Alteration in NCX-3 immunoreactivity within the gerbil hippocampus following spontaneous seizures

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Although NCX-3 is highly expressed in the brain, the distribution of NCX-3 in the epileptic hippocampus is still controversial. Therefore, to assess the distribution and pattern of NCX-3 expression in epileptic hippocampus, we performed a comparative analysis of NCX-3 immunoreactivities in the hippocampus of seizure-resistant (SR) and seizure-sensitive (SS) gerbils. In SR gerbils, NCX-3 immunoreactivity was higher than pre-seizure SS gerbils, particularly in the parvalbumin (PV)-positive interneurons. Three h post-ictal, NCX-3 immunoreactivity in the SS gerbil hippocampus was markedly elevated to the level of SR gerbils. Six h post-ictal, the expression of NCX-3 was reduced to the level of pre-seizure SS gerbils. Therefore, the results of the present study suggest that down-regulation of NCX-3 expression in the SS gerbil hippocampus may be involved in the hyperexcitability of SS gerbils due to an imbalance of intracellular Na⁺/Ca²⁺ homeostasis and Ca²⁺ concentration.

INTRODUCTION

The Na⁺/Ca²⁺ exchanger (NCX) is a cell membrane ion transporter for the homeostasis of intracellular Ca²⁺ levels. This exchanger transports three Na⁺ ions for one Ca²⁺ ion, and is driven by the Ca²⁺ and Na⁺ gradients over the plasma membrane (1, 2). In fact, Ca²⁺-dependent neurotransmitter release is affected by local Ca²⁺ homeostasis according to NCX activity (3, 4), thus NCX plays an important role in the intracellular Ca²⁺ concentration, which represent a major event in many pathophysiological processes.

Epilepsy is a chronic condition that is characterized by the presence of spontaneous episodes of neuronal discharges. Some reports have suggested that the spontaneous episodes of neuronal discharges may, at least in part, result from abnormal synaptic transmission, which may involve modulation of the transmembrane ion gradient (5, 6). In addition, previous studies have suggested the short- and long-term alterations in the intracellular Ca²⁺ levels and calcium homeostasis occur during epileptogenesis and are associated with the epileptic phenotype (7-9). Moreover, persistent alterations in calcium balance in patients with epilepsy and in animal models of temporal lobe epilepsy (TLE) have been described in voltage-activated calcium currents, glutamate-mediated calcium influx, and the expression of calcium-binding proteins (9-12). Previous studies also indicate that seizure-induced changes are accompanied by changes in the expression and/or function of calcium-extrusion proteins (13, 14).

Mongolian gerbils provide an opportunity to identify neurologic factors that correlate with seizure behavior, because seizure activity in Mongolian gerbils begins at ~2 month of age (15, 16). This animal model also exhibits seizure activity in response to a variety of stimuli without the neuronal degeneration associated with the use of neurotoxins such as kainate. Therefore, Mongolian gerbils allow an epileptic and non-epileptic animals to be directly compared, which allows differences in brain anatomy and electrophysiologic properties correlated with seizure behavior to be identified (17-19). In addition, the seizure activity in this animal model has been linked to the functional alteration of various ion channels/transporters (19-22). However, differences in the expression of NCX-3 immunoreactivity in the epileptic gerbil hippocampus remain to be clarified, specifically, it is not known whether or not the altered expression of NCX-3 correlates with seizure activity in the Mongolian gerbil. Therefore, in the present study, we provide the first comprehensive description of the immunohistochemical distribution of NCX-3 protein levels in the normal and epileptic hippocampi of gerbils and the association between NCX-3 and different sequelae of spontaneous seizures. This finding suggests that altered expression of NCX-3 in the hippocampus may be closely related with seizure activity and the recovery period in this animal model.

RESULTS AND DISCUSSION

NCX-3 immunoreactivity in the SR and pre-seizure SS gerbil hippocampus

As shown in Fig. 1A1, NCX-3 immunoreactivity in SR gerbils was detected in the pyramidal cell layer of the CA1-3 regions
Fig. 1. Immunohistochemical analysis of NCX-3 expression in SR and SS gerbil hippocampi. NCX-3 immunoreactivity in SR gerbil hippocampus was mainly detected in the neurons of the CA1-3 region and NCX-3 expression was also observed within the interneurons of the CA1-3 and hilar regions (A1-D1). As compared with SR gerbils, NCX-3 immunoreactivities in pre-seizure SS gerbils were rarely detected in the hippocampus (A2-D2). However, 3 h after the onset of seizure, NCX-3 immunoreactivity in the SS gerbil hippocampus was significantly enhanced in the principal neurons of the CA1-3 and hilar regions (A3-D3). At 6 h post-ictal, the expression of NCX-3 recovered to pre-seizure SS gerbil levels (A4-D4). Bar = 280 μm (panels A1-A4), 50 μm (panels B1-D4) and 25 μm (high magnification in panels B1-C4).

Fig. 2. Double immunofluorescent stainings for NCX-3 (green) and PV (red) in the gerbil hippocampus. NCX-3 immunoreactive interneurons of the CA1-3 and hilar regions in SR gerbils were parvalbumin (PV)-positive neurons (A1-C1). In the pre-seizure SS gerbil hippocampus, however, NCX-3 immunoreactive PV-positive interneurons were significantly down-regulated in the CA1-3 and hilar regions (A2-C2). At 3 h post-ictal, NCX-3 expression was detected to be similar to SR gerbil levels (A3-C3). Blue is DAPI counterstaining. Rectangles in panels A1, A3, B1, B3, C1, C2 and C3 indicate the high magnification in the right bottom of panels A1, A3, B1, B3, C1, C2 and C3. Bar = 50 μm (panels A1-C3) and 25 μm (high magnification of panels A1-C3).

(Fig. 1A1). In addition, the expression of NCX-3 was observed within the interneurons of the CA1-3 regions and the hilar region of the dentate gyrus (Fig. 1B1 and C1). The NCX-3 immunoreactive interneurons of CA1-3 and the hilar region were parvalbumin (PV)-positive interneurons (Fig. 2A1, B1 and C1).

In the rat hippocampus, NCX subtypes are highly expressed in a cell-type and region-specific manner (13). In the present study, however, there are some differences in the pattern of
Changes of NCX-3 in epileptic hippocampus

Dae-Kyoon Park, et al.

NCX-3 expression in the gerbil hippocampus compared to the rat hippocampus. Briefly, NCX-3 immunoreactivity is abundantly expressed in the mossy fibers projecting from the granule cells located in the dentate gyrus in the rat hippocampus (13, 14). In contrast, the expression of NCX-3 in the gerbil hippocampus was mainly observed in the CA2 pyramidal cells. In addition, the expression of NCX-3 is more intensely detected in the oriens and radiatum layers of the rat CA1 region (13). However, in the case of the current study, NCX-3 immunoreactivity in the CA1 region was mainly expressed within pyramidal cell bodies and interneurons in the CA1-3 regions and the subgranular layer. These discrepancies may be due to species-specific differences because a number of previous studies have shown the differential localization of some receptors, channels, and neurotransmitters in this animal species (23-26). Therefore, our findings confirm and extend the species-specific distribution of NXC-3 immunoreactivity in the rodent hippocampus.

As shown in Fig. 1A2, NCX-3 immunoreactivity in pre-seizure SS gerbils was rarely observed in the pyramidal cell layer of the CA1 region. In addition, NCX-3 expression in the CA2-3 and hilar regions was lower, compared to SR gerbils (Fig. 1A2, B2, C2 and D2). Moreover, NCX-3 expression in PV-positive interneurons was significantly down-regulated, particularly in the CA1 region (Fig. 2A2, B2 and C2). These immunohistochemical data were consistent with Western blot analysis of NCX-3 (Fig. 3 and 4).

Because some investigators have suggested that seizure-induced alterations of intracellular Ca^{2+} concentration are accompanied by changes in the expression and/or function of calcium-extrusion proteins (15, 21, 22), our findings indicate that down-regulation of NCX-3 expression may be closely related to spontaneous seizure activity in gerbils. Indeed, persistent alterations in the intracellular Ca^{2+} concentration and calcium homeostasis arise during epileptogenesis and associated with the epileptic phenotype (10, 15, 17). Moreover, the permanent changes in voltage-activated calcium currents, glutamate-mediated calcium influx, and expression of calcium-binding proteins in patients with epilepsy and animal models of temporal lobe epilepsy (TLE) have been described (17-20). Therefore, the results in the present study suggest that down-regulation of NCX-3 immunoreactivity may play an important role in the generation and spreading of spontaneous seizure activity.

Up-regulated NCX-3 immunoreactivity in the SS gerbil hippocampus following spontaneous seizures

As shown in Fig. 1A3, NCX-3 immunoreactivity in the SS gerbil hippocampus was enhanced in the pyramidal cell layer of CA1-3 and the hilar region 30 min-3 h post-ictal (Fig. 1B3, C3 and D3). In addition, the expression of NCX-3 was increased similar to SR gerbil levels (Fig. 3 and 4). Double immunofluorescence staining for NCX-3 and PV revealed that NCX-3 immunoreactivity within the PV-positive interneurons was dra-
matically increased (Fig. 2A3, B3 and C3). Six h post-ictal, NCX-3 immunoreactivities in the CA1-3 and hilar regions were recovered to pre-seizure SS gerbil levels (Fig. 1A4, B4, C4, D4 and 3). Similar to the immunohistochemical study, immunoblot analysis revealed the same alteration patterns of NCX-3 expression after the onset of seizures (Fig. 4).

Many previous studies have reported that spontaneous seizure activity in the gerbil increases alterations in the levels of expression of various channels and/or enzymes in the SS gerbil hippocampus (12). In addition, these seizure-induced alterations direct the reinforcements and/or normalization of inhibitory transmissions, which may play an important role in maintaining refractory periods (1-2 days) of seizure activity in this animal model. Moreover, NCX in the neurons affects the return of the elevated concentration of intracellular free Ca2+ to resting levels as a calcium-driven Ca2+-extrusion mechanism (1, 3). Furthermore, a previous study demonstrated that some anti-epileptic drugs inhibit Ca2+ influx through the NCX isoform of the cell membrane Na+/Ca2+ transporter, thus the control of NCX expression in the hippocampus can contribute to inhibit chemical-induced convulsions (27). Indeed, NCX regulates the intracellular Ca2+ concentration by releasing Ca2+ from the cell and bringing Na+ into the cell, and in the case of an increased concentration of intracellular Na+, NCX can reverse the concentration change (27, 28). Therefore, our findings suggest that the elevated NCX-3 immunoreactivity in the SS gerbil hippocampus following spontaneous seizures may be a compensatory response to control neuronal hyperexcitability due to abnormalities in the intracellular concentration of Ca2+ and calcium homeostasis. In contrast, NCX is well-suited for rapid recovery from high levels of intracellular Ca2+ and could therefore be important for restoration of basal intracellular Ca2+ after epileptic seizures (3). Thus, enhancement of NCX-3 immunoreactivity in PV-positive interneurons following the onset of seizure is suggestive of fast-adaptation of PV-positive inhibitory interneurons to reduce the hyperexcitability of epileptic hippocampus. Indeed, the immunoreactivity of diverse ion channels in GABAergic neurons are reduced in the hippocampus of pre-seizure SS gerbils and these changes have been normalized following the spontaneous onset of seizure and/or anti-epileptic drug treatment (24, 26). Moreover, intracellular Ca2+ signals in different neuronal compartments are shaped by the spatial organization of Ca2+ influx, buffering, and extrusion mechanisms (29, 30). With respect to these reports, our findings suggested that up-regulated NCX-3 immunoreactivity in GABAergic neurons increases the Ca2+ current, which plays an important role in support/maintenance of fast-spikeing properties after seizure activity (31).

In conclusion, the present data show that down-regulation of NCX-3 expression in SS gerbils may generate seizure activity due to abnormalities in the intracellular Ca2+ concentration and Ca2+ homeostasis, and up-regulated NCX-3 immunoreactivity within PV-positive interneurons following the onset of seizure may be compensatory responses to the hyperexcitability of GABAergic interneurons for the suppression of seizure activity.

MATERIALS AND METHODS

Experimental animals

These studies utilized the progeny of Mongolian gerbils (Meriones unguiculatus) obtained from the Experimental Animal Center of Hallym University in Chuncheon, South Korea. Animals were provided with a commercial diet and water ad libitum under controlled temperature, humidity and lighting conditions (22 ± 2°C, 55 ± 5%, and a 12:12 light/dark cycle). Procedures involving animals conformed to our institutional guidelines, which fully comply with international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 80-23, 1996). All efforts were made to minimize the number of animals used and animal suffering.

Seizure induction

To assess the spatiotemporal alterations of NCX-3 immunoreactivity following a seizure, SS adult gerbils were divided into the following 6 groups: pre-seizure group; and post-seizure groups I, II, III, IV, and V (n = 3 each) that recovered normally 30 min or-, 3, 6, 12 or 24 h after tonic-clonic generalized seizures. For seizure induction, each animal was stimulated by vigorous stroking on the back with a pencil, as described in previous studies (9, 16, 24). Pre-seizure SS gerbils had no seizure activity at least 36 h prior to perfusion (26, 32).

Tissue processing and immunohistochemistry

For immunohistochemistry, all animals were anesthetized (urethane, 1.5 g/kg, ip) and perfused via the ascending aorta with 200 ml of 4% paraformaldehyde in phosphate buffer (PB). The brains were removed, post-fixed in the same fixative for 4 hours, and rinsed in PB containing 30% sucrose at 4°C for 2 days. Thereafter, tissues were frozen and sectioned using a cryostat at 50 μm and consecutive sections were collected in six-well plates containing phosphate-buffered saline (PBS). These free-floating sections were first incubated with 10% normal serum for 30 minutes at room temperature, then incubated with goat anti-NCX-3 antibody (diluted 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBS containing 0.3% Triton X-100 and 2% normal serum overnight at room temperature. After washing 3 times for 10 min with PBS, the sections were incubated sequentially in rabbit anti-goat IgG (Vector Laboratories, USA) and ABC complex (Vector Laboratories, USA), diluted 1:200 in the same solution for the primary antiseraum. Between the incubations, the tissues were washed with PBS 3 times for 10 min each. The sections were visualized with 3,3′-diaminobenzidine (DAB) in 0.1M Tris buffer and mounted on gelatin-coated slides. The immunoreactions were observed under an Olympus BX50 microscope (Japan), and the images were captured using an Olympus.
DP72 digital camera and DP2-BSW microscope digital camera software.

**Double immunofluorescence**

Based on the results of the immunohistochemical studies, we performed double immunofluorescent staining for both goat anti-NCX-3 IgG (diluted 1 : 50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse anti-parvalbumin antibodies (PV, diluted 1 : 2,500; Chemicon, CA, USA) to confirm the cell type. Brain tissues were incubated in the mixture of antisera overnight at room temperature. After washing 3 times for 10 min with PBS, the sections were also incubated in a mixture of Cy2 conjugated donkey anti-goat IgG (1 : 200, Amersham, PA, USA) and Cy3 conjugated donkey anti-mouse IgG antibodies (1 : 200, Amersham, PA, USA) for 1 h at room temperature. The immunoreactions were observed under an Olympus BX50 microscope, and the images were captured using Olympus DP72 digital camera and DP2-BSW microscope digital camera software.

**Western blot**

Based on the immunohistochemical results, of the expression of NCX-3 protein was quantified in the gerbil hippocampus as in previous study (26, 32). Five animals in each experimental group (SR, pre-seizure SS, 3 and 6 h groups following spontaneous seizure activity) were used in the immunoblot study. For tissue preparation, the animals were decapitated, the hippocampi were removed, then each tissue was homogenized in 10 mM PB containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM PMSF. After centrifugation, the protein concentrations in the supernatants were determined using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, USA). Aliquots containing 30 μg of total protein were boiled with an equal volume of 2× SDS sample buffer and boiled for 3 min, then each mixture was loaded onto a 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Schleicher and Schuell, USA). To reduce background staining, the filters were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, sequentially incubated with primary anti-sera (1 : 200), peroxidase-conjugated anti-goat IgG antibody (Sigma, St. Louis, MO, USA) and then with an ECL kit (Amersham, USA). Optical densities were measured using NIH Image 1.59 software.

**Densitometry analysis of data**

The immunohistochemical data was quantified as previously described (33). Briefly, the images of each section on the monitor were captured (15 sections per animal). The mean gray value and its standard deviation were obtained from the selected images using Adobe Photoshop v. 8.0. Each image was normalized by assessing the mean gray value. After the regions (CA1-3 pyramidal cell layers and dentate granule cell layer) were outlined, 10 areas/gerbil (500 μm²/area) were selected from the hippocampus, and the gray values were measured. The intensity measurements were represented as the mean number of a 256-gray scale using NIH Image 1.59 software. The values of background staining were obtained from the corpus callosum. Optical density values were then corrected by subtracting the average values of background noise obtained from 15 image inputs.

**Statistical analysis**

All data obtained from the quantitative measurements were analyzed using one-way ANOVA to determine statistical significance. Bonferroni’s test was used for post-hoc comparisons. A P value <0.01 or 0.05 was considered statistically significant (9, 23, 24, 26, 32, 33).

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**REFERENCES**


