ELSEVIER

Cell Calcium 43 (2008) 482–491

cell calcium

www.elsevier.com/locate/ceca

Na^+/Ca^{2+} exchanger 2 is neuroprotective by exporting Ca^{2+} during a transient focal cerebral ischemia in the mouse

Daejong Jeon [a,b,1], Kon Chu [c,1], Keun-Hwa Jung [c,d], Manho Kim [c], Byung-Woo Yoon [c], C. Justin Lee [a], Uhtaek Oh [b,*], Hee-Sup Shin [a,**]

[a] *Center for Neural Science, Korea Institute of Science and Technology, Seoul 136-791, Republic of Korea*
[b] *Sensory Research Center, Creative Research Initiatives, Seoul National University, College of Pharmacy, Seoul 151-742, Republic of Korea*
[c] *Department of Neurology and Clinical Research Institute in Seoul National University Hospital, Seoul National University, Seoul 110-744, Republic of Korea*
[d] *Division of Epidemic Intelligence, Department of Disease Investigation and Surveillance, Korea Center for Disease Control and Prevention, Seoul, Republic of Korea*

Received 12 May 2007; received in revised form 28 July 2007; accepted 8 August 2007
Available online 19 September 2007

Abstract

Na^+/Ca^{2+} exchanger (NCX), by mediating Na^+ and Ca^{2+} fluxes bi-directionally, assumes a role in controlling the Ca^{2+} homeostasis in the ischemic brain. It has been suggested that the three isoforms of NCX (NCX1, 2 and 3) may be differentially involved in permanent cerebral ischemia. However, the role of NCX2 has not been defined in ischemic reperfusion injury after a transient focal cerebral ischemia. Furthermore, it is not known whether NCX2 imports or exports intracellular Ca^{2+} ($[Ca^{2+}]_i$) following ischemia and reperfusion. To define the role of NCX2 in ischemia and reperfusion, we examined mice lacking NCX2, *in vivo* and *in vitro*. After an *in vitro* ischemia, a significantly slower recovery in population spike amplitudes, a sustained elevation of $[Ca^{2+}]_i$ and an increased membrane depolarization were developed in the NCX2-deficient hippocampus. Moreover, a transient focal cerebral ischemia *in vivo* produced a larger infarction and more cell death in the NCX2-deficient mouse brain. In particular, in the wild type brain, NCX2-expressing neurons were largely spared from cell death after ischemia. Our results suggest that NCX2 exports Ca^{2+} in ischemia and thus protects neuronal cells from death by reducing $[Ca^{2+}]_i$ in the adult mouse brain.

Keywords: Cerebral ischemia; Na^+/Ca^{2+} exchanger; NCX2-deficient mice; $[Ca^{2+}]_i$; Population spike; Membrane depolarization

1. Introduction

An important event during ischemia–reperfusion injury is the loss of ionic homeostasis, which causes permanent cell injury. The major changes underlying neuronal injury are increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and Na^+ ($[Na^+]_i$) which trigger the process leading to cell death [1]. The Na^+/Ca^{2+} exchanger (NCX) is an antiporter located in the plasma membrane that electrogenically couples, in a bi-directional way, the mobilization of 1 Ca^{2+} and 3 Na^+ ions depending on the electrochemical gradients across the plasma membrane [2–4]. Under some conditions where a substantial elevation of $[Ca^{2+}]_i$ is affected, NCX, with its high turnover rates for Ca^{2+}, is considered as a relevant system to controlling $[Ca^{2+}]_i$ Normally the NCX serves as a Ca^{2+} extrusion mechanism driven by the Na^+ electrochemical gradient (forward mode). However, accumulation of $[Na^+]_i$ caused by inhibition of Na^+/K^+ ATPase activity due to decreased ATP production during ischemia can cause NCX to operate

* Corresponding author at: National CRI Center for Sensory Research, Seoul National University, College of Pharmacy, Seoul 151-742, Republic of Korea. Tel.: +82 2 880 7854; fax: +82 2 872 0596.
** Corresponding author at: Center for Neural Science, Korea Institute of Science and Technology, Seoul 136-791, Republic of Korea. Tel.: +82 2 958 6931; fax: +82 2 958 6937.
E-mail addresses: utoh@plaza.snu.ac.kr (U. Oh), shin@kist.re.kr (H.-S. Shin).
[1] These authors contributed equally to this work.

0143-4160/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ceca.2007.08.003

reversibly, leading to an influx of Ca^{2+}, which may contribute to cell damage (reverse mode) [2–4]. Although NCX working in the reverse mode would be harmful to neuronal survival, NCX activity can return to the forward mode during reperfusion supplying oxygen and glucose, which would be beneficial to neuronal survival [5].

Three different NCX isoforms (NCX1, 2 and 3) are encoded by distinct genes in mammals [6–8]. Although NCX isoforms share similar biophysical properties, they show different sensitivity to ATP levels and differentially expressed both during development and in adults [9–11]. Recently, substantial evidence has suggested that the transcripts and proteins of NCX isoforms are differentially regulated after a permanent middle cerebral artery occlusion (pMCAO) [12,13]. In the ischemic core region, the transcripts and proteins of all NCX isoforms were down-regulated. However, in the peri-infart area only NCX1 and NCX3 transcripts were up-regulated. Moreover, antisense oligodeoxynucleotides (AS-ODNs) for NCX1 and NCX3 worsened the severity of the neuronal damage after pMCAO, but the AS-ODN for NCX2 did not change the infarct size [12]. These results suggest that NCX1 and NCX3 play a major role in the development of permanent focal cerebral ischemia, but the role of NCX2 remains undefined.

In a transient ischemic injury model, NCX1 and NCX3 protein was also degraded after transient MCAO (tMCAO), whereas NCX2 protein levels were preserved [14,15]. However, it is unknown what roles the persistent NCX2 proteins after tMCAO play in ischemia–reperfusion injury. In addition, there have been no studies defining the mode of action of NCX2, forward or reverse, during ischemia and reperfusion.

To better understand the cellular role played by NCX2 in ischemia–reperfusion injury in the brain, we performed tMCAO on mice lacking NCX2 [16], the predominant isoform in adult brains and analyzed the consequences. Furthermore, on brain slices after an *in vitro* ischemia we measured the changes in population spike (PS) amplitudes, $[Ca^{2+}]_i$ and membrane potentials to define the role of NCX2 in the Ca^{2+} homeostasis during ischemic insults. Our results suggest that NCX2 has a protective role in ischemic cell death by reducing $[Ca^{2+}]_i$ in the adult mouse brain.

2. Materials and methods

2.1. Animals

The generation of mice lacking NCX2 was described in our previous report [16]. NCX2 heterozygous (NCX2+/−) mice were backcrossed into two inbred mice, C57BL/6J and 129S4/SvJae, each over nine generations. NCX2 wild-type (NCX2+/+) and NCX2-deficient (NCX2−/−) mice used for analysis were obtained from interbreeding NCX2+/− mice of the two backgrounds. Animal care and handling were carried out according to institutional guidelines. The mice were maintained with free access to food and water under a 12:12 h light:dark cycle. All experiments were performed on 10–13-week-old mice, except for Ca^{2+} imaging and patch-clamp recording, for which mice 3–5 weeks old were used. All experiments were performed in a blind manner with respect to the genotype.

2.2. Extracellular recording on hippocampal slices

Preparation of hippocampal slices and the method of recording have been described previously [16]. Hippocampal slices (400 μm) were prepared in oxygenated cold artificial cerebrospinal fluid (ACSF) (124 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$ and 10 mM glucose at pH 7.4). Slices were then placed at a warm, humidified (32 °C, 95% O_2–5% CO_2) recording chamber containing oxygenated ACSF and maintained for 1 h prior to experiments. A bipolar stimulating electrode was placed in the stratum radiatum and population spike (PS) amplitudes were recorded in the stratum pyramidale in the CA1 region using a glass microelectrode (borosilicate glass, 3–5 MΩ, filled with 3 M NaCl). Test responses were elicited at 0.033 Hz. The baseline stimulation was delivered at an intensity that evoked a response approximately 60% of the maximum evoked response. *In vitro* ischemia was induced by changing the atmospheric gas composition from 95% O_2–5% CO_2 to 95% N_2–5% CO_2 (oxygen and glucose deprivation, OGD) in the ACSF in which glucose was replaced by mannitol. OGD was induced for 5, 7.5 and 10 min. One hour after ischemic insult, slices were stained with hematoxylin and eosin for histological analysis. Photographs were taken using a microscope-mounted camera with an interference-contrast filter. Cell numbers were determined by counting all viable cells within the principal cell body layer contained in a 200× field of view, at six different positions within the hippocampal formation, including three equally spaced areas from CA1. Total counts in these sampling regions were converted into cell densities for quantification and data were analyzed using Student's *t*-test.

2.3. Measurement of intracellular Ca^{2+} changes and whole-cell patch-clamp recording

Preparation of hippocampal slices (250 μm) and the method of recording in CA1 pyramidal neurons were described in our previous report [17]. Visually guided whole-cell patch recordings were obtained from CA1 pyramidal neurons in the current-clamp configuration. Pipettes were filled with a solution containing 140 mM Cs-gluconate, 4 mM NaCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM EGTA, 5 mM HEPES, 2 mM Mg-ATP and 0.3 mM Na-GTP, and the pH was adjusted to 7.4 by CsOH. For measurement of intracellular Ca^{2+} changes from a cell body, cell impermeant Fura-2 pentapotassium salt (100 μM, Molecular Probes) was added to the internal solution and allowed to diffuse for 20 min after entering whole-cell mode. Ratio of Fura-2 was taken at 340 and 380 nm excitation wavelengths and images were acquired

every 2 s using Axon Imaging Workbench version 5.2. Ca^{2+} signals (340/380 nm) were normalized by the baseline levels measured during 1 min period just before OGD. An ischemic insult *in vitro* was induced by 2 min OGD, and within 2 min of OGD all the recorded neurons showed peak $[Ca^{2+}]_i$ values. Simultaneously, membrane potentials were recorded and digitized at 10 kHz (Axon Instruments, Multiclamp 700A, DIGIDATA 1322A). The amplitudes of membrane potential were analyzed with the pCLAMP version 9.2 (Axon Instruments) and the Mini Analysis Program (Synaptosoft).

2.4. Focal cerebral ischemia with reperfusion, and preparation of brain tissue

Focal cerebral ischemia was induced using the intraluminal thread occlusion of middle cerebral artery (MCAO) for 120 min, as previously described [18]. After 120 min of MCA occlusion, the reperfusion of the MCA was initiated by removing the MCA occlusive filament. Animals were allowed for food and water ad libitum post-operatively. Rectal temperature was maintained at $37 \pm 0.5\,^{\circ}C$ using a thermistor-controlled heating blanket. Mean arterial blood pressure, arterial blood gases, pH, blood glucose and rectal temperatures were recorded. Mean arterial blood pressures were determined during occlusion and the first 30 min of reperfusion. Arterial blood samples obtained with use of a femoral catheter were analyzed at baseline and at the end of ischemia.

2.5. Measurement of infarct volumes

The brain was removed and cut from the frontal tip in 1 mm slices which were then immersed in a 2% solution of 2,3,7-triphenyltetrazolium chloride (TTC), as described previously [18]. Stained slices were then fixed in phosphate-buffered 4% paraformaldehyde, and the infarcted and total hemispheric areas of each section were traced and measured using an image analysis system (Image-Pro Plus, Media Cybernetics). Infarction volumes were calculated using three-dimensional reconstruction software, and were determined by two independent investigators blind to the type of section.

2.6. Histology and TUNEL assay

Immunostaining was performed as described previously [19]. On day 1 post-ischemia, rats were anesthetized and perfused through the heart with 50 ml of cold saline and 50 ml of 4% paraformaldehyde, in 0.1 mol/l PBS. Brains were then removed and either cryopreserved as tissue blocks for cryostat sectioning at 30 μm. Coronal sections through the striatum were selected. To count neurons, samples were stained with the following antibodies: anti-NCX2 (1:500, Santa Cruz); anti-NeuN (1:200, Chemicon). FITC-conjugated anti-sheep IgG (1:100, Biodesign) or Cy3-conjugated anti-mouse IgG antibodies (1:300, Jackson Immunoresearch) were used as secondary antibodies. Colocalizations were analyzed by laser scanning confocal microscopy using a Bio-Rad MRC 1024 unit (argon and krypton). The fluorescence signals were detected at excitation/emission wavelengths of 550/570 nm (Cy3, red) and 488/522 (FITC, green). The TUNEL procedure was used for *in situ* detection of DNA fragmentation. Cell density counts were performed on sections counterstained with toluidine blue. Five coronal sections through the center of the infarct lesion were analyzed. Square sampling regions (300 μm × 300 μm) in the medial striatum were used for cell counting. Five sampling regions were placed along the infarct border. Total counts in these sampling regions were converted into cell densities for quantification.

2.7. Statistical analysis

Data are given as mean ± S.E.M. (electrophysiological study) or S.D. (other studies). Student's *t*-test was used for statistical analyses. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Reduced PS amplitudes in the NCX2−/− after OGD

PS recorded from the CA1 pyramidal cell layer represent the healthiness of the brain slice, and the degree of proper synaptic transmission and cell firing. To test whether NCX2 has a role in ischemia–reperfusion injury, we recorded and assessed the changes in field responses before and after ischemic insult *in vitro*. Hippocampal slices from both NCX2+/+ and NCX2−/− mice were subjected to OGD for 5, 7.5 or 10 min, after which O_2 and glucose supplies were re-established. In the 5 min ischemia experiments, in both genotypes, PS amplitudes were completely depressed by the initiation of OGD, but then fully recovered (Fig. 1A). However, NCX2−/− slices ($n = 10$) showed a significantly slower recovery of the PS amplitudes to the normal level than NCX2+/+ slices ($n = 13$) (+/+, 99.5 ± 6.7% of baseline at 20 min; −/−, 78.9 ± 7.8%, $p < 0.0001$, Student's *t*-test) (Fig. 1A). In the 7.5 min experiments, the recovery was not complete for both genotypes. However, there was a significantly less recovery of PS amplitudes in NCX2−/− slices ($n = 15$) than in NCX2+/+ slices ($n = 12$) (+/+, 66.7 ± 18.4% of baseline at 20 min; −/−, 20.8 ± 8.9%, $p < 0.00001$, Student's *t*-test) (Fig. 1B). These data indicate that NCX2−/− slices were more vulnerable to *in vitro* ischemic injury compared to wild-type tissue. After 10 min of ischemic insult there was no PS recovery in either genotype tissue (data not shown).

One hour after the 7.5 min ischemia, slices were stained with hematoxylin and eosin to determine cell damage in the CA1 region histologically by counting all viable cells within the principal cell body layer contained in a 200× field of view (Fig. 1C). Compared to the control, non-ischemic tissue, both

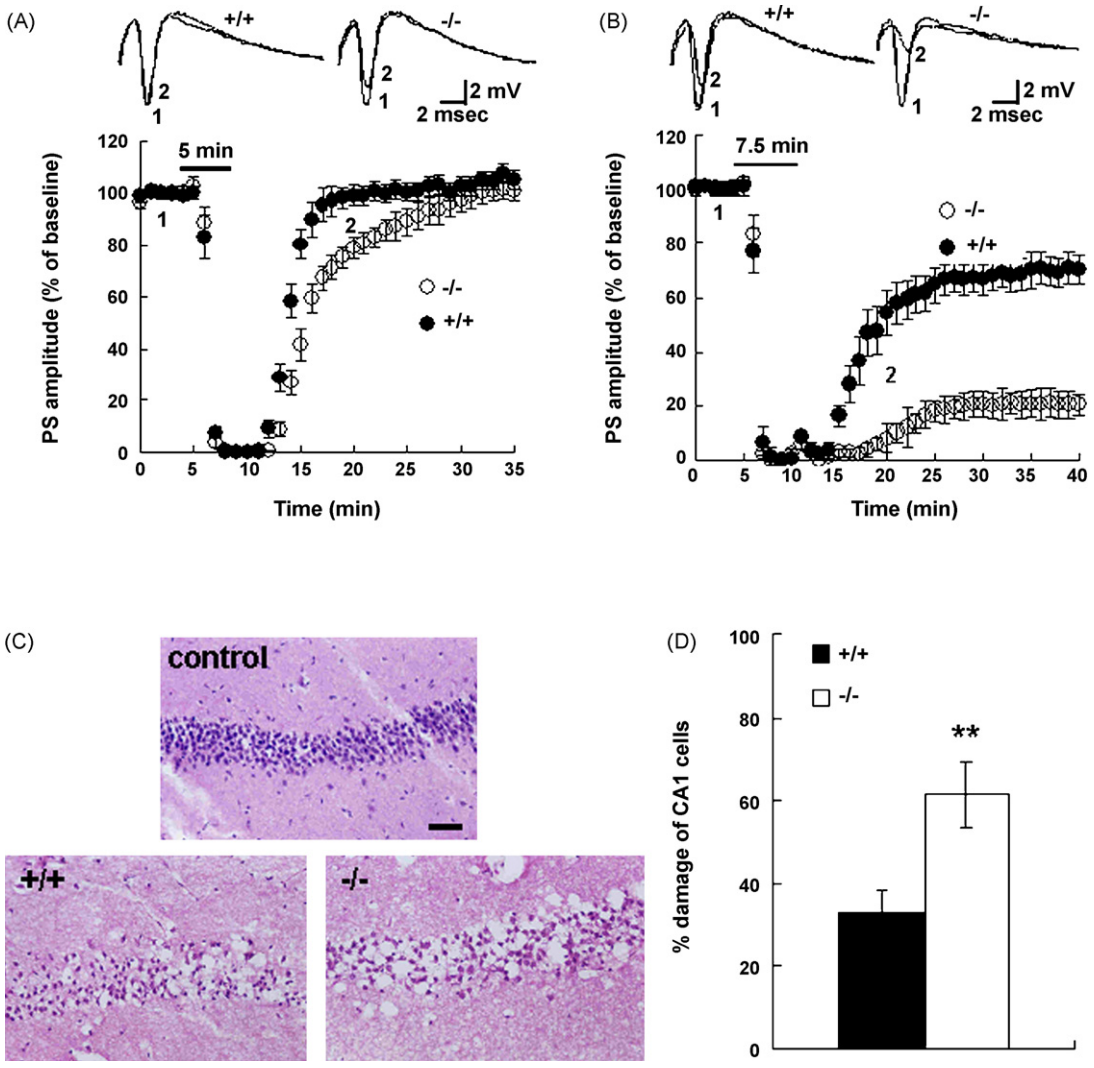

Fig. 1. Reduced recovery of field responses in the NCX2−/− hippocampal slices after an ischemic insult *in vitro*. (A) Compared to NCX2+/+, NCX2−/− hippocampal slices showed a delayed recovery of PS amplitudes after 5 min OGD (+/+, $n = 13$; −/−, $n = 10$). Upper traces indicate PS samples before and after OGD. (B) After 7.5 min OGD, there was no full recovery of PS amplitudes in either genotype, indicating cell damage (+/+, $n = 12$; −/−, $n = 15$). NCX2−/− clearly showed reduced recovery in PS amplitudes compared to NCX2+/+. Upper traces indicate PS samples before and after OGD. (C) Hippocampal slices were subjected to *in vitro* ischemia for 7.5 min and then stained with hematoxylin/eosin. The scale bar equals to 50 μm. (D) After the 7.5 min ischemia, there was 30% more damage to the CA1 region of NCX2−/− tissue ($n = 15$) compared to wild-type ($n = 12$). $^{**}p < 0.01$.

NCX2+/+ ($n = 12$) and NCX2−/− ($n = 15$) tissue showed evidence of profound damage. However, in the NCX2−/− tissue the regions of damage were greater and there was sparser distribution of neurons compared to NCX2+/+ tissues in the CA1 region (−/−, 61.2 ± 7.9; +/+, 32.9 ± 5.1 neurons, $p < 0.01$, Student's *t*-test) (Fig. 1D).

3.2. Sustained elevation of intracellular Ca^{2+} and higher membrane potential in the NCX2−/− after OGD

To determine how NCX2 contributes to changes in $[Ca^{2+}]_i$ during ischemia–reperfusion injury, we used cell impermeant Fura-2 dye and monitored the changes of the Fura-2 intensity. By whole-cell patch recording in CA1 pyramidal neurons from both NCX2+/+ and NCX2−/− mice, we simultaneously measured the changes in $[Ca^{2+}]_i$ and membrane potential after OGD for 2 min. Fig. 2A shows hippocampal CA1 pyramidal layer (upper) and a CA1 pyramidal neuron (lower) patched with cell impermeant Fura-2 included in the glass pipette. Within 2 min OGD, both genotypes displayed a rapid increase in $[Ca^{2+}]_i$ (upper) and rapid depolarization (lower) as shown in Fig. 2B. There was no difference in the peak $[Ca^{2+}]_i$ between the two genotypes (+/+, 1.19 ± 0.05; −/−, 1.19 ± 0.03, $p = 1.0$, Student's *t*-test) (Fig. 2C). However, NCX2−/− neurons ($n = 9$) showed more sustained elevation of $[Ca^{2+}]_i$ than NCX2+/+ neurons ($n = 9$) (+/+, 1.11 ± 0.03 at 10 min; −/−, 1.19 ± 0.02, $p < 0.05$, Student's *t*-test) (Fig. 2D). Moreover, NCX2−/− neurons exhibited more prolonged depolarization than NCX2+/+ neurons (+/+, -39 ± 2 mV at 10 min; −/−, 28 ± 4 mV, $p < 0.05$, Student's

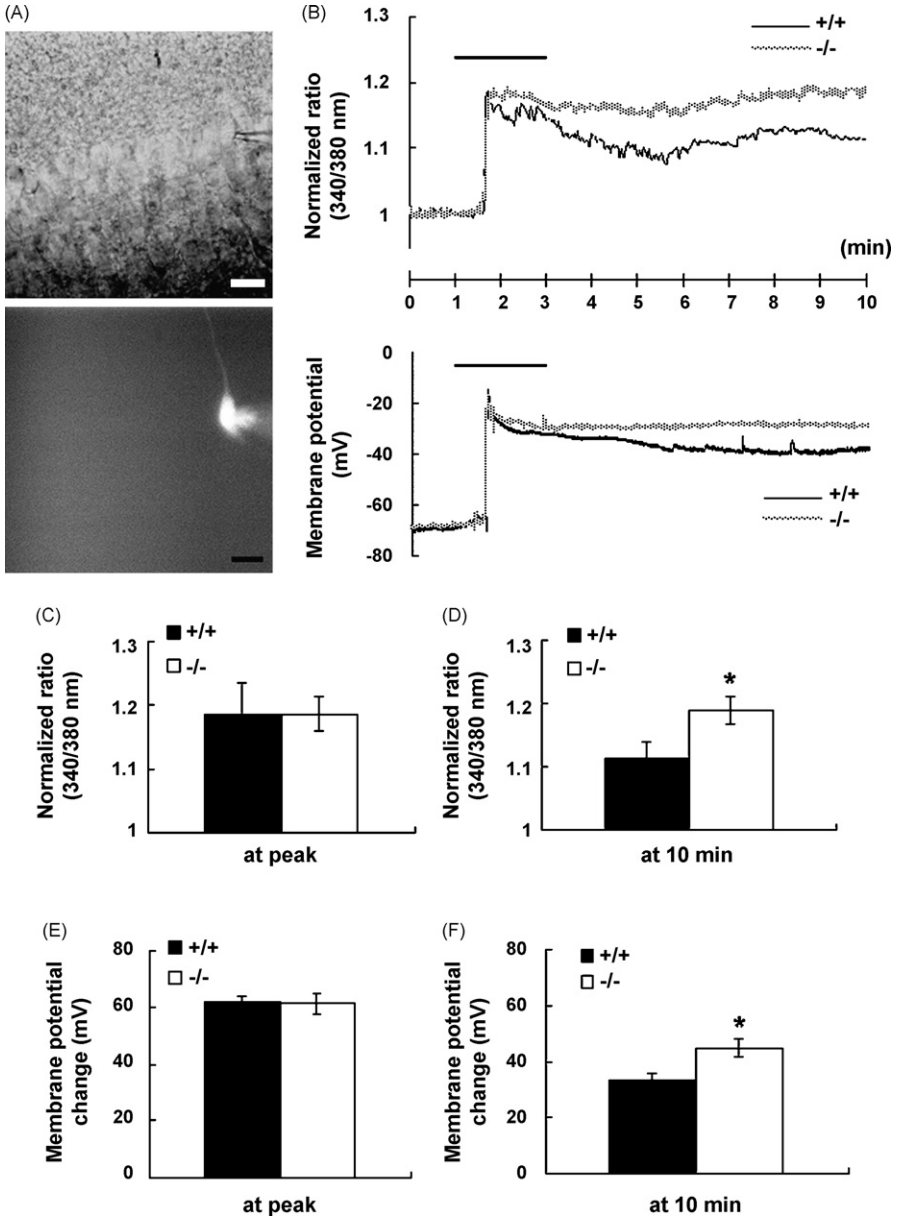

Fig. 2. Changes in the ratio of cell impermeant Fura-2 fluorescence intensities and in simultaneously recorded membrane potentials. (A) The DIC image of hippocampal CA1 layer (upper) and the fluorescence image of a CA1 pyramidal neurons (lower) loaded with Fura-2 through a patch pipette after breaking the gigaohm seal. Scale bars equal to 10 μm. (B) Representative traces of the changes in the ratio of Fura-2 fluorescence intensities at 340 and 380 nm excitation wavelengths, and in membrane potentials of the same CA1 pyramidal neuron of each genotype after an ischemic insult. Bars indicate the OGD period. (C–F) Although both genotypes showed increased $[Ca^+]_i$ and membrane potentials, and similar peak values in $[Ca^+]_i$ (C) and membrane potentials (D), NCX2−/− neurons exhibited more prolonged elevation of $[Ca^+]_i$ (E) and depolarization (F) ($n = 9$ for NCX2+/+, $n = 9$ for NCX2−/−; $^*p < 0.05$).

t-test) (Fig. 2E), without difference in amplitudes of depolarization at the peak (+/+, -12 ± 2 mV; −/−, -12 ± 3 mV, $p = 0.89$, Student's *t*-test) (Fig. 2F). These data support that NCX2 plays a significant role in the Ca^{2+} extrusion during ischemic injury.

3.3. Infarction volume after a transient focal cerebral ischemia

To assess the contribution of NCX2 to neuronal damage *in vivo*, NCX2+/+ and NCX2−/− mice were subjected to a 2 h MCA occlusion followed by 22 h reperfusion, after which brains were removed and infarct volumes measured using the TTC staining as described in Section 2. In NCX2−/− mice, the infarct area covered a wide region from the basal ganglia to the cerebral cortex (Fig. 3A), whereas the infarct area in NCX2+/+ mice was mainly confined to the basal ganglia (Fig. 3B). Quantitatively, the infarct volume was 30% greater in NCX2−/− mice ($n = 17$) than in NCX2+/+ mice ($n = 16$) (−/−, 88.5 ± 11.4 mm^3; +/+, 57.5 ± 10.1 mm^3, $p < 0.01$) (Fig. 3C). Physiological variables including mean arterial blood pressure (MABP), MCA occlusion (MCAO),

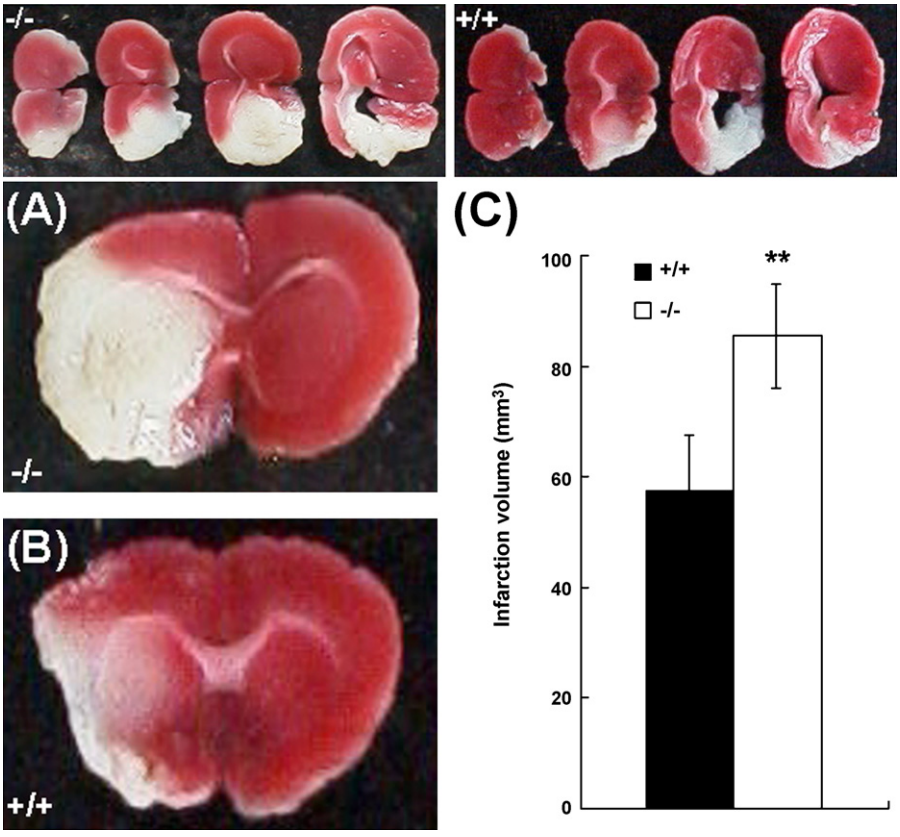

Fig. 3. Measurement of infarction volumes. NCX2−/− (A) and wild-type (B) mice were subjected to a 2 h MCA occlusion followed by a 22 h reperfusion, after which brains were removed and infarct areas were visualized using the TTC staining. (C) The infarction volume was measured using an image analysis system ($n = 16$ for NCX2+/+, $n = 17$ for NCX2−/−; $^{**}p < 0.01$).

blood gases and glucose concentration were not distinguishable between the two genotypes (Table 1).

3.4. Neuronal loss after a transient focal cerebral ischemia

To investigate whether NCX2 affects neuronal death *in vivo*, we evaluated neuronal survival in NCX2+/+ and NCX2−/− mice 1 day after ischemia by counting the number of NeuN-immunopositive ($NeuN^+$) cells in the striatum. In the contralateral hemisphere, numerous $NeuN^+$ cells were found throughout the striatum in both genotypes (data not shown). In contrast, $NeuN^+$ cells were only occasionally observed in the ischemic (ipsilateral) striatum (Fig. 4A and B). In NCX2−/− mice ($n = 6$, Fig. 4B), the number of $NeuN^+$ cells in the medial region of the striatum was significantly less than that found in the comparable region of the NCX2+/+ mice ($n = 6$, Fig. 4A) (−/−, 42.1 ± 14.3 cells/mm^2; +/+, 94 ± 28.1 cells/mm^2, $p < 0.01$) (Fig. 4C). In addition, the number of TUNEL-positive ($TUNEL^+$) cells in the infarct areas was significantly higher in NCX2−/− mice ($n = 6$, Fig. 5B) compared with NCX2+/+ mice ($n = 6$, Fig. 5A) (−/−, 70.6 ± 5.1/field; +/+, 43.7 ± 7.1/field, $p < 0.01$, field: 0.09 mm^2, Fig. 5C). These results demonstrate that ischemia caused greater neuronal damage and neuronal loss in NCX2−/− mice compared to NCX2+/+ mice.

Table 1
Physiological parameters before and after *in vivo* focal cerebral ischemia

	NCX2−/−		NCX2+/+	
	Baseline	At 2 h after MCAO	Baseline	At 2 h after MCAO
pH	7.42 ± 0.03	7.40 ± 0.02	7.42 ± 0.01	7.40 ± 0.07
PaO_2 (mmHg)	108.2 ± 11.1	106.2 ± 7.4	107.1 ± 6.5	111.1 ± 8.9
$PaCO_2$ (mmHg)	39.2 ± 1.4	38.9 ± 1.4	38.8 ± 1.9	38.9 ± 1.6
Rectal temperature (°C)	36.9 ± 0.3	36.8 ± 0.2	36.7 ± 0.3	36.6 ± 0.1
MABP (mmHg)	89 ± 7	84 ± 3	87 ± 3	86 ± 2
Glucose (mg/dl)	82 ± 11	81 ± 9	81 ± 14	82 ± 7

MCAO indicates MCA occlusion; MABP, mean arterial blood pressure. No significant difference for any parameter.

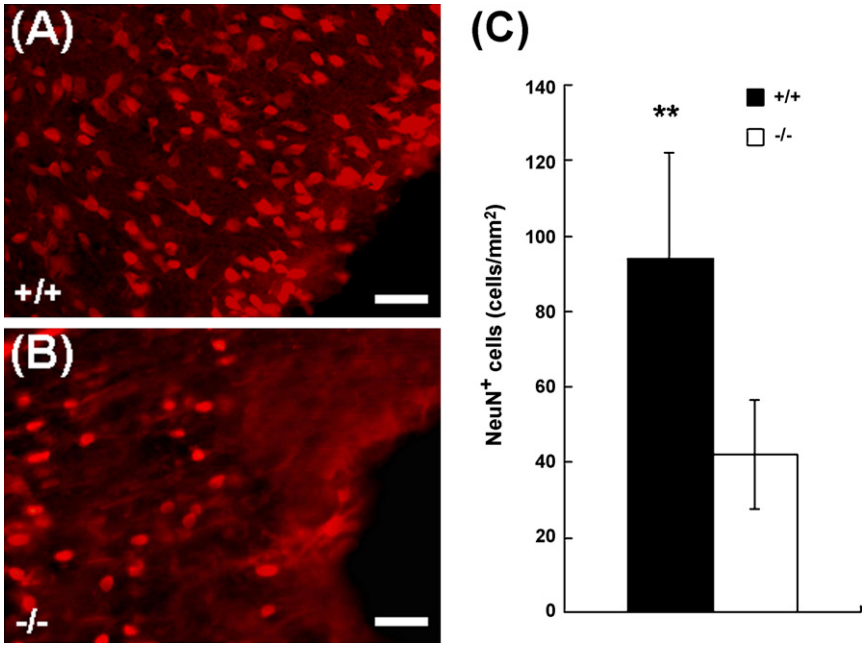

Fig. 4. Evaluation of neuronal loss. Photomicrographs showing NeuN$^+$ cells (red) in the medial region of the ischemic striatum of wild-type (A) and NCX2−/− (B) mice 22 h after reperfusion. (C) Density of NeuN-immunopositive cells in the five subregions of striatum (**$p < 0.01$). Scale bars equal to 50 μm.

3.5. Cells expressing NCX2 and their viability in ischemia

To evaluate the viability of cells expressing NCX2 in ischemia, immunostaining with antibodies (anti-NCX2, anti-NeuN) was performed on the sections from the striatum of normal brain and ischemic brain of the wild-type mice, and we compared the number of NCX2-immunopositive (NCX2$^+$) cells and NeuN$^+$ cells between normal brain and ischemic brain (Fig. 6A–D). We found that there was no significant difference in the number of NCX2$^+$ cells in the comparable medial regions of the striatum between

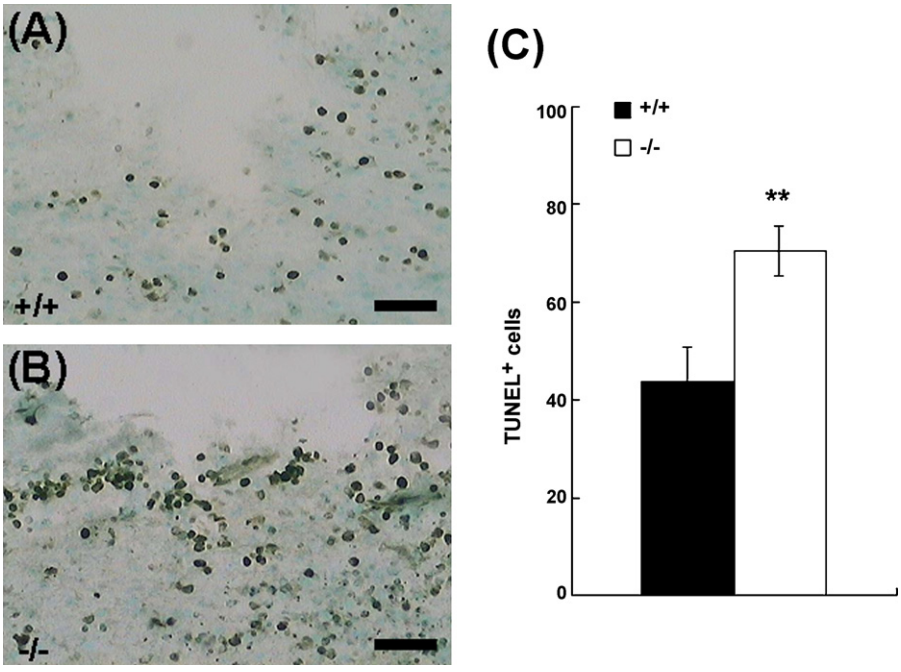

Fig. 5. Evaluation of cell death. Detection of TUNEL reaction products in the infarct region 1 day after ischemia in NCX2+/+ (A) and NCX2−/− (B) mice. (C) The number of TUNEL$^+$ cells in the infarct area 1 day after ischemia. Data represent the number of positive cells/field (field: 0.09 mm^2; **$p < 0.01$). Scale bars equal to 50 μm.

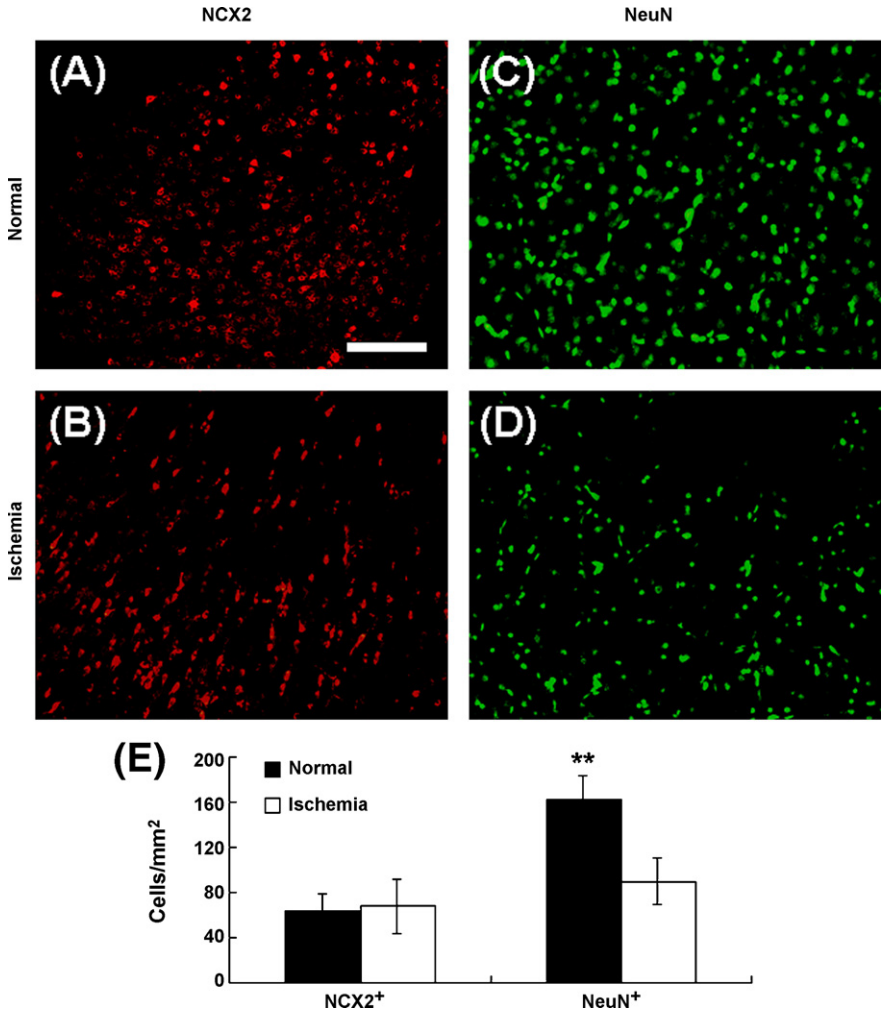

Fig. 6. The viability of NCX2$^+$ cells. Representative digital photomicrographs demonstrating NCX2-immunopositive cells (red) in the striatum of the normal (A) and the ischemic brain (B). NeuN-immunopositive cells (green) in the striatum of the normal (C) and the ischemic brain (D). Scale bars equal to 100 μm. (E) The comparison of the numbers of NCX2$^+$ cells and NeuN$^+$ cells in the peri-infarct area 1 day after ischemia with those from the normal brain (**$p < 0.01$).

two brains (normal, $n = 5$, 63 ± 16.0 cells/mm^2; ischemia, $n = 5$, 67.8 ± 23.9 cells/mm^2, $p = 0.72$), whereas the number of NeuN$^+$ cells was reduced in ischemic brains (normal, 162.4 ± 21.01 cells/mm^2; ischemia, 90 ± 21.1 cells/mm^2, $p < 0.01$) (Fig. 6E). These results indicate that the neurons expressing NCX2 were selectively protected from ischemia.

4. Discussion

In a permanent ischemic injury model, NCX2 was regarded less important than other NCX isoforms, since NCX2 proteins were known to be down-regulated in all ischemic insult regions (core and peri-infart regions) [12,13]. In a transient ischemic reperfusion injury model, the expression of NCX2 was not altered after tMCAO [14,15]. However, there was no direct evidence for the role of NCX2 in ischemia and reperfusion. Further, the mode of action, forward versus reverse, of NCX2 is not known under the ischemia–reperfusion. Thus, it is valuable to investigate the relevant role of NCX2 in brain ischemia.

The present results have shown that NCX2−/− mouse hippocampus displayed a significantly delayed or reduced recovery of PS amplitudes, a sustained increase in $[Ca^+]_i$ and increased membrane depolarization after an *in vitro* ischemic insult. However, the peak values of $[Ca^+]_i$ in the NCX2−/− neurons were similar to those of NCX2+/+ neurons. If NCX2 operates in a reverse mode to import Ca^{2+} during ischemia, NCX2−/− neurons would have shown a reduced $[Ca^+]_i$. The results indicated that NCX2 was necessary to remove Ca^{2+} after ischemic insult. From these results we suggest that the forward mode, Ca^{2+} extrusion, is the primary action of the NCX2 during ischemia. In fact, NCX2-transfected BHK cells displayed a constant $[Ca^+]_i$ under a chemical hypoxia insult without reoxygenation [20]. However, we do not know whether the accumulation of $[Na^+]_i$ in *in vitro* ischemic conditions is enough to induce NCX2 to work in reverse mode. At least, after the similarly elevated $[Ca^+]_i$ levels both in NCX2+/+ and NCX2−/− neurons after

ischemic insults, NCX2 is required for extrusion of $[Ca^+]_i$. In support of our results, it has been shown that the falling phase of the $[Ca^+]_i$ signal after an *in vitro* ischemic exposure is considered to be primarily dependent on reactivation of Na^+/K^+ ATPase [2,4]. In the recovery period when O_2 and glucose are re-established, activation of Na^+/K^+ ATPase and the Na^+/H^+ exchanger could be restored to pre-ischemic conditions, which quickly leads to NCX working in the forward mode (Na^+ influx–Ca^{2+} efflux pathway) in the hippocampus [5]. Thus, the extrusion of cytosolic Ca^{2+} through NCX2 may be directly involved in rapid reduction of $[Ca^+]_i$ after ischemic insult. In addition, it has been reported that the increase in $[Ca^+]_i$ in ischemic insult is correlated with the membrane depolarization [21], and the inhibition of NCX enhances the depolarization induced by OGD [22]. These results indicate that NCX2 working as Na^+ influx–Ca^{2+} efflux pathway plays a pivotal protective role in the pathophysiology of cerebral ischemia.

Consistent with our *in vitro* results, we found that the lack of NCX2 resulted in an increased infarct volume and increased neuronal loss in a transient focal cerebral ischemia in the mouse brain. Moreover, we observed that the neurons expressing NCX2 in peri-infarct areas were selectively protected from ischemic injury compared to NCX2-negative neurons. Although NCX2 works in the reverse mode in the ischemic core region owing to a decreased ATP production and accumulation of $[Na^+]_i$, the forward activity of NCX2 may be necessary to delimit the infarct volume to the initial injury site, as well as peri-infarct areas.

In conclusion, a genetic deletion of NCX2 increased ischemic neuronal injury *in vitro* and *in vivo*, due to a dysfunction in the regulation of $[Ca^+]_i$ by extrusion. These results provide evidence that NCX2 has a neuroprotective role in ischemic cell death in the adult brain. It is possible that different NCX isoforms could be differentially involved in brain ischemia, and thus the pharmacological inhibition of all three NCX isoforms' activity could complicate the efforts to understand the role played by NCX in brain ischemia. Further studies of mice lacking NCX1 or NCX3 during reperfusion injury will reveal its role in ischemic reperfusion damage in the adult brain. Overcoming the specificity problem of NCX activator or inhibitor will be required for the treatment of ischemic neuronal cell death. In particular, drugs improving forward activity of NCX2 may be helpful to limit the severity of neuronal damage after brain ischemia.

Acknowledgments

This work was supported by the National Honor Scientist program of Korea and grants from Korea Institute of Science and Technology, and the National Creative Research Initiatives of the Ministry of Science and Technology of Korea. The authors thank Dongho Woo for help in Ca^{2+} imaging.

References

[1] P. Lipton, Ischemic cell death in brain neurons, Physiol. Rev. 79 (1999) 1431–1568.
[2] M.P. Blaustein, W.J. Lederer, Sodium/calcium exchange: its physiological implications, Physiol. Rev. 79 (1999) 763–854.
[3] K.D. Philipson, D.A. Nicoll, Sodium–calcium exchange: a molecular perspective, Annu. Rev. Physiol. 62 (2000) 111–133.
[4] L. Annunziato, G. Pignataro, G.F. Di Renzo, Pharmacology of brain Na^+/Ca^{2+} exchanger: from molecular biology to therapeutic perspectives, Pharmacol. Rev. 56 (2004) 633–654.
[5] E. Tanaka, H. Uchikado, S. Niiyama, K. Uematsu, H. Higashi, Extrusion of intracellular calcium ion after in vitro ischemia in the rat hippocampal CA1 region, J. Neurophysiol. 88 (2002) 879–887.
[6] D.A. Nicoll, S. Longoni, K.D. Philipson, Molecular cloning and functional expression of the cardiac sarcolemmal Na^+–Ca^{2+} exchanger, Science 250 (1990) 562–565.
[7] Z. Li, S. Matsuoka, L.V. Hryshko, D.A. Nicoll, M.M. Bersohn, E.P. Burke, R.P. Lifton, K.D. Philipson, Cloning of the NCX2 isoform of the plasma membrane Na^+–Ca^{2+} exchanger, J. Biol. Chem. 269 (1994) 17434–17439.
[8] D.A. Nicoll, B.D. Quednau, Z. Qui, Y.R. Xia, A.J. Lusis, K.D. Philipson, Cloning of a third mammalian Na^+–Ca^{2+} exchanger, NCX3, J. Biol. Chem. 271 (1996) 24914–24921.
[9] L. Yu, R.A. Colvin, Regional differences in expression of transcripts for Na^+/Ca^{2+} exchanger isoforms in rat brain, Brain Res. Mol. Brain Res. 50 (1997) 285–292.
[10] B. Linck, Z. Qiu, Z. He, Q. Tong, D.W. Hilgemann, K.D. Philipson, Functional comparison of the three isoforms of the Na^+/Ca^{2+} exchanger (NCX1, NCX2, NCX3), Am. J. Physiol. 274 (1998) C415–C423.
[11] M. Papa, A. Canitano, F. Boscia, P. Castaldo, S. Sellitti, H. Porzig, M. Taglialatela, L. Annunziato, Differential expression of the Na^+–Ca^{2+} exchanger transcripts and proteins in rat brain regions, J. Comp. Neurol. 461 (2003) 31–48.
[12] G. Pignataro, R. Gala, O. Cuomo, A. Tortiglione, L. Giaccio, P. Castaldo, R. Sirabella, C. Matrone, A. Canitano, S. Amoroso, G. Di Renzo, L. Annunziato, Two sodium/calcium exchanger gene products, NCX1 and NCX3, play a major role in the development of permanent focal cerebral ischemia, Stroke 35 (2004) 2566–2570.
[13] F. Boscia, R. Gala, G. Pignataro, A. de Bartolomeis, M. Cicale, A. Ambesi-Impiombato, G. Di Renzo, L. Annunziato, Permanent focal brain ischemia induces isoform-dependent changes in the pattern of Na^+/Ca^{2+} exchanger gene expression in the ischemic core, periinfarct area, and intact brain regions, J. Cereb. Blood Flow Metab. 26 (2006) 502–517.
[14] D. Bano, K.W. Young, C.J. Guerin, R. Lefeuvre, N.J. Rothwell, L. Naldini, R. Rizzuto, E. Carafoli, P. Nicotera, Cleavage of the plasma membrane Na^+/Ca^{2+} exchanger in excitotoxicity, Cell 120 (2005) 275–285.
[15] L.L. Li, L.N. Sun, H.Y. Zhou, Z.B. Li, X.L. Wang, Selective alteration of expression of Na^+/Ca^{2+} exchanger isoforms after transient focal cerebral ischemia in rats, Neurosci. Lett. 404 (2006) 249–253.
[16] D. Jeon, Y.M. Yang, M.J. Jeong, K.D. Philipson, H. Rhim, H.S. Shin, Enhanced learning and memory in mice lacking Na^+/Ca^{2+} exchanger 2, Neuron 38 (2003) 965–976.
[17] D. Jeon, C. Kim, Y.M. Yang, H. Rhim, E. Yim, U. Oh, H.S. Shin, Impaired long-term memory and long-term potentiation in N-type Ca^{2+} channel-deficient mice, Genes Brain Behav. 6 (2007) 375–388.
[18] S.H. Lee, M. Kim, B.W. Yoon, Y.J. Kim, S.J. Ma, J.K. Roh, J.S. Lee, J.S. Seo, Targeted hsp70.1 disruption increases infarction volume after focal cerebral ischemia in mice, Stroke 32 (2001) 2905–2912.
[19] K.H. Jung, K. Chu, S.W. Jeong, S.Y. Han, S.T. Lee, J.Y. Kim, M. Kim, J.K. Roh, HMG-CoA reductase inhibitor, atorvastatin, promotes sensorimotor recovery, suppressing acute inflammatory reaction after experimental intracerebral hemorrhage, Stroke 35 (2004) 1744–1749.

[20] A. Secondo, R.I. Staiano, A. Scorziello, R. Sirabella, F. Boscia, A. Adornetto, V. Valsecchi, P. Molinaro, L.M. Canzoniero, G. Di Renzo, L. Annunziato, BHK cells transfected with NCX3 are more resistant to hypoxia followed by reoxygenation than those transfected with NCX1 and NCX2: possible relationship with mitochondrial membrane potential, Cell Calcium 42 (2007) 521–535.

[21] E. Tanaka, S. Yamamoto, Y. Kudo, S. Mihara, H. Higashi, Mechanisms underlying the rapid depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons in vitro, J. Neurophysiol. 78 (1997) 891–902.

[22] P. Calabresi, G.A. Marfia, S. Amoroso, A. Pisani, G. Bernardi, Pharmacological inhibition of the Na^+/Ca^{2+} exchanger enhances depolarizations induced by oxygen/glucose deprivation but not responses to excitatory amino acids in rat striatal neurons, Stroke 30 (1999) 1687–1694.