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Preferential involvement of Na^+/Ca^{2+} exchanger type-1 in the brain damage caused by transient focal cerebral ischemia in mice

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ABSTRACT

The Na^+/Ca^{2+} exchanger (NCX), an ion-transporter located in the plasma membrane of neuronal cells, contributes to intracellular Ca^{2+} homeostasis. Within the brain, three isoforms (NCX1, NCX2, and NCX3) are widely distributed. However, it is not clear to what extent these isoforms are involved in ischemic brain damage in mammals. We therefore used genetically altered mice and isoform-selective NCX inhibitors in a model of transient focal ischemia to investigate the role of each NCX isoform in ischemic brain damage. NCX isoform-mutant mice ($NCX1^{+/-}$, $NCX2^{+/-}$, and $NCX3^{+/-}$) and wild-type mice were subjected to 90 min of middle cerebral artery occlusion (MCAO) followed by 24 h of reperfusion. One of three NCX inhibitors [SN-6, KB-R7943, or SEA0400 (3 or 10 mg kg^{-1}, i.p.)] was administered to ddY mice at 30 min before more prolonged (4-h) MCAO followed by 24 h of reperfusion. After transient MCAO reperfusion, the cerebral infarcts in $NCX1^{+/-}$ mice, but not those in $NCX2^{+/-}$ or $NCX3^{+/-}$ mice, were significantly smaller than those in wild-type mice. SN-6 and SEA0400, which are more selective for the NCX1 isoform, significantly reduced the infarct volume at 10 mg/kg. In contrast, KB-R7943, which is more selective for NCX3, did not. These results suggest that the NCX1 isoform may act preferentially (vs. the NCX2 and NCX3 isoforms) to exacerbate the cerebral damage caused by ischemic insult in mice, and that NCX1-selective inhibitors warrant investigation as a potential therapeutic agents for stroke.

1. Introduction

The Na^+/Ca^{2+} exchanger (NCX) is an ion-transporter located in the plasma membrane of many cells, and it mediates effects on intracellular Ca^{2+} homeostasis. Three different isoforms (NCX1, NCX2, and NCX3) have been identified in mammals [1]. Of these, NCX1 is expressed ubiquitously in many tissues (including heart, brain, and kidney), whereas NCX2 and NCX3 are expressed in both brain and skeletal muscle [2]. Under normal conditions, NCX is thought to extrude Ca^{2+} to the outside of the cell using the Na^+ concentration gradient across the cell membrane. Under pathological conditions, such as ischemia/reperfusion injury, the exchanger is thought to cause Ca^{2+} overload due to elevated levels of intracellular Na^+ (Na_i^+), leading to cell damage [3].

Potent and selective inhibitors are used to study the physiological and pathological roles of NCX isoforms. Such inhibitors may have therapeutic potential for several ischemic diseases, arrhythmias, heart failure, and essential hypertension [4–6]. Although, some recent studies have reported an association between NCX and cerebral ischemia/reperfusion injury [7–9], its role in ischemic brain damage remains controversial, and the underlying mechanisms remain unclear.

To investigate the possible involvement of the three NCX isoforms in cerebral ischemia/reperfusion injury, we used three types

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of NCX heterozygous mice (NCX1$^{+/-}$, NCX2$^{+/-}$, and NCX3$^{+/-}$) with the same C57BL/6 background, and we performed transient middle cerebral artery occlusion (tMCAO). We also compared the effects of various NCX inhibitors (SN-6, KB-R7943, and SEA0400) on ischemic brain damage in mice. These NCX inhibitors have a common benzyloxyphenyl structure, but exhibit different selectivities toward the NCX isoforms. KB-R7943 (2-[2-[4-(4-nitrobenzyloxy) phenyl]ethyl]isothiourea), which was developed as a prototype selective NCX inhibitor, is more effective against NCX3 than against the NCX1 and NCX2 isoforms [4,10]. KB-R7943 has been reported to reduce ischemia/reperfusion injury in heart [11] and brain [12]. On the other hand, SN-6 (2-[4-(4-nitrobenzyloxy) benzyl]thiazolidine-4-carboxylic acid ethyl ester) and SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline) are each more effective against NCX1 than against the NCX2 and NCX3 isoforms [13,14]. Indeed, SEA0400 has no effect at all on NCX3. SN-6 has been reported to reduce ischemia/reperfusion injury in the heart [15], while SEA0400 has been reported both to reduce infarct volume in a rat transient MCAO model [16] and to attenuate several ischemia/reperfusion injuries in heart failure [17] and renal failure [18] models.

2. Materials and methods

2.1. Animals

NCX1$^{+/-}$, NCX2$^{+/-}$, and NCX3$^{+/-}$ mice, and control wild-type mice (SLC, Shizuoka, Japan), each with a C57BL/6J genetic background, and ddY mice (Kiwa Laboratory Animals Co., Ltd, Wakayama, Japan) were used in the experiments. All animals used were males and they were housed under diurnal lighting conditions (light period 8:00 a.m. to 8:00 p.m.) and allowed access to food and water ad libitum. The experimental designs and all procedures were conducted in accordance with the Animal Care Guidelines of the Animal Experimental Committees of Gifu Pharmaceutical University and Fukuoka University.

2.2. Drugs

SEA0400, SN-6, and KB-R7943 were synthesized [13,16]. The other drugs used and their sources were as follows: arabic gum (Wako Pure Chemical Industries, Osaka, Japan), isoflurane (Merck Hoei Co., Ltd., Tokushima, Japan), 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma–Aldrich, St Louis, MO, USA).

2.3. Ischemic model

Anesthesia was induced by means of 2.0% isoflurane, then maintained using 1% isoflurane in 70% N_2O and 30% O_2. For this, an animal general anesthesia machine (Soft Lander; Sin-ei Industry Co., Ltd., Saitama, Japan) was employed. The body temperature was maintained between 37.0 and 37.5 °C with the aid of a heating pad and heating lamp. A filament occlusion of the left middle cerebral artery (MCA) was carried out as previously described [19]. Briefly, the left MCA was occluded with an 8-0 nylon monofilament (Ethicon, Somerville, NJ, USA) coated with a mixture of silicone resin (Xantopren; Bayer Dental, Osaka, Japan). This coated filament was introduced into the internal carotid artery through the common carotid artery, and passed up to the anterior cerebral artery *via* the internal carotid artery, so as to occlude the MCA. Ninety minutes (for C57BL/6J-background mice) or 4 h (for ddY mice) after the start of this ischemia, animals were briefly re-anesthetized with isoflurane and the filament withdrawn through the common carotid artery. Thus, the MCA, posterior communicating artery, and common carotid artery, were reperfused.

2.4. Brain infarction

Brain infarction was analysed as in a previous report [19] (see in the Supplemental information).

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining

TUNEL staining was performed in brains at 24 h after tMCAO, NCX1$^{+/-}$ and wild-type mice (see in the Supplemental information).

2.6. Statistical analysis

The data are presented as the means ± S.E.M. Statistical comparisons were made using a one- or two-way ANOVA followed by a Student's *t*-test or Dunnett's test. For this, STAT VIEW version 5.0 (SAS Institute Inc., Cary, NC, USA) was used. $P < 0.05$ was considered to indicate statistical significance.

3. Results

In this study, three types of heterozygous mice (NCX1$^{+/-}$, NCX2$^{+/-}$, and NCX3$^{+/-}$) were consistently utilized (except in the inhibitor study; Section 3.4). Although NCX1$^{-/-}$ and NCX2$^{-/-}$ mice died before birth [20], NCX3$^{-/-}$ mice were viable and fertile.

3.1. Expression of NCX isoforms in brains isolated from NCX1$^{+/-}$, NCX2$^{+/-}$, and NCX3$^{+/-}$ mice

We examined NCX1, NCX2, and NCX3 mRNA expressions in brains isolated from the three heterozygous strains, and compared them with those from wild-type C57BL/6J mice. Real-time PCR revealed that the expression of the targeted NCX isoform in each heterozygous strain (NCX1$^{+/-}$, NCX 2$^{+/-}$, and NCX3$^{+/-}$) was significantly reduced (to between about one-third and two-thirds that of the wild-type brain), whereas the expressions of the other two isoforms were not altered vs. those in the wild-type brain (Supplemental Fig. 1A). Furthermore, we confirmed, using Western blotting, that the NCX1 protein expression of the NCX1$^{+/-}$ brain was about 50% that of the wild-type brain, whereas the NCX1 expressions of NCX2$^{+/-}$ and NCX3$^{+/-}$ brains were similar to that of the wild-type brain (Supplemental Fig. 1B). Thus, these three NCX heterozygous mice can be used experimentally as NCX isoform-specific deficient mice with the same C57BL/6J background.

3.2. Attenuation of MCAO-induced brain infarction in NCX1$^{+/-}$ mice, but not in NCX2$^{+/-}$ or NCX3$^{+/-}$ mice

To explore the involvement of NCX isoforms in ischemic brain injury, age-matched NCX1$^{+/-}$, NCX2$^{+/-}$, and NCX3$^{+/-}$ mice, and control wild-type mice were challenged with the same experimental conditions (90-min MCAO and 24-h reperfusion). Twenty-four hours after the start of reperfusion, the mice had developed infarcts affecting both the cortex and striatum. Infarction area and volume were smaller in NCX1$^{+/-}$ mice than in wild-type mice, but this was not the case in either NCX2$^{+/-}$ or NCX3$^{+/-}$ mice (Fig. 1). Actually, the infarct area in the NCX1$^{+/-}$ mice was smaller (vs. the wild-type control) in 2 of the 5 coronal sections (6 and 10 mm). There were no significant differences in the physiological parameters (arterial blood pressure, heart rate, pO_2, pCO_2, or pH) between the wild-type and NCX1$^{+/-}$ mice (Supplemental Table 1).

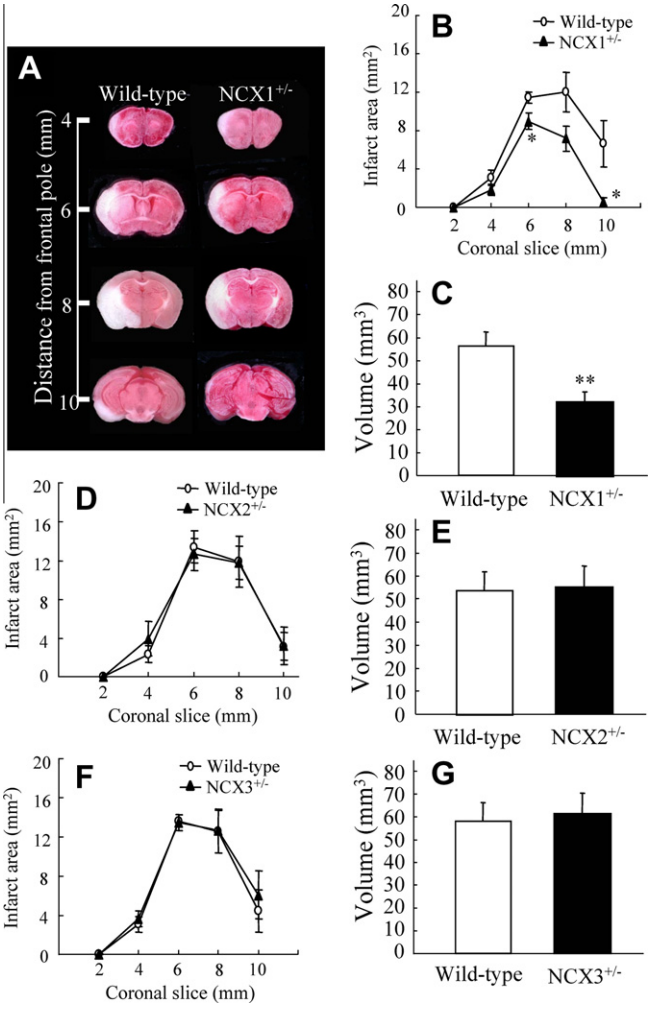

Fig. 1. Decreased infarct size in NCX1$^{+/-}$ mice. (A) 2,3,5-Triphenyltetrazolium chloride (TTC) staining of coronal sections of brains taken at the end of 24-h reperfusion after 90-min MCAO in wild-type mice and in NCX1$^{+/-}$ mice. After TTC staining, damaged tissue appears white, whereas viable tissue appears reds. NCX1$^{+/-}$ mice had reduced infarcts compared to wild-type mice. (B) Distribution of infarct areas in different brain slices in experimental mice. The area of infarction was smaller in brain slices from NCX1$^{+/-}$ mice (n = 12 or 13; $^{*}p < 0.05$ vs. wild-type mice). (C) The infarct volume was reduced in NCX1$^{+/-}$ mice too (n = 12 or 13; $^{**}p < 0.01$ vs. wild-type mice). However, NCX2$^{+/-}$ and NCX3$^{+/-}$ mice showed no improvement in either infarct area (C, D) or infarct volume (F, G) (n = 6–8).

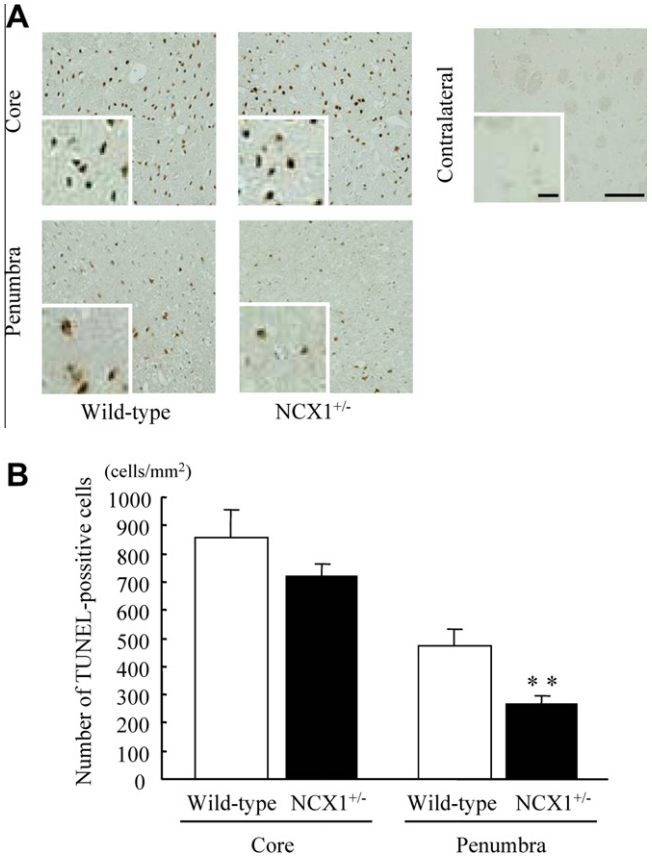

Fig. 2. NCX1$^{+/-}$ mice displayed reduced MCAO-induced apoptosis in the penumbra. (A) Representative images of TUNEL staining in the infarct core and penumbra, and on the side contralateral to the infarction. Insets are higher-magnification images. NCX1$^{+/-}$ mice exhibited a reduced number of MCAO-induced TUNEL-positive cells in the penumbra. Scale bars: higher-magnification panels, 20 μm; lower-magnification panels, 100 μm. (B) Cell-counting analysis revealed that NCX1$^{+/-}$ mice had a significantly reduced number of TUNEL-positive cells in the penumbra (n = 6–10; $^{**}p < 0.01$ vs. wild-type mice).

3.3. Attenuation of MCAO-induced apoptosis in the penumbra in NCX1$^{+/-}$ mice

Detailed observation of TTC-stained ischemic brains showed that the reduction of the infarct area in NCX1$^{+/-}$ mouse brains was predominantly due to a reduction in the size of the penumbra, rather than to a reduced core area. Therefore, we investigated the apoptotic reactions in the penumbra of NCX1$^{+/-}$ brains. TUNEL staining of ischemic brains from wild-type mice revealed a greater number of TUNEL-positive cells in the core and penumbra areas on the ipsilateral side than on the contralateral side (Fig. 2A). However, the number of TUNEL-positive cells in the ipsilateral penumbra area was significantly smaller in NCX1$^{+/-}$ mouse brains than in wild-type brains (Fig. 2A and B).

3.4. Effects of NCX inhibitors on MCAO-induced infarct volume

To investigate the effects of the NCX inhibitors SN-6, KB-R7943, and SEA0400 on ischemic brain injury, we administered one of these inhibitors (at 3 or 10 mg/kg, i.p.) to ddY mice, and then subjected them to prolonged (4-h) MCAO and 24-h reperfusion. Compared with vehicle-treatment, both SN-6 and SEA0400 at 10 mg kg^{-1}, i.p., significantly reduced the infarct volume, while SN-6 at 3 mg kg^{-1}, i.p. tended (non-significantly) to reduce it. In contrast, neither KB-R7943 at 3 or 10 mg kg^{-1}, i.p., nor SEA0400 at 3 mg kg^{-1}, i.p., had any effects (Fig. 3).

4. Discussion

NCX exists as three different isoforms (NCX1, NCX2, and NCX3), and all three isoforms are expressed in the brain. In the present study, the mRNA expression level of NCX1 in brain was higher than those of NCX2 and NCX3. A previous study noted that the NCX1 isoform is more intensively expressed in the pyramidal neurons of the cerebral cortex than the NCX2 isoform [21]. In contrast, the NCX2 isoform seems to be expressed preferentially in somatosensory neurons [21]. In the hippocampus, the three NCX isoforms are strongly expressed in the granular cell layers of the dentate gyrus and in the pyramidal cells of the CA1 to CA4 subfields [21]. Thus, the three NCX isoforms seem to be differentially distributed among CNS regions, and presumably have different functional roles in the brain. In this study, the targeted NCX isoform in NCX1$^{+/-}$, NCX 2$^{+/-}$, and NCX3$^{+/-}$ mice was significantly reduced (to between about one-third and two-thirds that seen in the wild-type brain), with the expressions of the other two isoforms not being altered

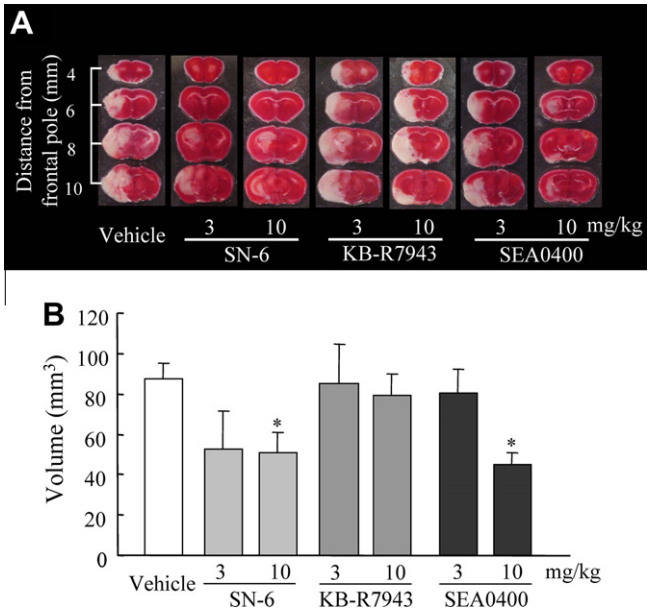

Fig. 3. Effects of various NCX-inhibitors on infarction after MCAO and reperfusion in ddY mice. (A) TTC staining of coronal sections of brains taken at the end of 24-h reperfusion after prolonged (4-h) MCAO in representative mice. (B) Effects of various NCX inhibitors (SN-6, KB-R7943, and SEA0400) on infarction volume (measured at the end of 24-h reperfusion) (n = 3–10; $^{*}p < 0.05$ vs. Vehicle).

compared with those in the wild-type brain. These results indicate that each heterozygous mouse is useful for examinations of the pathological roles of NCX-isoforms. We should, however, mention that Molinaro et al. [9] reported that NCX3$^{+/-}$ and NCX3$^{-/-}$ mice displayed increased expressions of both NCX1 and NCX2 mRNAs in cortex and hippocampus. The reason for this discrepancy is unclear, and further study will be needed to elucidate it.

In this study, the cerebral infarcts measured at 24 h after transient MCAO (tMCAO) were smaller in NCX1$^{+/-}$ mice than in wild-type mice, but this was not true in either NCX2$^{+/-}$ or NCX3$^{+/-}$ mice. However, some previous reports conflict with our findings: (i) cerebral infarction in NCX1$^{+/-}$ mice did not differ from that in the controls at 24 h after tMCAO [7,22]; (ii) NCX2$^{-/-}$ mice displayed greater brain damage than their controls at 24 h after tMCAO [8]; (iii) both NCX3$^{+/-}$ and NCX3$^{-/-}$ mice exhibited exacerbated brain infarction (vs. controls) at 24 h after tMCAO [9]; and (iv) in rats, knockdown of NCX1 or NCX3, but not of NCX2, using antisense oligodeoxynucleotides (AS-ODNs) was associated with increased brain damage at 24 h after permanent MCAO (pMCAO) [23]. To obtain result (i) above, Luo et al. [7,22] produced brain damage in NCX1$^{+/-}$ mice by inposing 30- or 60-min MCAO and 24-h reperfusion using mice with a 129/SV background, while we employed 90-min MCAO plus 24-h reperfusion and used C57BL/6J-background mice for all the NCX-knockout groups. Further, Pignataro et al. [23] found in a rat pMCAO model that NCX1 knockdown (around 50% of the control protein level) achieved using NCX1 AS-OGNs increased the brain damage [see result (iv) above]. Some previous studies have reported that C57BL/6 mice are more susceptible to brain ischemic injuries than 129/SV mice in global and focal ischemia models [24,25]. This difference is considered to be distinct differences in intracranial cerebrovascular anatomy: The posterior communicating artery was poorly developed or absent in 93% of C57BL/6 mice and 50% of 129/SV mice [26]. Furthermore, other biochemical differences have also been reported in these strains [27,28]. Accordingly, these discrepancies in NCX isoforms may be due to the differences in strain and/or ischemic condition, but further studies will be needed to clarify it. On the other hand, Luo et al. [7,22] also demonstrated that NCX1$^{+/-}$ neurons exhibited a lower mortality rate than their controls after 3-h oxygen and glucose deprivation and 21-h reoxygenation *in vitro*, suggesting that an NCX1 reverse mode of action (i.e., Ca^{2+} influx into cells) may play some role in the neuronal death resulting from neuronal ischemia/reperfusion. Our NCX2-knockout mice failed to yield NCX2$^{-/-}$ mice by crossbreeding between male and female NCX2$^{+/-}$ mice, each with a C57BL/6J genetic background, suggesting embryonic lethality, although Jeon et al. [29] succeeded in creating NCX2$^{-/-}$ mice by interbreeding between NCX2$^{+/-}$ mice with 129/SV and C57BL/6J genetic backgrounds. Their NCX2$^{-/-}$ mice exhibited greater brain damage than their controls in a tMCAO model, but such an effect was not seen either in the NCX2$^{+/-}$ mice used in the present study or as a result og the NCX2-knockdown (around 20% of the control protein level) achieved using NCX2 AS-OGNs [23] in tMCAO and rat pMCAO models, respectively. Together, these results suggest that the pathological role of NCX2 in stroke may be smaller than that of NCX1. Furthermore, NCX3$^{+/-}$ mice did not exhibit altered brain damage (vs. wild-type mice) after tMCAO in the present study, whereas brain damage was worse than control both in NCX3-knockout mice (both NCX3$^{+/-}$ and NCX3$^{-/-}$) [9] and as a result of the NCX3 knockdown (around 60% of the control protein level) achieved using NCX3 AS-OGNs [23] in tMCAO and pMCAO models, respectively. Interestingly, both pMCAO and tMCAO led to persistent decreases in the protein expressions of NCX1 and NCX3 in the ischemic region [23,30], while NCX3-deficient mice (NCX3$^{+/-}$ and NCX3$^{-/-}$) exhibited a markedly increased NCX1 protein level in the cortex [9] and use of NCX3 AS-OGNs tended to lead to increased NCX1 and NCX2 protein levels in non-ischemic brain tissue [23]. These findings suggest that compensatory increases in other NCX isoforms may lead to a worsening of ischemic brain damage in NCX3-deficient mice. However, in the present study the expressions of the other NCX isoforms (NCX1 and NCX2) in the brains of NCX3$^{+/-}$ mice were not different compared with those of wild-type mice. Further extensive study will be required to explain the differences between our results and the ones previously reported in NCX3-knockout mice.

Here, to confirm the involvement of NCX isoforms in cerebral ischemia, we used three NCX inhibitors and evaluated their effects in a more severe ischemia model. At 10 mg kg^{-1}, i.p., SN-6 and SEA0400, which have high selectivity for NCX1, but not KB-R7943 at 10 mg kg^{-1}, i.p., significantly reduced the infarct volume at 24 h after mouse tMCAO. These data are in line with a previous report that SEA0400 reduced infarct volumes in rat cerebral cortex after tMCAO [16]. During cerebral ischemia, there is excessive extracellular accumulation of glutamate and a subsequent impairment of intracellular Na^+ and Ca^{2+} homeostasis, leading to neuronal cell death [31]. The reduced of the energy supply leads to a cascade of events involving depolarization, influx of Na^+, and a subsequent reverse operation of the membrane protein, and this induces intracellular Ca^{2+} overload and irreversible neuronal injury [32]. Reportedly, the NCX inhibitors used in this study all inhibit the reverse mode of action (i.e., Ca^{2+} influx) of NCX much more effectively than the forward mode (i.e., Ca^{2+} efflux) [13,14]. In the present study, we reveal an involvement of Ca^{2+} overload *via* NCX1 in the mechanism responsible for the neuronal cell injury that follows transient cerebral ischemia, at least in mice. These findings suggest that Ca^{2+} overload *via* the reverse mode of action of NCX1 plays an important role in the pathogenesis of cerebral ischemic injury. In the present study, we used SN-6 and SEA0400 as highly selective inhibitors of NCX1. At a high dose (10 mg kg^{-1}), the two inhibitors did not differ in the efficacy with which they reduced cerebral ischemic damage. However, at a low dose (3 mg kg^{-1}) SN-6, but not SEA0400, tended to reduce the cerebral infarction. It has been reported that SN-6 also eliminates oxygen free radicals [15]. Therefore the possibility exists that SN-6 is

effective against cerebral ischemic damage *via* the dual actions of inhibiting NCX and scavenging oxygen free radicals. Against that idea is our finding that KB-R7943, which exerts broad NCX inhibition, had no effect on the tMCAO-induced brain damage. KB-R7943 is reportedly 3-fold more effective against NCX3 than against either NCX1 or NCX2 [33]. Therefore, the present ineffectiveness of KB-R7943 may be due to its poor selectivity for NCX1, and if so our result suggests a weak contribution, if any, of NCX3 to the brain damage seen after tMCAO.

In conclusion, the above findings indicate that NCX1 plays a pivotal role in cerebral ischemic injury, and suggest that selective NCX1 inhibitors may hold promise as potential therapeutic agents for stroke.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.114.

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