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Searching for a Role of NCX/NCKX Exchangers in Neurodegeneration

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Abstract Control of intracellular calcium signaling is essential for neuronal development and function. Maintenance of Ca^{2+} homeostasis depends on the functioning of specific transport systems that remove calcium from the cytosol. Na^+/Ca^{2+} exchange is the main calcium export mechanism across the plasma membrane that restores resting levels of calcium in neurons after stimulation. Two families of Na^+/Ca^{2+} exchangers exist, one of which requires the co-transport of K^+ and Ca^{2+} in exchange for Na^+ ions. The malfunctioning of Na^+/Ca^{2+} exchangers has been related to the development of pathological conditions in the regulation of neuronal death after hypoxia–anoxia, brain trauma, and nerve injury. In addition, the Na^+/Ca^{2+} exchanger function has been associated with impaired Ca^{2+} homeostasis during aging of the brain, as well as with a role in Alzheimer's disease by regulating β-amyloid toxicity. In this review, we summarize the current knowledge about the Na^+/Ca^{2+} exchanger families and their implications in neurodegenerative disorders.

Keywords Calcium homeostasis · Ischemia · Alzheimer disease · Spinal cord injury · DREAM

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Introduction

Ca^{2+} signaling plays a crucial role in mediating numerous cellular processes in plants and animals. Ca^{2+} enters the cell through a number of finely regulated plasma membrane channels and binds to a multiplicity of sensor proteins, which decode the Ca^{2+} signal to activate specific effector pathways. Subsequent Ca^{2+} extrusion is mediated by membrane transporter systems (reviewed in [1, 2]. In the plasma membrane, Ca^{2+}-ATPases (PMCA) and sodium/calcium exchangers (NCX) are involved in the extrusion of cytosolic-free Ca^{2+}. The homeostasis of cytosolic Ca^{2+} is also regulated through sequestering of free Ca^{2+} into organelles by the endoplasmic reticulum Ca^{2+}-ATPase (SERCA), the Golgi Ca^{2+}-ATPase, and the mitochondrial Ca^{2+} uniporter [1]. PMCA is a pump with high affinity in the sub-micromolar Ca^{2+} concentration range and limited transport capacity that function as a fine-tuner of intracellular-free Ca^{2+} concentration ($[Ca^{2+}]_i$). The major contribution, however, to cytosolic Ca^{2+} clearance is made by sodium/calcium exchangers that have a large capacity to exchange Na^+ for Ca^{2+} or for Ca^{2+} and K^+ ions. Thus, in stimulated neurons, when the $[Ca^{2+}]_i$ reaches levels higher than 500 nM, Ca^{2+} extrusion across the plasma membrane is mainly accomplished by Na^+/Ca^{2+} exchangers [3].

The Na^+/Ca^{2+} exchangers belong to a superfamily of membrane proteins characterized by the presence of two highly conserved regions known as α-repeats 1 and 2, which contain amino acid residues critical for ion binding and translocation [4–9]. Molecular and functional studies have revealed the existence of two families of plasma membrane Na^+/Ca^{2+} exchangers, the NCX and NCKX families [10–12]. Three NCX and five NCKX isoforms have been identified by molecular cloning, and each isoform displays specific tissue and cellular distribution

patterns, suggesting that they play specialized and non-overlapping roles in Ca^{2+} signaling [13–18]. Both NCX and NCKX are single-chain proteins with a mass of 100–180 kDa that show an absolute selectivity for Na^+ over any other alkali cation. NCKX proteins differ from NCX proteins in their absolute requirement for K^+ and their lower Ca^{2+} transport rates [12]. The direction of ion transport via Na^+/Ca^{2+} exchangers depends on the plasma membrane potential, the concentration gradient for each ion transported, and the stoichiometry. The stoichiometry is generally thought to be either three or four Na^+ ions per one Ca^{2+} in the NCX family [19, 20] and four Na^+ ions per one Ca^{2+} plus one K^+ in the NCKX family [21, 22]. Under physiological conditions, the plasma membrane potential is more negative than the reversal potential of NCX and NCKX exchangers. This causes the exchangers to operate electrogenically in the forward mode, i.e., to remove Ca^{2+} from the cell. The exchangers may reverse and mediate Ca^{2+} influx into the cytosol when their equilibrium potential drops below the plasma membrane potential, which may happen under pathological conditions, i.e., when Na^+,K^+-ATPase activity is compromised (reviewed in [12]).

NCX and NCKX proteins share similar overall topologies containing 10 (NCX) or 11 (NCKX) transmembrane-spanning segments (TMs), the first of which (TM0) is cleaved by a signal peptidase. The remaining TMs are divided into two clusters connected by a large cytoplasmic loop with important regulatory functions (Fig. 1) [23–27]. Surprisingly, sequence similarity between NCX and NCKX gene families is limited to the α-repeat regions, which are regions essential for the transport function in both protein families [4, 28]. Whereas all members of the NCX family share about 70% overall amino acid identity [29–31], the homology among the various NCKX proteins depends on the isoforms that are compared. For example, NCKX3 and 4 are closely related, with 71% overall amino acid sequence identity but only about 40% identity with NCKX1 and 2 [14, 24, 30, 32].

The NCX Exchanger Family

The NCX gene family (named SLC8 for the human family) includes the three mammalian proteins NCX1 [29], NCX2 [31], and NCX3 [30] and the recently identified NCX4 from fish species, as well as other NCX genes from non-vertebrate species [33]. The NCX proteins range in size from 861 to 973 amino acids. NCX1 displays 65 and 73% sequence identity with NCX2 and NCX3, respectively, whereas NCX2 shows 75% sequence identity with NCX3 [30]. The family diversity is enlarged at the post-transcriptional level through alternative splicing to yield 17 NCX1 and 3 NCX3 isoforms, whereas no splice variants for NCX2 have been so far identified [34]. The variants arise from a region in the large intracellular loop [35], which is encoded by six small exons, that are used in different combinations in a tissue-specific manner [36, 37]. Brain expression of the three exchanger genes and of many of their splice variants has been studied in detail [34]. The presence of multiple isoforms underlines the critical role of the exchanger in the control of cytosolic Ca^{2+} concentration in neurons, although the kinetic properties of NCX1, NCX2, and NCX3 activation by Na^+ and Ca^{2+} are apparently similar [38], at least to the currently available detection methods.

The most recent topological model predicted for NCX proteins (Fig. 1) indicates the existence of nine transmembrane α helices (TMS) that can be divided into two clusters: an N-terminal hydrophobic domain (TMS 1–5) and a C-terminal hydrophobic domain (TMS 6–9). These two hydrophobic clusters are important for the binding and ion transport, and are connected by a large hydrophilic cytoplasmic loop of approximately 500 residues [39–41]. All members of the NCX family have two amphipathic regions, designated as the α-1 and α-2 repeats, which are internally homologous and oppositely oriented with respect to the plasma membrane [42]. These α-repeat regions include TMS2–TMS3 and TMS7–TMS8, respectively [43].

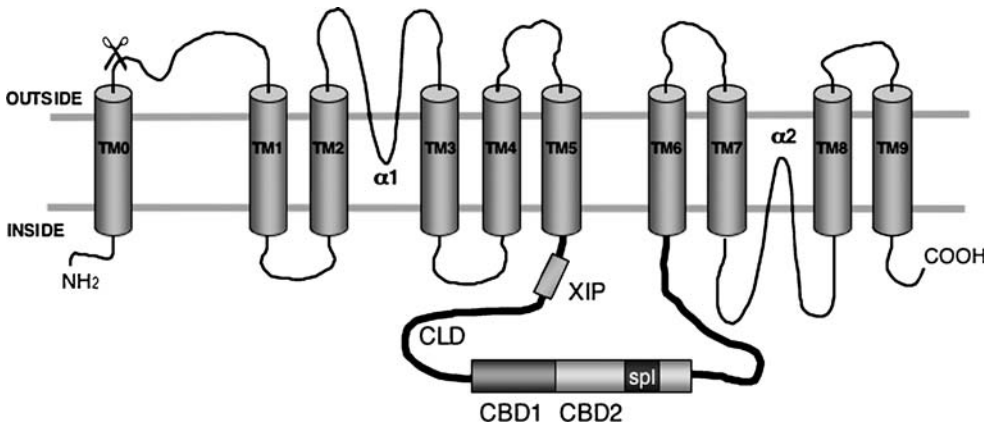

Fig. 1 Topological model for the plasma membrane Na^+/Ca^{2+} exchanger family. The localization of the two α-regions (α-1 and α-2) involved in the ion translocation is shown. The large cytosolic loop contains two Ca^{2+}-binding sites (CBD1 and CBD2) and the region where alternative splicing occurs (*spl*). A α-catenin-like domain (*CLD*) connects the two CBDs to the N-terminal end of the cytoplasmic loop. The N-terminal portion of the loop includes an autoinhibitory domain (XIP)

Mutation of one amino acid residue (N125C) in the α-1 region reduces the Ca^{2+} affinity of NCX1 [9, 39], whereas mutation of acidic or hydroxyl-containing amino acid residues within the putative transmembrane segment of the α-repeats results in loss of or a large reduction in the exchanger activity [7]. These data indicated that α-repeats are involved in the ion translocation. The large hydrophilic intracellular loop is responsible for the regulation of NCX activity by intracellular messengers such as Ca^{2+} and Na^{+} ions, NO, PKC, PKA, and ATP (reviewed in [44]). Two consecutive Ca^{2+}-binding domains (CBD1 and CBD2), arranged antiparallel to each other, are located in the mid-portion of the loop [35]. An α-catenin-like domain (CLD) that connects the two calcium-binding domains to the N-terminal end of the cytoplasmic loop has been proposed [41]. CLD, CBD1, and CBD2 form the regulatory exchanger loop where CBD1 functions as the primary Ca^{2+} sensor, detecting slight increases in cytosolic Ca^{2+}, and CBD2 binds Ca^{2+} at elevated Ca^{2+} concentrations. A small autoinhibitory domain named exchange inhibitory peptide (XIP) identified at the N-terminal end of the cytoplasmic loop has been suggested to bind calmodulin [45].

Transcription of NCX genes is differentially regulated by calcium during development as well as in adult neurons. Whereas transcription of the NCX1 gene is controlled by three separate promoters and is independent of calcium levels [37, 46, 47], the regulation of NCX2 and NCX3 genes is strictly dependent on calcium, although in opposite directions. NCX2 and NCX3 transcripts are, thus, rapidly downregulated and upregulated after membrane depolarization, respectively. The downregulation of NCX2 is dependent on the activity of the Ca^{2+}-dependent phosphatase calcineurin, whereas the induction of NCX3 is calcineurin independent [48]. Recently, it has been described that the Ca^{2+}-dependent transcriptional repressor downstream regulatory element antagonist modulator (DREAM) participates in the regulation of the NCX3 gene. DREAM binds, in a Ca^{2+}-dependent manner, to a site composed by two repeats of the downstream regulatory element (DRE) located downstream from the TATA box in the NCX3 promoter [49]. Changes in intracellular Ca^{2+} levels in neurons, thus, directly regulate the amount and kind of exchanger protein produced. The physiological relevance of this elaborated cellular mechanism to modulate NCX protein diversity is nevertheless not fully understood, as the function of individual exchangers cannot be discriminated by current methods.

In the last years, knockout mice have been generated to discriminate the functional role of the NCX genes. Homozygous NCX1 knockout mice are not viable and die during embryonic development, most likely because of heart-beating failure [50]. Mice deficient for the NCX2 gene, the major isoform in the forebrain, exhibit an improvement in several hippocampal-dependent learning and memory tasks [51]. In contrast, mice lacking the NCX3 gene, which is highly expressed in the cerebellum and in the peripheral nervous system, exhibit reduced motor activity and weakness of forelimb muscles [52].

The K^{+}-Dependent NCKX Exchanger Family

The NCKX gene family (named SLC24 for the human family) includes at least five distinct exchanger molecules: NCKX1 [42], NCKX2 [14], NCKX3 [16], and NCKX4 [53], whereas NCKX5 has been identified but not yet characterized as a functional exchanger [10]. An additional protein named NCKX6 [54] has now been reclassified outside the NCKX family [24, 54]. NCKX proteins range in size from 605 to 1,098 amino acids [55]. As reported for the NCX exchanger family, all full-length members of the NCKX gene family are predicted to contain two multiple transmembrane spanning clusters separated by a large hydrophilic intracellular loop. This cytosolic loop and another large hydrophilic domain at the extracellular N-terminal show reduced sequence similarity among members of the NCKX family. Deletion of the two hydrophilic domains, which eliminates about 60% of the protein, does not affect the transport function of the bovine NCKX1 exchanger [56], indicating that these hydrophilic regions are not involved in the protein function. NCKX contain the two α-repeat regions, the only sequence domain that is shared between members of the NCKX and NCX proteins [10]. Scanning mutagenesis of the α-repeats of human NCKX2 show that mutation of about 25% of these residues leads to impaired NCKX2 proteins, identifying six residues that contribute to the major cation-binding site of the exchanger [4].

Little is known about the regulation of NCKX genes and proteins. Alternative splicing of NCKX1 [57] and NCKX2 [55] has been described to provide heterogeneity in this family of proteins, although its functional significance is not presently understood. At the post-translational level, a redox mechanism regulates dimerization and membrane expression of the NCKX2 protein [25], and a cleavage-signal peptide has been defined at its N-terminal extracellular loop [23]. More important, inactivation of the NCKX1 exchanger has been described after a short period of operation at full rate. This mechanism prevents intracellular-free Ca^{2+} depletion, lower than 2 mM, and is important to desensitize photoreceptors exposed to prolonged activation during bright daylight illumination [58, 59]. Whether a similar desensitization mechanism operates for other NCKX exchanger in other cell types is presently not characterized.

The specific contribution of each of the NCKX proteins to neuronal Ca^{2+} homeostasis and/or physiology remains

largely unexplored. Analysis of NCKX2 knock-out mice, however, revealed a significant reduction in Ca^{2+} flux in cortical neurons, a profound loss of long-term potentiation and an increase in long-term depression at hippocampal Schaffer/CA1 synapses. These mice also showed deficits in specific tests of motor learning and spatial working memory. Surprisingly, there was no obvious loss of photoreceptor function in cones, where expression of the NCKX2 protein has been previously reported. These data emphasize the critical and non-redundant role of NCKX2 in the local control of neuronal Ca^{2+} homeostasis and the essential role in development of synaptic plasticity associated with learning and memory [60].

Brain Distribution of Na^+/Ca^{2+} Exchanger Isoforms

NCX1 protein was found first in the plasma membranes of cardiac cells [61], however, expression in many other tissues including brain, skeletal, and smooth muscle, kidney, eye, and blood cells has been since then reported [37]. Expression of NCX2 and NCX3 have been found only in brain and in skeletal muscle [37]. The three NCX isoforms are highly expressed in the mammalian brain, suggesting that NCX proteins may play a role in the regulation of neuronal function. In cerebral cortical areas, NCX1 isoforms are highly expressed in pyramidal neurons of layers III–V within the molecular layer of motor cortex. In contrast, NCX2 is expressed preferentially in somatosensory cortical areas. Within the hippocampal formation, antibodies against all three NCX protein isoforms show a strong labeling in most neuronal populations, including the granular cell layer of the dentate gyrus and pyramidal cells in the CA1, CA2, CA3, and CA4 subfields [18, 62]. NCX1 protein expression is particularly high in CA3, and the granule cells of the dentate gyrus, whereas expression of NCX3 is higher in the CA3 subregion and in the oriens, radiatum, and lacunoso-moleculare layers of the hippocampus. This widespread distribution in hippocampus suggests that different NCX isoforms may play a role in controlling intracellular Na^+ and Ca^{2+} homeostasis in the major hippocampal projections. NCX isoforms are also expressed in areas critical for the extrapyramidal control of motor coordination, including substantia nigra pars compacta, striatum, and nucleus accumbens [18, 62]. In the cerebellum, NCX3 expression is predominant in the molecular layer, whereas NCX1 is localized in excitatory mossy fibers that project to the granule cell layer [18, 62]. NCX isoforms are also expressed in other brain areas such as thalamus, amygdala, and several hypothalamic nuclei [18].

Although the K^+-dependent Na^+/Ca^{2+} exchange function was originally found in photoreceptors, recent work have shown that several members of the NCKX family are expressed in nonretinal tissues [28]. Indeed, with the exception of the specific expression of NCKX1 in rod photoreceptors, all NCKX proteins are expressed in the brain, NCKX2 being the major neuronal isoform [14, 17, 54]. In contrast with NCX proteins, especially NCX1 that shows a high expression in glial cells, NCKX proteins are expressed mainly in neurons [63], with a peak expression in cultured hippocampal neurons that coincides with their full maturation in vitro, 2 weeks after plating [64].

Pathophysiological Implications of Na^+/Ca^{2+} Exchanger Function

The importance of Na^+/Ca^{2+} exchanger function becomes evident when deregulation of Ca^{2+} and Na^+ homeostasis is directly involved in neuronal and glial damage. Regarding the NCKX exchanger family, a clear physiological role was first established in retinal rod and cone photoreceptors, where NCKX are the only Ca^{2+} extrusion proteins present in the plasma membrane [65]. At least six pathogenic mutations in the sequence of the rod-specific NCKX1 gene have been reported in patients with hereditary retinal disease. In contrast, mis-sense mutations found in the human cone-specific NCKX2 gene are unlikely to be pathogenic [66]. In the brain, little is known about pathophysiological involvement of NCKX proteins; however, Ca^{2+} influx via reversed K^+-dependent exchanger function was several times more rapid in CA1 neurons than in forebrain neurons or cerebellar granules, suggesting a role in neuronal death following global brain ischemia to which CA1 neurons are particularly vulnerable. Because NCKX reversal inhibitors are not yet available, the precise role of NCKX proteins in ischemic death of CA1 neurons remains to be established [67].

The involvement of NCX exchangers in brain pathologies has been related to at least three main conditions including cerebral hypoxia–anoxia episodes, nerve injury, and neurodegenerative disease (reviewed in [44]).

Hypoxia/Anoxia In brain ischemia, gating of postsynaptic glutamate receptors and other membrane channels triggers intracellular Ca^{2+} overload and cellular loss. During ischemia, NCX exchangers are cleaved by caspases and other Ca^{2+}-activated proteases, such as calpains, which could contribute to Ca^{2+} overload and cause neurons to switch from apoptosis to necrosis [68, 69]. Nevertheless, the role played by NCX proteins in cell injury in different experimental models of cerebral ischemia remains controversial. For instance, selective inhibition of NCX proteins exacerbates brain ictus [70], whereas activation of the exchanger with redox agents reduces the infarction area [71] after middle cerebral artery occlusion. Other inves-

tigations, however, have shown that selective inhibition of NCX reduces brain injury in the model of transient middle cerebral artery occlusion [72] and has a protective effect in ischemic spinal cord [73]. Taken together, at present, it is not clear whether the function of NCX exchangers mediates positive or negative effects after ischemic episodes. Studies using in vitro models have not helped to solve the controversy, as different groups have reported that NCX can mediate either an increase or a decrease in neuronal death elicited by glutamate in cerebellar granule neurons in culture [74, 75]. Our results in cerebellar granular neurons showed that reduced NCX3 expression has a protective effect under low depolarizing conditions and exacerbates neuronal death in high extracellular K^+ culture conditions [49]. On this basis, the NCX3 protein should have a protective role in a scenario of ischemia-associated generalized depolarization.

Consistent with the idea of a differential function for the three NCX proteins, their pathophysiological implications may as well be distinct. Thus, expression of the three genes is differentially regulated after permanent middle cerebral artery occlusion with a massive downregulation of NCX2 expression throughout the brain and increased accumulation of NCX1 and NCX3 messenger RNAs (mRNAs), both at the periinfarct area, as well as in nonischemic surrounding brain regions [76]. These data suggest that if NCX proteins are neuroprotective during ischemic injury, the major role is prevalently exerted by the NCX1 and NCX3 gene products.

Nerve Injury Several reports suggest that NCX proteins play a role in mediating Ca^{2+}-induced white matter injury after anoxia or trauma [77]. The inhibition of NCX activity, thus, reduces white matter degeneration in different experimental models of axonal injury, such as optic nerve anoxia [77, 78], spinal cord injury [79, 80], and stretch-injured axons [81]. In addition, in experimental allergic encephalomyelitis and multiple sclerosis, degenerating axons of the spinal cord show increased expression of voltage-gated Na(v)1.6 sodium channels that colocalize with NCX proteins. The proposed mechanism involves massive sodium influx into axons that triggers reverse operation of the exchanger and subsequent influx of damaging levels of intra-axonal calcium that is associated with axonal injury [82, 83].

Aging and Neurodegenerative Disease Impairment of Ca^{2+} homeostasis is closely related to the process of aging of the brain. Indeed, studies performed on cortical nerve endings of aged rats have demonstrated that NCX activity is significantly decreased due to a reduction in the affinity of the exchanger for Ca^{2+} ions [84, 85]. The situation can get worse in aged brain showing accumulation of aggregated β-amyloid peptides because it has been shown that aggregated peptides could interact with the hydrophobic intracellular loop of the exchanger inhibiting its calcium-extruding function [86]. Furthermore, β-amyloid peptides can disrupt intracellular Ca^{2+} homeostasis in several ways, including an enhancement of calcium influx through voltage-dependent and ligand-gated calcium channels and an impairment of membrane ATPases and glucose and glutamate transporters [87].

Concluding Remarks

An increasing number of studies suggest that both NCX and NCKX proteins play a critical role in maintaining intracellular Na^+ and Ca^{2+} homeostasis in the brain under physiological and pathological conditions. However, due to conflicting results of the regulatory effects of NCX proteins, it still remains to be fully clarified whether the function of the exchanger yields beneficial or harmful effects on a number of degenerative diseases. Regarding NCKX exchangers, an important goal will be to clarify their role in the nonretinal tissues where they are expressed. The development of pharmacological tools able to selectively block or activate each NCX and NCKX protein remains as a desirable track to obtain new insights into their functionality and their pathophysiological role.

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