ng/ml doxycycline. Among hygromycin-resistant clones, several clones were randomly selected, and they were transferred into six-well tissue culture plates for cell expansion. To evaluate Akt activity, the double-stable PC-12 Tet-Off cell lines, containing Akt D+ or Akt D−, were screened for Akt expression and phospho-GSK3 evaluation.

Cell Treatments and Western Blot Analysis. PC-12 Tet-Off wild-type cells were first seeded on plastic dishes and then, after 24 h, they were treated with 100 ng/ml NGF or 25 μ M LY294002. Next, to induce Akt D+ and Akt D− gene expression after seeding, PC-12 Tet-Off Akt D+ and PC-12 Tet-Off Akt D− were deprived of doxycycline for 48 h. MG-132 (20 μ M) was used for 12 and 24 h (Lee et al., 2005).

For Western blot analysis, cells were washed in phosphate-buffered saline and collected by gentle scraping in ice-cold lysis buffer containing protease inhibitor cocktail II (Calbiochem). For Akt and GSK3 expression, proteins (50 μ g) were separated on 12% SDS-polyacrylamide gels and transferred onto Hybond ECL nitrocellulose membranes (Amersham). Membranes were blocked with 5% nonfat dry milk in 0.1% Tween 20 (Sigma) (2 mM Tris-HCl and 50 mM NaCl, pH 7.5) for 2 h at room temperature, and then they were incubated overnight at 4°C in the blocking buffer with the 1:1000 polyclonal antibody against Akt, p-Akt, GSK3 β , or p-GSK3 β . For

NCX1, NCX2, NCX3, PMCA, CREB1, and p-CREB1 expression, proteins (100 μ g) were separated on 8% SDS-polyacrylamide gels and transferred onto Hybond ECL nitrocellulose membranes (Amersham). Membranes were blocked with 5% nonfat dry milk in 0.1% Tween 20 (Sigma) (2 mM Tris-HCl and 50 mM NaCl, pH 7.5) for 2 h at room temperature, and then they were incubated overnight at 4°C in blocking buffer containing either monoclonal antibody (1:500) against NCX1; polyclonal antibody (1:200) against NCX2 and NCX3, respectively; monoclonal antibody (1:1000) against β -actin (Sigma); monoclonal antibody (1:1000) against PMCA ATPase (clone 5F10; ABR-Affinity BioReagents, Golden, CO); monoclonal antibody (1:1000) against CREB; polyclonal antibody (1:1000) against GSK3 β and p-GSK3 β (Cell Signaling Technology Inc., Danvers, MA); or polyclonal antibody (1:1000) against p-CREB (ser133) (Cell Signaling Technology Inc.). Finally, after the incubation with primary antibodies, membranes were washed with 0.1% Tween 20, followed by incubation with secondary antibodies for 1 h at room temperature. Immunoreactive bands were detected with the ECL reagent (Amersham). The optical density of the bands (normalized to β -actin in the experiments on NCX isoform expression) was determined by Chemi-Doc Imaging System (Bio-Rad, Hercules, CA).

Chemical Hypoxia. Chemical hypoxia was reproduced by adding to the cells, for 1 to 3 h, 5 μ g/ml oligomycin plus 2 mM 2-deoxyglucose in glucose-free medium composed of 145 mM NaCl, 5.5 mM KCl, 1.2 mM MgCl₂, 1.5 mM CaCl₂, and 10 HEPES, pH 7.4, as

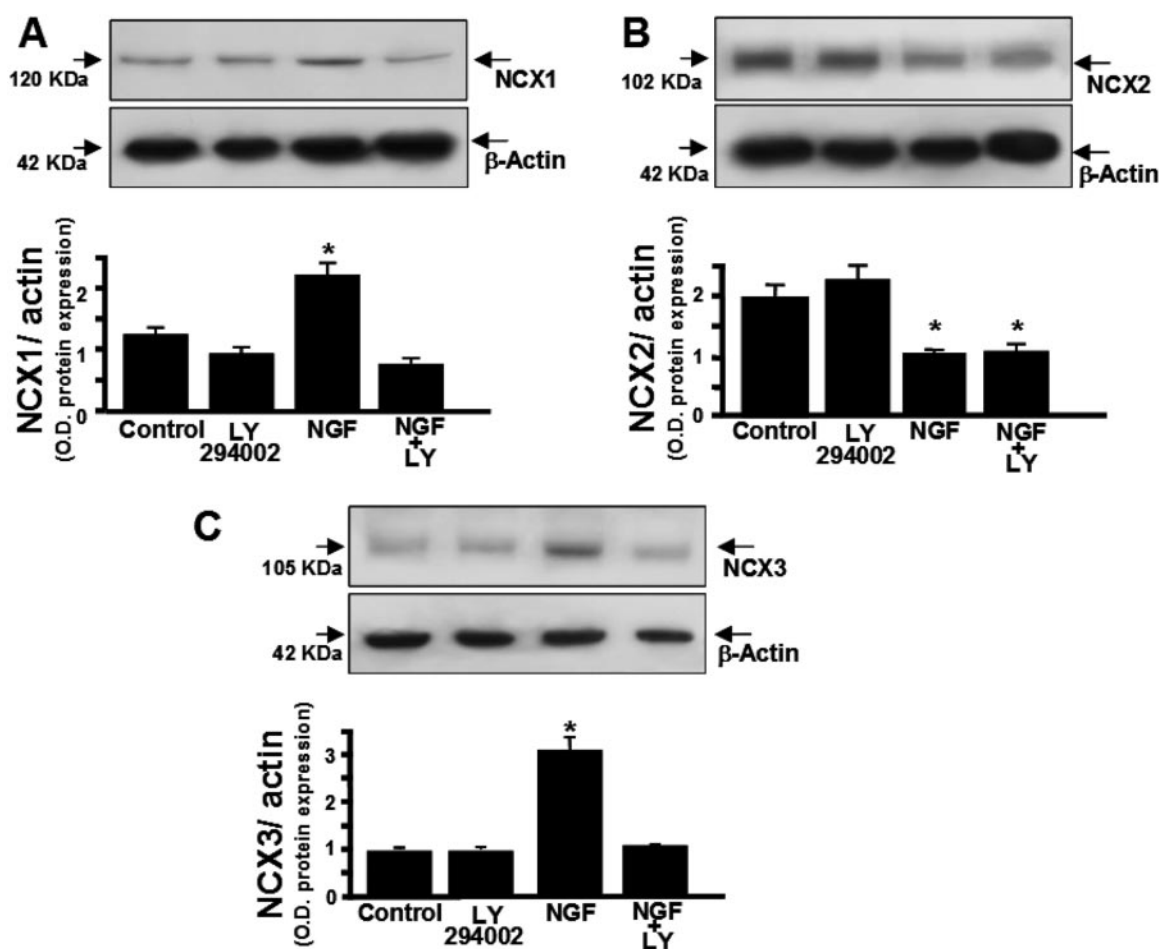


Fig. 2. Effect of the inhibition of the PI3-K/Akt pathway on NGF-induced NCX1, NCX2, and NCX3 expression regulation in PC-12 Tet-Off wild-type cells. A, representative Western blot of 100 ng/ml NGF for 24 h on NCX1 expression in the presence (25 μ M) or in the absence of LY294002. The bar graph shows the quantification of NGF effect on NCX1 expression, normalized to β -actin, and reported as optical density values. *, $p < 0.05$ versus all the other experimental groups. B, representative Western blot of 100 ng/ml NGF for 24 h on NCX2 expression in the presence (25 μ M) or in the absence of LY294002. *, $p < 0.05$ versus control and LY294002 groups. C, representative Western blot of 100 ng/ml NGF for 24 h on NCX3 expression in the presence (25 μ M) or in the absence of LY294002. *, $p < 0.05$ versus all the other experimental groups. All Western blots were performed in at least three different experimental sessions.

described previously (Amoroso et al., 1997, 2000). Control cells were exposed for the same amount of time to normal Krebs's solution composed of 145 mM NaCl, 5.5 mM KCl, 1.2 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4.

Determination of Cell Death. Cell death was evaluated by measuring the number of dead and living cells after each treatment. PC-12 Tet-Off wild-type and PC-12 Tet-Off Akt D+ cells were plated on six-well plates (approximately 500,000 cells in each well). To quantify cell death, PC-12 Tet-Off wild-type and PC-12 Tet-Off Akt D+ cells were washed with normal Krebs's solution and double stained with 36 μ M FDA and 7 μ M PI in a phosphate-buffered solution for 20 min at 37°C. Stained cells were examined immediately with a standard inverse fluorescence microscope at 480 and 546 nm (Amoroso et al., 1997). PI-positive and FDA-positive cells were counted in three representative high-power fields of independent cultures, and cell death was determined by calculating the ratio of the number of PI-positive cells/PI + FDA-positive cells (Wei et al., 2000).

Measurement of NCX Activity Evaluated as Na⁺-Dependent ⁴⁵Ca²⁺ Uptake and ⁴⁵Ca²⁺ Efflux. Na⁺-dependent ⁴⁵Ca²⁺ uptake into cells was measured by following the method described previously (Iwamoto et al., 2004), but with slight modifications (Secondo et al., 2007). PC-12 Tet-Off wild-type and PC-12 Tet-Off Akt D+ were plated on six-well plates (approximately 500,000 cells in each well). After 48 h, cells were incubated at 37°C for 10 min in normal Krebs's solution (5.5 mM KCl, 145 mM NaCl, 1.2 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, and 10 HEPES-NaOH, pH 7.4) containing 1 mM ouabain and 10 μ M monensin. Then, ⁴⁵Ca²⁺ uptake was initiated by switching the normal Krebs medium to Na⁺-free *N*-methyl-D-glucamine (5.5 mM KCl, 147 mM *N*-methyl glucamine, 1.2 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, and 10 mM HEPES-NaOH, pH 7.4) containing 10 μ M ⁴⁵Ca²⁺ (74 kBq/ml) and 1 mM ouabain. After 30-s

incubation, cells were washed with an ice-cold solution containing 2 mM La³⁺ to stop ⁴⁵Ca²⁺ uptake. Cells were subsequently solubilized with 0.1 N NaOH, and aliquots were taken to determine radioactivity and protein content. To measure ⁴⁵Ca²⁺ efflux, cells were loaded with 10 μ M ⁴⁵Ca²⁺ (74 kBq/ml) together with 1 μ M ionomycin for 60 s in normal Krebs's solution. Next, cells were exposed for 10 s to a Ca²⁺- and Na⁺-free solution, a condition that blocks both intracellular ⁴⁵Ca²⁺ efflux and extracellular Ca²⁺ influx. Intracellular ⁴⁵Ca²⁺ content in this condition represents a 0% efflux. ⁴⁵Ca²⁺ efflux was started by using Ca²⁺-free Na⁺-containing normal Krebs's solution plus 2 mM EGTA. To irreversibly prevent Ca²⁺ entrance in intracellular calcium stores and to cause a transient increase in calcium concentration, both Ca²⁺- and Na⁺-free solutions were added together with 1 μ M thapsigargin (Iwamoto et al., 2004). Cells were subsequently solubilized with 0.1 N NaOH, and aliquots were taken to determine radioactivity and protein content. Bradford method determined protein content (Bradford, 1976).

Small Interfering RNA. The mammalian expression vector pSUPER.retro.puro (OligoEngine, Seattle, WA) was used to express siRNA against NCX1 and NCX3 in PC12 Tet-Off Akt D+. This vector has the significantly increasing ability to integrate siRNA expression cassettes into the genome of mammalian cells. To prepare siRNA against NCX1, a 60-base oligonucleotide and another oligonucleotide with the complementary sequence were annealed and inserted into pSUPER.retro.puro, previously digested with BglII and XhoI according to the manufacturer's instructions. The gene-specific insert contained a 19-nucleotide sequence corresponding to the nucleotides 2000 to 2018 downstream of the transcription start site of rat NCX1 (GenBank accession no. NM_019268), whose specificity was verified by BLAST. A mismatch sequence cloned in the same vector and pSUPER.retro.puro vector itself were used as experimental controls.

To express siRNA against NCX3, the gene-specific insert con-

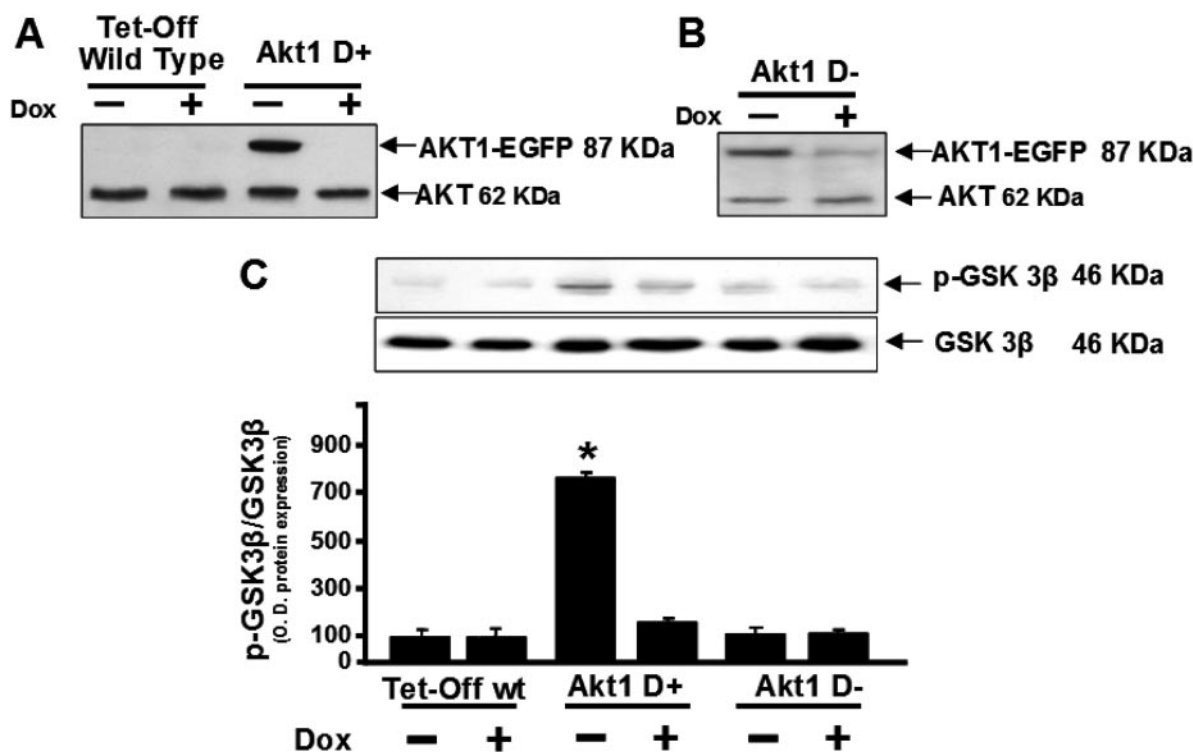


Fig. 3. Selection of positive and inactive mutants of double-stable Tet-Off PC-12 cells expressing Akt-1. A, representative Western blot of Akt-EGFP expression in positive mutants of double-stable cells (Akt D+), obtained after a 48-h exposure to a medium without doxycycline. B, representative Western blot of Akt-EGFP in inactive mutants of double-stable cells (Akt D-), obtained after a 48-h exposure to a medium lacking in doxycycline. C, typical Western blot of p-GSK3 β expression in PC-12 Tet-Off wild-type, Akt D+, and Akt D- cells incubated in the presence (10 ng/ml) or in the absence of doxycycline for 48 h. The bar graph at the bottom depicts the effect of doxycycline removal on p-GSK3 β , normalized to GSK3 β , in PC-12 Tet-Off wild-type, Akt D+, and Akt D- cells. *, $p < 0.05$ versus all the other experimental groups. All Western blots were studied in at least three different experimental sessions.

tained a 19-nucleotide sequence corresponding to coding region +124 to +142 relative to the first nucleotide of the start codon of rat NCX3 (GenBank accession no. U53420), whose specificity was verified by BLAST, was used. A mismatch sequence cloned in the same vector and pSUPER.retro.puro vector itself were used as experimental controls.

To verify the insertion of these specific sequences into the pSUPER vectors, the purified vectors were digested with BglII, thus rendering them unable to cut positive clones because the site was destroyed upon ligation. Therefore, successful ligation of the sequence resulted in a restriction pattern that was distinct from that of the unligated vectors. siRNA-CREB1 and siCONTROL nontargeting siRNA were from Dharmacon RNA Technologies.

After 24-h plating, PC-12 Tet-Off Akt D+ cells were transfected with pSUPER-NCX1, pSUPER-NCX3, pSUPER.retro.puro vectors, pSUPER-mismatch sequences, or siRNA-CREB and its control by means of the Ca^{2+} phosphate transfection standard method (Toyofuku et al., 1994). After 48 h, cells were lysed and used to quantify NCX1 or NCX3 protein expression together with PMCA protein expression, and then used as further control of silencing, by Western blot analysis, as described above.

RT-PCR Analysis. Total RNA was extracted from PC-12 Tet-Off wild-type and PC-12 Tet-Off Akt D+ with TRIzol (Invitrogen), following the instruction procedures of the supplier (Invitrogen). Total RNA was treated with ribonuclease-free deoxyribonuclease I (In-

vitrogen) for 15 min 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 RT-PCR (Invitrogen). Using 1/10 of the cDNAs as a template, the PCR was carried out under the following conditions: an initial denaturation at 95°C for 3 min, followed by 30 reaction cycles (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min) and by a final extension at 72°C for 10 min. The pairs of nucleotide used were 5'-ACCACAA-GACTACAGTGC-3' and 5'-TTGGAAGCTGGTCTGTCTCC-3' for NCX1 forward and reverse primer, respectively (Yu and Colvin, 1997); 5'-GCGTGTGGGCGATGCTCA-3' and 5'-GACCTCGAGGC-GACAGTTC-3' for NCX2 forward and reverse primer, respectively; and 5'-CTGGAAGAGGGGATGACCC-3' and 5'-GTTTAGGGTGT-TCACCCAATA-3' for NCX3 forward and reverse primer, respectively (Quednau et al., 1997); and 5'-CCTGCTGGATTACATTAAAGCACTG-3' and 5'-CCTGAAGTACTCATTATAGTCAAGG-3' for hypoxanthine-guanine phosphoribosyl transferase (HPRT). PCR products were analyzed by electrophoresis on 1.5% agarose gel. The optical density of the bands was determined by revising the Gel Image Analysis System (ChemiDoc Imaging System; Bio-Rad). Optical density measurements were normalized to the optical density of the HPRT band used as internal standard.

Statistical Analysis. Data are expressed as mean \pm S.E.M. Statistical comparisons between controls and treated experimental groups were performed using one-way analysis of variance,

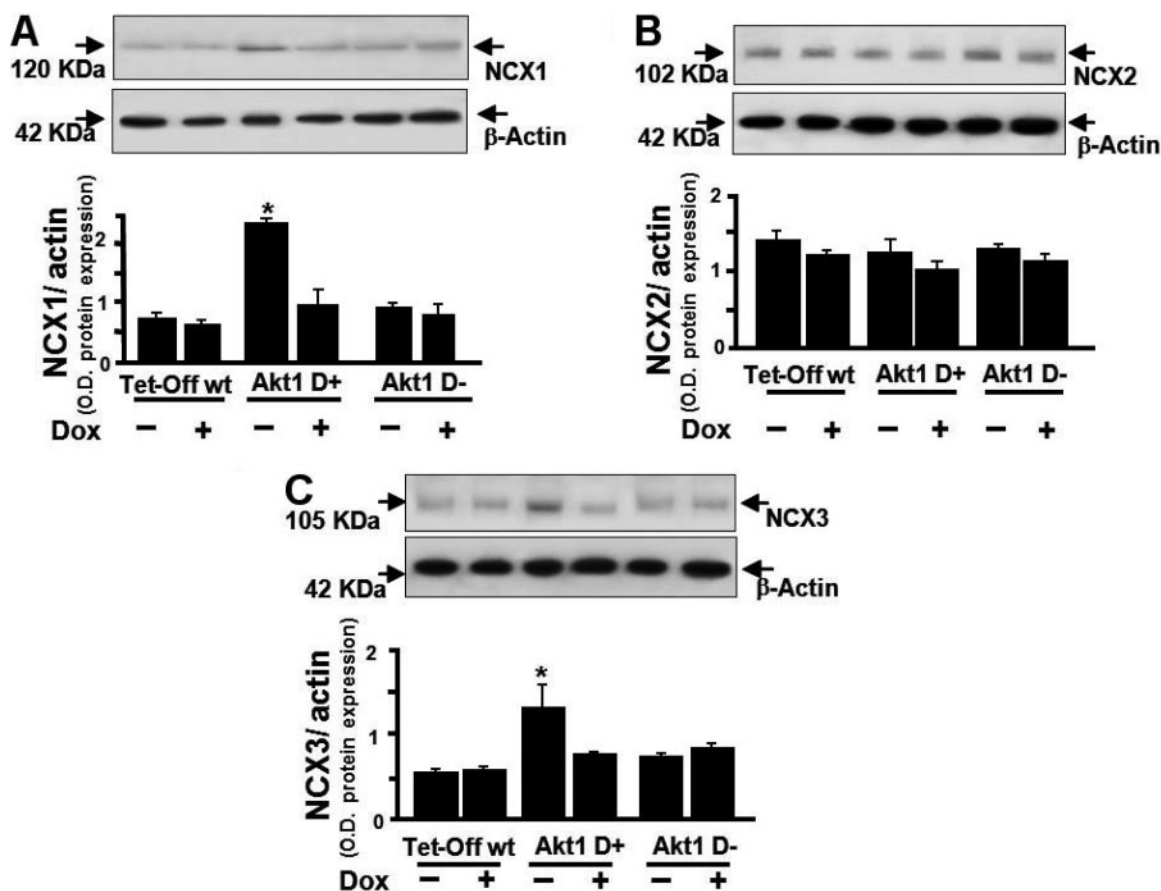


Fig. 4. Effect of doxycycline removal on NCX1, NCX2, and NCX3 protein expression in positive and inactive mutants of double-stable Tet-Off PC-12 cells expressing Akt1. A, representative Western blot of NCX1 expression in the presence (10 ng/ml) or in the absence of doxycycline in PC-12 Tet-Off wild-type positive mutants of double-stable cells (Akt D+), and inactive mutants of double-stable cells (Akt D-). The bar graph shows the quantification of NCX1 expression, normalized to β -actin, in PC-12 Tet-Off wild-type, Akt D+, and Akt D- cells, reported as optical density values. *, $p < 0.05$ versus all the experimental groups. B, representative Western blot of NCX2 expression in the presence (10 ng/ml) or in the absence of doxycycline in PC-12 Tet-Off wild-type, Akt D+, and Akt D- cells. The bar graph shows the quantification of NCX2 expression, normalized to β -actin, in PC-12 Tet-Off wild-type, Akt D+, and Akt D- cells, reported as optical density values. C, representative Western blot of NCX3 expression in the presence (10 ng/ml) or in the absence of doxycycline in PC-12 Tet-Off wild-type, Akt D+, and Akt D- cells. The bar graph shows the quantification of NCX3 expression, normalized to β -actin, in PC-12 Tet-Off wild-type, Akt D+, and Akt D- cells, reported as optical density values. *, $p < 0.05$ versus all the other experimental groups. All Western blots were studied in at least three different experimental sessions.

followed by Newman-Keuls test. $P < 0.05$ was considered statistically significant.

Results

Effect of the Inhibition of PI3-K/Akt Pathway on NGF-Induced Regulation of NCX1, NCX2, and NCX3 Expression in Tet-Off Wild-Type PC-12 Cells. NGF (100 ng/ml) induced a time-dependent increase in Akt phosphorylation, which peaked at 24 h and subsequently declined to basal values (Fig. 1). After 24-h incubation, NGF induced an up-regulation of NCX1 and NCX3 expression and a down-regulation of NCX2 (Fig. 2). The putative specific PI3-K inhibitor LY294002 at 25 μ M reverted NGF-induced NCX1 and NCX3 up-regulation (Fig. 2, A and C), whereas it did not revert NGF-induced NCX2 down-regulation (Fig. 2B).

Screening for Positive and Inactive Mutants in Double-Stable Tet-Off PC-12 Cells Expressing Akt1. In Akt1 active and inactive mutants, doxycycline removal induced the expression of a band at 87 kDa that corresponded to Akt1-EGFP, whereas it did not modify the endogenous 62-kDa Akt band expression (Fig. 3, A and B). In addition, upon removal of doxycycline, a 7-fold increase in the phosphorylation GSK3 β , a well known target of Akt, occurred only in Akt1-positive mutants, whereas no change was detectable in the inactive mutants (Fig. 3C).

NCX1, NCX2, and NCX3 mRNA and Protein Expression in Double-Stable Tet-Off PC-12 Cells Expressing Akt1. Akt1 active form expression induced by doxycycline removal caused an overexpression of NCX1 and NCX3 gene product, but not of NCX2 (Fig. 4, A–C). In contrast, the

overexpression of the Akt1 inactive form failed to modify the expression of all three NCX isoforms (Fig. 4, A–C).

RT-PCR analysis revealed that an increase of NCX1 but not of NCX2 and NCX3 transcripts occurred in the Akt1-positive mutants. (Fig. 5, A–C).

Effect of siRNA against CREB1 on NCX1 Expression in Double-Stable Tet-Off PC-12 Cells Expressing Akt1. Knocking down gene expression of CREB1, a well known Akt target (Fukunaga and Kawano, 2003), by sequence-specific siRNA prevented the increase of NCX1 protein expression in Akt1-positive mutants, whereas no reduction in NCX1 protein expression was observed in the presence of the specific control of siRNA against CREB (Fig. 6, A–C).

Effect of the Proteasome Inhibitor MG-132 on NCX3 Protein Expression in Wild-Type Tet-Off PC-12 Cells and in Double-Stable Tet-Off PC-12 Cells Expressing Akt1. MG-132 (20 μ M), the proteasome inhibitor of protein degradation (Chung Soo Lee et al., 2005), increased the expression of NCX3 in wild-type Tet-Off PC-12 cells. In contrast, in Akt1-positive mutants, in which there was an up-regulation of NCX3, the proteasome inhibitor did not cause a further increase of NCX3 protein levels (Fig. 7A). By contrast, the proteasome inhibitor did not cause any increase of NCX1 protein levels both in wild-type Tet-Off PC-12 cells and in Akt1-positive mutants (Fig. 7B). These results suggested that only the NCX3 up-regulation is induced by Akt1 activation through an inhibition of the ubiquitin-proteasome system.

NCX Activity in the Forward and in the Reverse Mode of Operation in Akt1-Positive Mutants and Tet-Off Wild-Type PC-12 Cells. NCX activity in Tet-Off wild-

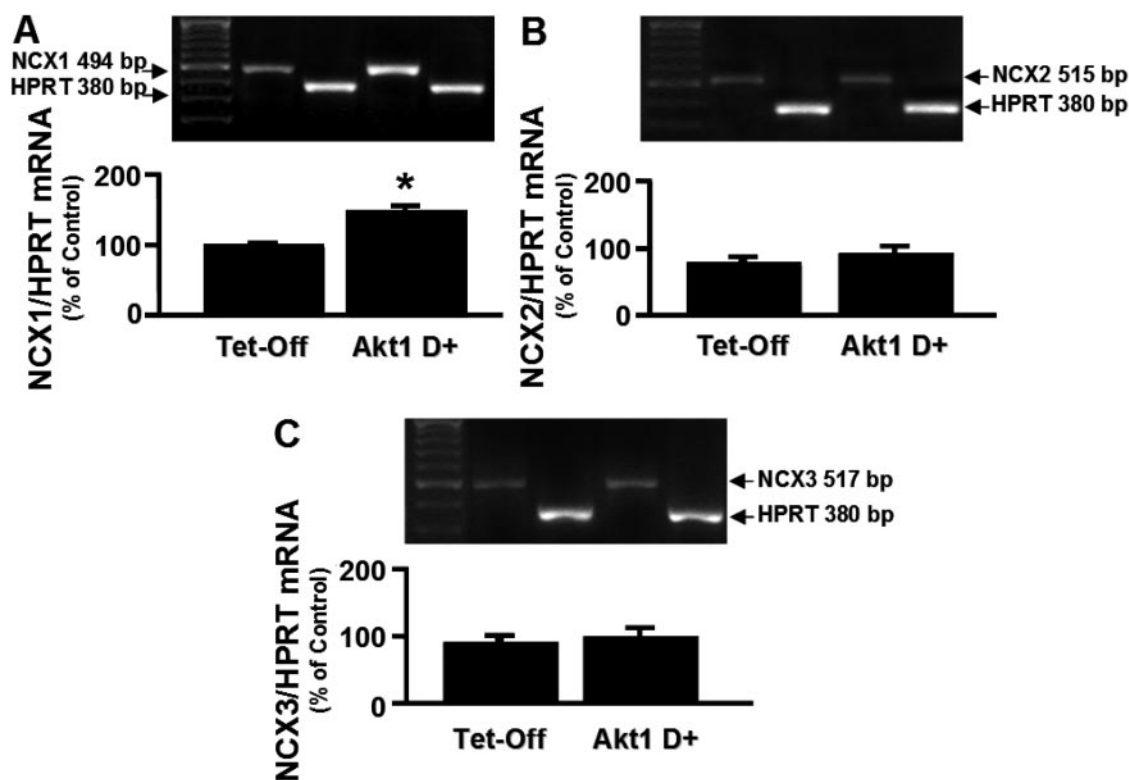


Fig. 5. Effect of doxycycline removal on NCX mRNA expression in PC-12 Tet-Off wild-type cells and positive mutants of double-stable cells (Akt D+). A, representative bar graph of NCX1 mRNA expression in PC-12 Tet-Off wild-type cells and Akt D+ cells. B, representative bar graph of NCX2 mRNA expression in PC-12 Tet-Off wild-type cells and Akt D+ cells. C, representative bar graph of NCX3 mRNA expression in PC-12 Tet-Off wild-type cells and Akt D+ cells. Data are normalized to HPRT mRNA. *, $p < 0.05$ versus Tet-Off wild-type cells.

type PC-12 cells and in Akt1-positive mutants was evaluated in the forward and reverse mode of operation with the help of $^{45}\text{Ca}^{2+}$ radiotracer flux (as reported under *Materials and Methods*). When NCX was induced to operate in the reverse mode of operation by extracellular Na^+ removal, its activity remarkably increased in doxycycline-deprived Akt1-positive mutants compared with Tet-Off wild-type cells (Fig. 8A). When NCX was induced to operate in the forward mode of operation, its activity was consistently and significantly increased in doxycycline-deprived Akt1 positive mutants compared with Tet-Off wild-type PC-12 cells (Fig. 8B).

Effect of NCX Silencing and NCX Pharmacological Paninhibition on Akt1-Increased Resistance to Chemical Hypoxia in PC-12 Tet-Off-Positive Mutants. As expected, when Akt1-positive mutants were exposed to chemical hypoxia for 1 or 3 h, they were more resistant (fluorescein-positive cells) than Tet-Off wild-type PC-12 cells (Fig. 9). It is noteworthy that the aspecific pharmacological paninhibitors of NCX isoform activity, the isothiourea derivative inhibitor KB-R7943 (Iwamoto and Shigekawa, 1998) and the amiloride analog 5-(N-4-chlorobenzyl)-2',4'-dimethylbenzamil (Amoroso et al., 1997; Annunziato et al., 2004), were all

able to completely revert the neuroprotective action exerted in the presence of the Akt1 active form (Fig. 10C). Moreover, RNA silencing of NCX1 or NCX3 gene product reduced the increased cell survival induced by the expression of the Akt1 active form (Fig. 10, A–C).

Discussion

The data emerging from the present study demonstrate that the overexpression of constitutively active Akt1, the most highly diffused and expressed isoform in the brain (Hanada et al., 2004; Hui et al., 2005), induces an up-regulation of NCX1 and NCX3. Regarding NCX1 up-regulation induced by Akt1 activation, this occurs at the transcriptional level because there was an increase in NCX1 transcript and it was counteracted by CREB1 inhibition through siRNA strategy. In contrast, Akt1-induced NCX3 up-regulation recognized a post-transcriptional mechanism occurring at the proteasome level because 1) NCX3 transcript did not increase and 2) the proteasome inhibitor MG-132 did not further enhance NCX3 protein levels in Akt1 active mutants as it would be expected if the ubiquitin-proteasome complex was

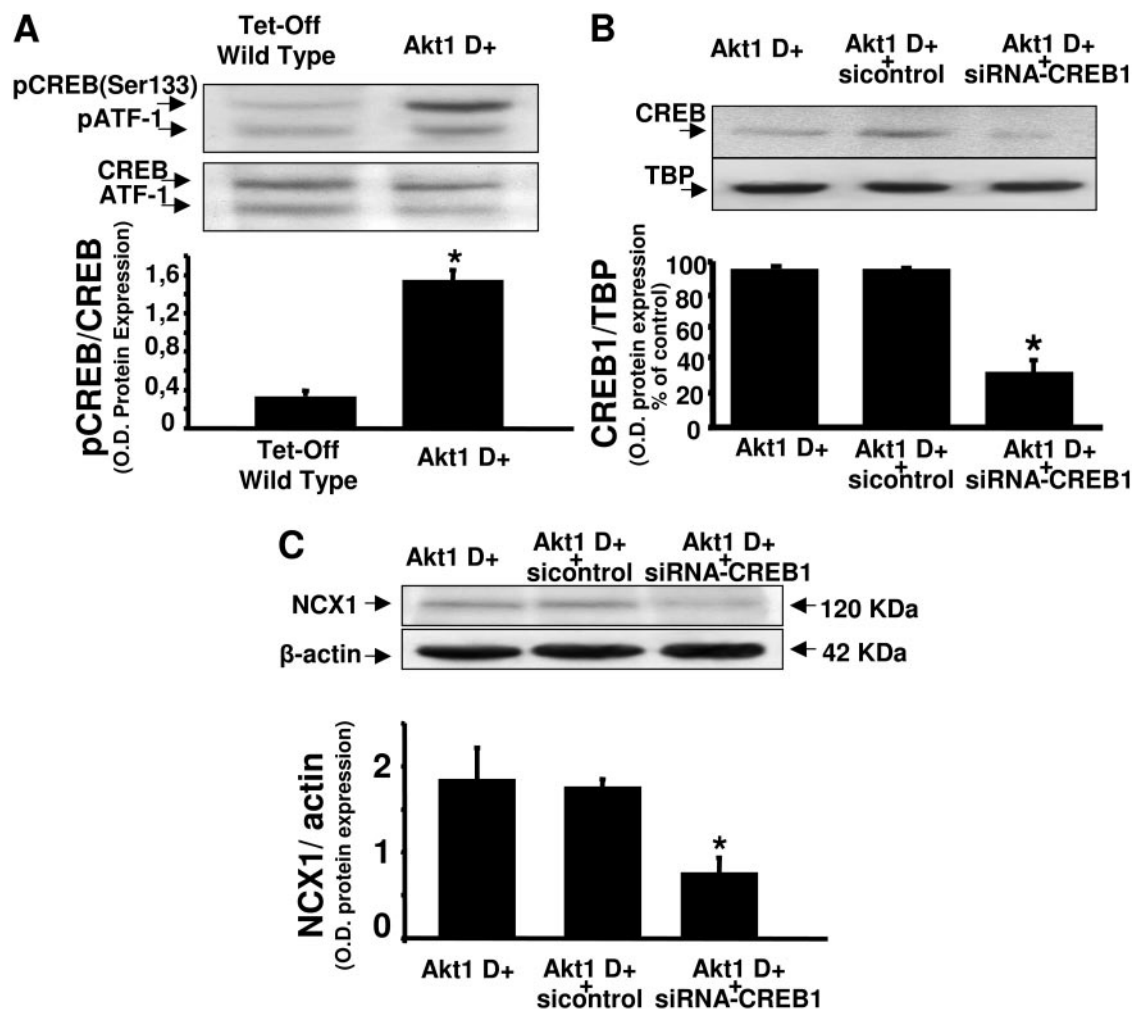


Fig. 6. Effect of CREB1 silencing on NCX1 protein expression in Akt1-positive mutants. A, Western blots representative of three different experiments of pCREB protein expression (top) and CREB protein expression (bottom) in PC-12 Tet-Off wild-type cells and Akt1-positive mutants. B, Western blot representative of three different experiments of the effect of siRNA-CREB1 and scrambled on CREB1 protein expression in Akt1-positive mutants. C, representative bar graph of the effect of the siRNA-CREB1 and scrambled on NCX1 protein expression in Akt1-positive mutants. *, $p < 0.05$ versus all the experimental groups.

not already blocked by Akt1 pathway. As expected, in PC-12 Tet-Off wild-type cells MG-132 enhanced NCX3 protein levels. NCX1 and NCX3 overexpression is accompanied by an increased activity of the exchanger isoform when it operates either in the forward or in the reverse mode. It is noteworthy that we demonstrated that the overexpression of NCX1 and NCX3 isoforms induced by this serine-threonine kinase was only partially responsible for the 80% reduction of hypoxia-induced cell death by Akt1-positive mutants, because siRNA-NCX1 and siRNA-NCX3 reduced this neuroprotective effect to values of ~26 and ~28%, respectively. This result suggested that Akt1, besides activating several neuroprotective pathways, produces an overexpression of NCX1 and NCX3 that may contribute to the reduction of hypoxia-induced cell death through an improvement of intracellular Ca^{2+} and Na^{+} dysregulation. Together, these findings indicate that NCX1 and NCX3 constitute novel and additional targets of Akt neuroprotective action.

The participation of NCX in neuroprotection and neurodegeneration during brain ischemia and neuronal anoxia is a recently emerging concept in neuroscience owing to the cru-

cial role played by this antiporter in the maintenance of intracellular Na^{+} and Ca^{2+} homeostasis (Amoroso et al., 1997, 2000; Annunziato et al., 2004). In fact, reduction in NCX1 and NCX3 expression by specific antisense oligonucleotides exacerbates ischemia-induced damage in permanent middle cerebral artery occlusion, whereas NCX2 knockdown does not (Pignataro et al., 2004). In contrast, the stimulation of NCX activity by redox agents (Reeves et al., 1986) reduces the extension of brain infarct volume (Pignataro et al., 2004).

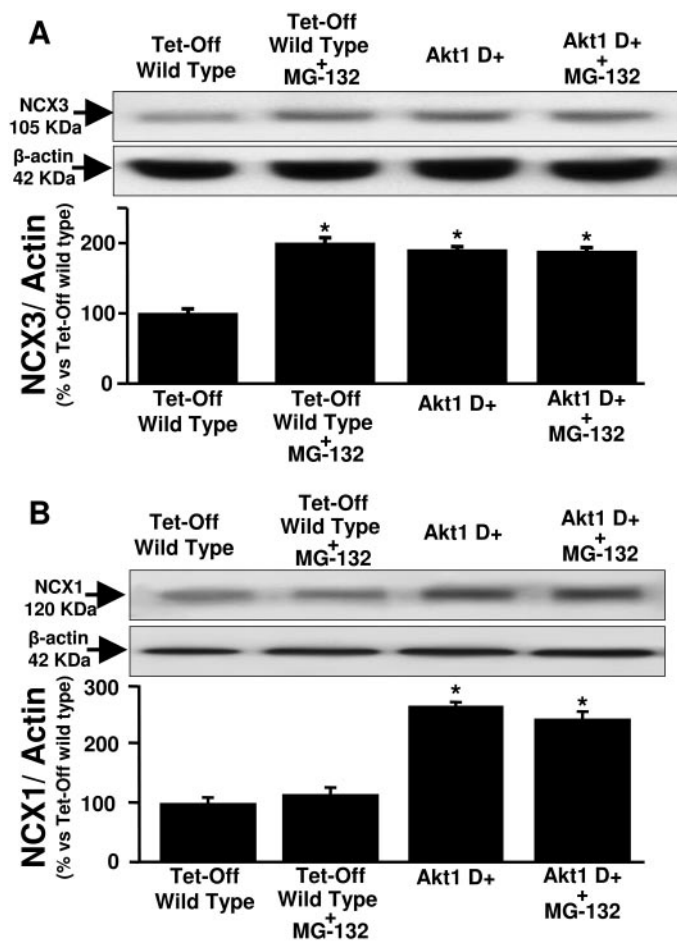


Fig. 7. Effect of proteasome inhibitor MG-132 on NCX3 and NCX1 protein expression in PC-12 Tet-Off wild-type cells and Akt1-positive mutants. A, Western blots representative of three different experiments of MG-132 effect on NCX3 protein expression (top) and β -actin protein expression (bottom) in Akt1-positive mutants and PC-12 Tet-Off wild-type cells. B, Western blots representative of three different experiments of MG-132 effect on NCX1 protein expression (top) and β -actin protein expression (bottom) in Akt1-positive mutants and PC-12 Tet-Off wild-type cells. *, $p < 0.05$ versus all the experimental groups.

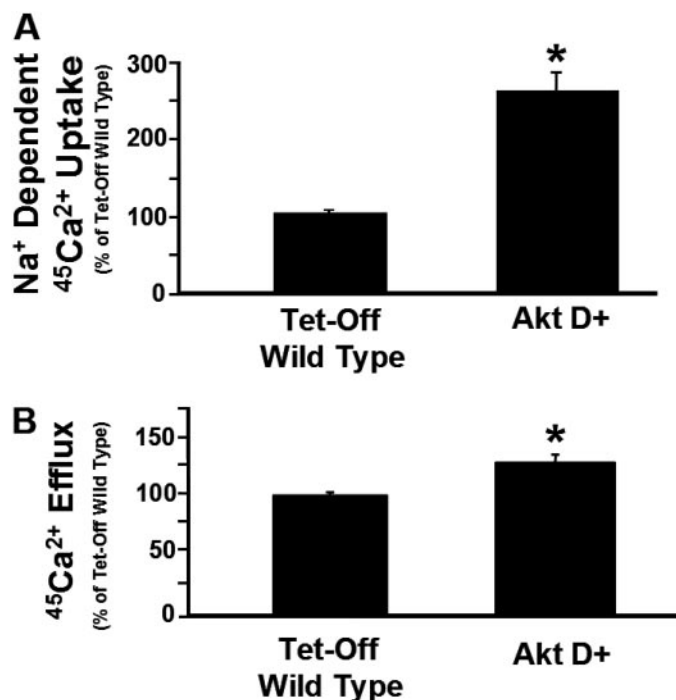


Fig. 8. Effect of doxycycline removal on NCX activity in the reverse and in the forward mode of operation evaluated as Na^{+} -dependent $^{45}\text{Ca}^{2+}$ uptake and $^{45}\text{Ca}^{2+}$ efflux, respectively, in PC-12 Tet-Off wild-type cells and positive mutants of double-stable cells (Akt D+). A, representative bar graph of NCX reverse mode activity evaluated as reported under *Materials and Methods* in PC-12 Tet-Off wild-type cells and Akt D+ cells. Data are normalized to protein content, and they are reported as percentage of the control (PC-12 Tet-Off wild-type cells). *, $p < 0.05$ versus control group (Student's t test). B, representative bar graph of NCX forward mode activity evaluated as reported under *Materials and Methods* in PC-12 Tet-Off wild-type cells and Akt D+ cells. Data are normalized to protein content and reported as percentage of the control (PC-12 Tet-Off wild-type cells). *, $p < 0.05$ versus control (Student's t test). The experiments were repeated in at least four experimental sessions.

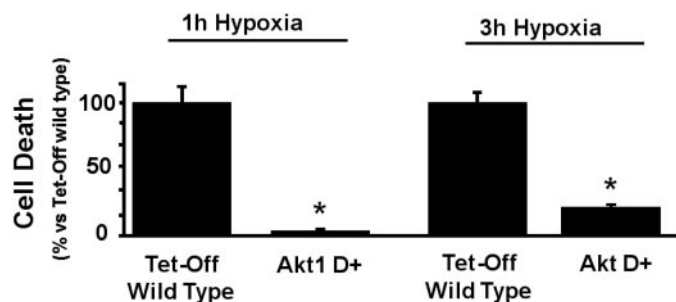


Fig. 9. Effect of chemical hypoxia on cell viability in PC-12 Tet-Off wild-type and positive mutants of double-stable cells (Akt D+). Bar graph represents the time course of the effect of chemical hypoxia on cell death in PC-12 Tet-Off wild-type and Akt D+ cells. *, $p < 0.05$ versus PC-12 Tet-Off wild-type cells (Student's t test). The experiments were repeated in at least four experimental sessions.

It is noteworthy that NCX1 and NCX3 transcripts are significantly up-regulated in the peri-infarct area (Boscia et al., 2006), a zone where a large population of neurons is able to survive the hypoxic insult because of the prevalence of neurotrophic factors (Lee et al., 1998). The relevance of the activity of NCX1 and NCX3 isoforms in brain ischemia has been further confirmed by the evidence that, during transient middle cerebral artery occlusion, the two exchanger isoforms NCX1 and NCX3 are cleaved in the cortex by caspases and calpains, respectively (Bano et al., 2005). In addition, it has recently been shown that the overexpression of the transcriptional repressor of the NCX3 gene promoter, named downstream regulatory element antagonist modulator, determines an increase in intracellular Ca^{2+} concentration and renders cerebellar granule cells more vulnerable to

the increased Ca^{2+} influx after partial opening of voltage-gated plasma membrane Ca^{2+} channels (Gomez-Villafuertes et al., 2005).

Actually, the relevance of the increased expression and activity of NCX1 and NCX3 in protecting Tet-Off PC-12 Akt1-positive mutants from chemical hypoxia lies in the role played by these two antiporter isoforms in handling the intracellular dysregulation of Na^+ and Ca^{2+} ions (Reuter et al., 2003). In fact, during severe anoxic conditions, the blockade of the two ATP-dependent pumps, Na^+/K^+ ATPase and Ca^{2+} ATPase, produces an intracellular overload of Na^+ ions that, in turn, triggers NCX1 and NCX3 to operate in the reverse mode. As a result, Na^+ homeostasis is re-established, and neuronal swelling and microtubule disorganization are prevented (Syntichaki and Tavernara-

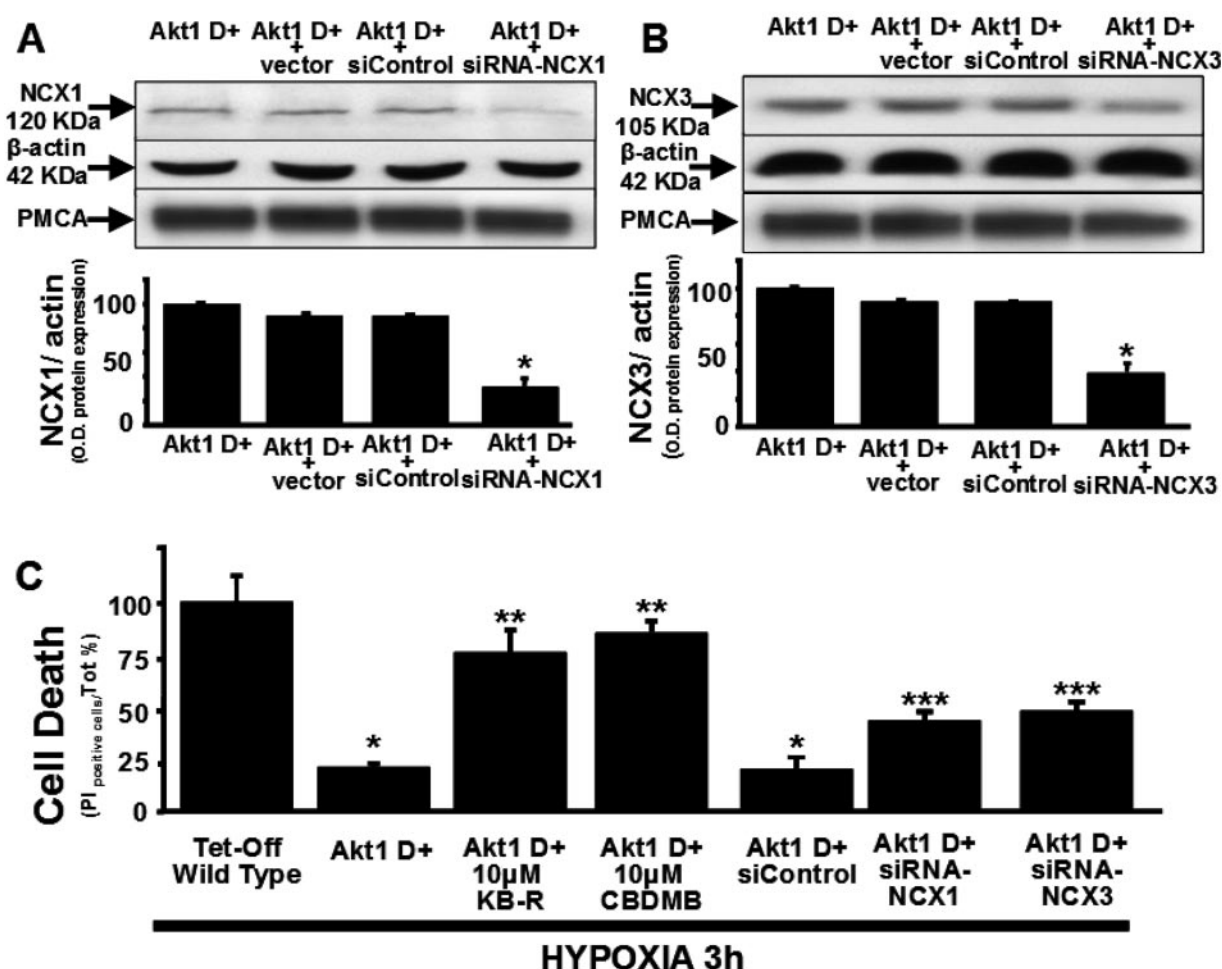


Fig. 10. Effect of NCX1 and NCX3 silencing and NCX pharmacological paninhibition on Akt-1-induced resistance to chemical hypoxia in positive Akt D+ mutants. Akt D+ cells were transiently transfected with siRNA control vector or with the vector targeting NCX1 or a mismatch sequence and then lysed and immunoblotted with anti-NCX1 as reported under *Materials and Methods*. A, Western blots representative of three different experiments of the effect of siRNA-NCX1 on NCX1 (top), PMCA ATPase (middle), and β -actin protein expression (bottom) in Akt D+ cells. At the bottom of A, a bar graph representing the quantification of the effects of siRNA against NCX1 and its controls on protein expression is reported as percentage of reduction (*, $p < 0.05$ versus basal expression). Akt D+ cells were transiently transfected with siRNA control vector or with the vector targeting NCX3 or a mismatch sequence, and then they were lysed and immunoblotted with anti-NCX3 as reported under *Materials and Methods*. B, Western blots representative of three different experiments of the effect of siRNA-NCX3 on NCX3 (top) PMCA ATPase (middle), and β -actin protein expression (bottom) in Akt D+ cells. At the bottom of B, a bar graph representing the quantification of the effects of siRNA against NCX3 and its controls on protein expression is reported as percentage of reduction (*, $p < 0.05$ versus basal expression). C, representative bar graph of the effect of the NCX inhibitors KB-R7943 (10 μM) and 5-(*N*-4-chlorobenzyl)-2',4'-dimethylbenzamil (10 μM), siRNA against NCX1, siRNA against NCX3, and, finally, of the vector (pSUPER.retro.puro) on chemical hypoxia-induced cell death in Akt D+ cells (see *Materials and Methods*). The experiments were repeated in at least four experimental sessions. *, $p < 0.05$ versus PC-12 Tet-Off wild-type; **, $p < 0.05$ versus PC-12 Tet-Off wild-type and Akt D+ groups; and ***, $p < 0.05$ versus all the other experimental groups.

kis, 2003). Instead, in neuronal cells that are exposed to a milder anoxic insult, a condition characterized by a lesser ATP depletion, NCX1 and NCX3 may work in the forward mode by extruding Ca^{2+} ions.

It is noteworthy that studies performed on cardiomyocytes overexpressing the constitutively active heterotrimeric protein $\text{G}\alpha_q$ —a protein coupled with seven transmembrane receptors activated by norepinephrine, prostaglandin $\text{F}_{2\alpha}$, endothelin, and angiotensin II—showed that these cardiac cells display a $\text{G}\alpha_q$ -induced decrease in Akt and NCX1 expression that leads to apoptotic cell death (Miyamoto et al., 2005).

The functional relationship between Akt and NCX1 and NCX3 expression is further highlighted by the results obtained with NGF in Tet-Off wild-type PC-12 cells. Indeed, when Trk-A receptors were stimulated by NGF (Chao, 2003), we observed an up-regulation of NCX1 and NCX3 expression that was prevented by LY294002, an inhibitor of PI3-K, the upstream modulator of Akt. These findings indicate that this NGF/Trk-A/Shc/PI3-K/Akt/NCX1-NCX3 transductional pathway is most likely operative in conditions in which an enhanced NGF synthesis and release occurs. Conversely, NGF/Trk-A/Shc/PI3-K/Akt is not involved in the modulation of the third isoform NCX2,

because in Akt1-positive mutants this gene product is not changed upon doxycycline removal. In addition, the NCX2 decrease observed upon NGF exposure is not modulated by this transductional pathway because the PI3-K inhibitor LY294002 did not revert this down-regulation.

That Akt may influence plasma membrane and intracellular proteins involved in ionic intracellular homeostasis such as L-type voltage-gated channels (Blair et al., 1999; Viard et al., 2004), sarco(endo)plasmic reticulum Ca^{2+} ATPase (Rota et al., 2005), and ryanodine receptors (Barac et al., 2005) has been reported previously.

Therefore, the novelty of the present study lies in the fact of having highlighted other relevant targets for the prosurvival action of Akt/PKB pathway: NCX1 and NCX3 gene products (Fig. 11). Our efforts, together with those of previous and emerging research on the key role played by NCX in regulating intracellular ionic homeostasis (Herschel et al., 2002), may lead to a more insightful understanding of the mechanisms underlying neuronal survival. These observations might contribute to the development of targeted compounds that could reduce brain damage induced by ischemia.

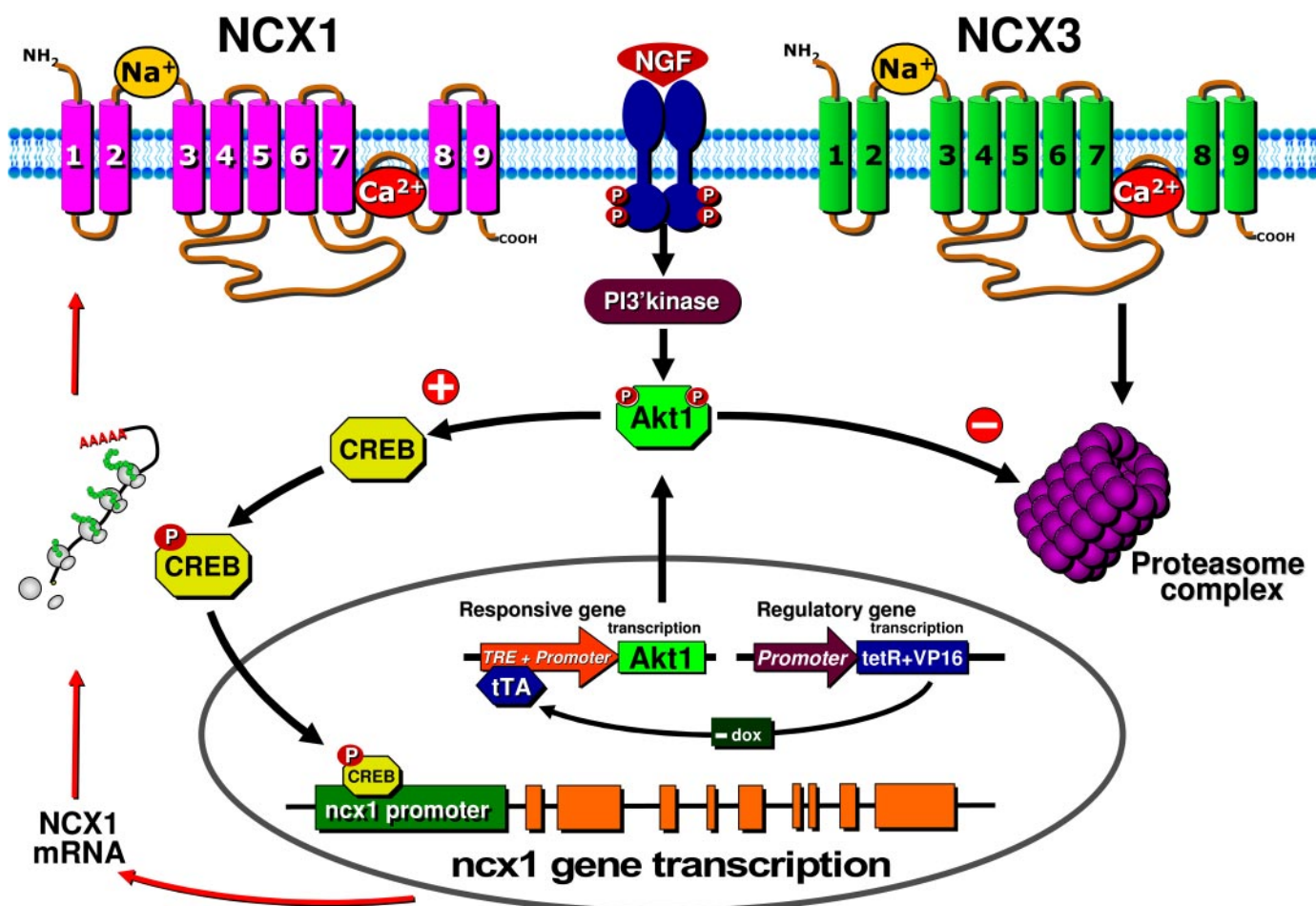


Fig. 11. Scheme representing the effect of PI3-K/Akt1 transductional pathway activated by NGF or by Akt1-positive mutant on *ncx1* gene expression and NCX3 post-translational regulation. Tet-Off strategy, used to regulate Akt1-positive dominant expression is schematically represented into the nucleus. Promoter, cytomegalovirus promoter; tetR, tetracycline repressor gene; TetR + VP16, tetracycline-controlled tTA; TRE, tetracycline-responsive element; dox, doxycycline.

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