Recent Structural and Functional Insights into the Family of Sodium Calcium Exchangers

Vishal Sharma,1,2 and Damien M. O’Halloran1,2*

1Department of Biological Sciences, The George Washington University, Washington, DC
2Institute for Neuroscience, The George Washington University, Washington, DC

Received 10 September 2013; Revised 4 December 2013; Accepted 8 December 2013

Summary: Maintenance of calcium homeostasis is necessary for the development and survival of all animals. Calcium ions modulate excitability and bind effectors capable of initiating many processes such as muscular contraction and neurotransmission. However, excessive amounts of calcium in the cytosol or within intracellular calcium stores can trigger apoptotic pathways in cells that have been implicated in cardiac and neuronal pathologies. Accordingly, it is critical for cells to rapidly and effectively regulate calcium levels. The Na\(^{+}\)/Ca\(^{2+}\) exchangers (NCX), Na\(^{+}\)/Ca\(^{2+}\)/K\(^{+}\) exchangers (NCKX), and Ca\(^{2+}\)/Cation exchangers (CCX) are the three classes of sodium calcium antiporters found in animals. These exchanger proteins utilize an electrochemical gradient to extrude calcium. Although they have been studied for decades, much is still unknown about these proteins. In this review, we examine current knowledge about the structure, function, and physiology and also discuss their implication in various developmental disorders. Finally, we highlight recent data characterizing the family of sodium calcium exchangers in the model system, Caenorhabditis elegans, and propose that C. elegans may be an ideal model to complement other systems and help fill gaps in our knowledge of sodium calcium exchange biology. genesis 52:93–109. © 2013 Wiley Periodicals, Inc.

Key words: sodium calcium exchange; sodium calcium potassium exchange; Caenorhabditis elegans; calcium homeostasis; antiporter

INTRODUCTION

Calcium ions participate in diverse signaling pathways in animals that control a variety of cellular processes, such as neurotransmission, muscular contraction, lymphocyte activation, and apoptosis. Despite its important regulatory functions, excessive overload of intracellular calcium has an adverse effect on cell health. Accordingly, organisms have evolved mechanisms to rapidly extrude intracellular Ca\(^{2+}\) through transporter proteins. Na\(^{+}\)/Ca\(^{2+}\) exchangers (NCX), Na\(^{+}\)/Ca\(^{2+}\)/K\(^{+}\) exchangers (NCKX), and Ca\(^{2+}\)/Cation exchangers (CCX) are the primary conduits of cytosolic Ca\(^{2+}\) extrusion in animals (Baker et al., 1969; Cervetto et al., 1989; Blaustein, 1968; Palty et al., 2004; Reeves and Hale, 1984). NCX, NCKX, and CCX exchangers are low affinity/high capacity ion transporters and can rapidly expel calcium ions from the cell (Palty et al., 2004; Reeves and Hale, 1984; Sheng et al., 2000). Another transport mechanism for the extrusion of intracellular calcium is the Plasma Membrane Calcium ATPase (PMCA) pump, which removes one calcium ion for every ATP hydrolyzed (Niggli et al., 1982). Cells can also modulate cytosolic calcium levels by regulating calcium release in the mitochondria, endoplasmic reticulum, and the Golgi network through ion transport proteins embedded in organelle membranes (Contreras and Sarria, 2009; Ginger et al., 2008; Ma et al., 2004). Three NCX genes (Solute like carrier 8A family) and five NCKX genes (Solute like carrier 24A family) have been cloned and identified in mammals. Under physiological conditions, NCX utilize the energy stored in the transmembrane sodium gradient to allow influx
of sodium ions and extrusion of one calcium ion (Reeves and Hale, 1984). Studies of Drosophila melanogaster NCX suggest that forward mode activity in retinal cells is initiated when cytosolic calcium levels reach 500 nM (Wang et al., 2005). The exchange mechanism of the NCKX varies slightly as it extrudes one calcium ion and one potassium ion with the influx of four sodium ions (Dong et al., 2001). The human NCKX6 was initially classified as a potassium dependent Na\(^+\)/Ca\(^{2+}\) exchanger but is now understood to be a Na\(^+\), Li\(^+\)/Ca\(^{2+}\) exchanger and considered a member within the calcium cation (CCX) exchanger branch (Cai and Lytton, 2004b; Palty et al., 2004). The CCX exchanger can couple the extrusion of one calcium ion with influx of three lithium or sodium ions.

**STRUCTURE**

**Domains**

The NCX proteins have a general topology composed of 10 transmembrane domains (TM) (Ren and Philipson, 2013), with a large intracellular loop between TM5 and TM6 (Fig. 1). The large intracellular loop contains the calcium binding domain 1 (CBD1) and calcium binding domain 2 (CBD2), which are regulatory domains required for intracellular ion sensing and binding; the intracellular loop also contains a XIP domain that confers sodium inactivation properties. In the canine NCX1, CBD1 and CBD2 are located between TM5 and TM6 (Hilge et al., 2006). CBD1 is the primary sensor for calcium in canine NCX1. Binding of four calcium ions to the CBD1 domain of NCX1 triggers a conformation change from disordered to a more rigid structure, resulting in activation of the NCX1 transporter (Hilge et al., 2006, 2009). Calcium has a low affinity for CBD2 and binds at elevated levels. CBD1 has a sevenfold higher affinity for calcium binding than CBD2 in canine NCX1 (Hilge et al., 2006). CBD2 may play a more selective role than CBD1. Thus, CBD1 and CBD2 function as “switches” for NCX transporters by detecting the appropriate mode of action through calcium influx or extrusion. This switch mechanism is evident in NCX1, which is inactivated once calcium dissociates from CBD1 due to decreased intracellular levels of calcium (Hilge et al., 2009). CBD1 and CBD2 are connected by a short linker sequence, which forms a regulatory tandem domain (Giladi et al., 2010). Calcium binding to CBD1 triggers conformational changes that restrict flexibility of the regulatory tandem domain formed by CBD1 and CBD2. Amino acids Asp\(^{499}\) and Asp\(^{560}\) on CBD1 and Asp\(^{565}\) on CBD2 form inter-domain salt bridges with Arg\(^{532}\) on CBD2, which tether the two regulatory domains and restrict flexibility (Giladi and Khananshvili, 2013). The Gly\(^{503}\) residue within the CBD1-CBD2 linker of canine NCX1 is necessary for calcium-mediated coupling of the CBD domains (Giladi et al., 2012). Linker-dependent interaction of the domains dramatically inhibits the dissociation of calcium and keeps the exchanger active for a prolonged...
state. Within the same intracellular loop that harbors CBD1 and CBD2 is the XIP motif: the XIP domain is a small, 20 amino acid sequence located on the N-terminus of the cytosolic loop that confers sodium dependent inactivation of the exchanger protein (Matsuoka et al., 1997). Domain linkers between TM2 and TM3 form the α1-repeat, and linkers between TM7 and TM8 form the α2-repeat. The α-repeat domains contain residues essential for cation liganding and transport (Winkfein et al., 2003). NCX and NCKX proteins share structural similarities. Structural analysis of the human retinal cone NCKX transporter, NCKX2, reveals the presence of 10 transmembrane domains (Kinjo et al., 2003), two α-repeat sequences and a large intracellular loop (Kinjo et al., 2003). The transmembrane domains are symmetrically divided in half and the two halves of five membrane-spanning helices are separated by a large cytosolic loop (Kinjo et al., 2003). The cytosolic loop contains regulatory domains that sense intracellular calcium and sodium levels and regulate protein activation or inactivation similar to the NCX proteins (Kinjo et al., 2003). Amino acids with oxygen-bearing polar side chains (e.g., serine and asparagine) or negatively charged side chains (e.g., aspartic acid and glutamic acid) located within transmembrane domains seem to affect the affinity of cations for transmembrane-binding pockets (Altimimi et al., 2010; Kang et al., 2005a). Glu188 in α1 repeat and Asp548 in α2 repeat are the key residues in determining the affinity of calcium, sodium, and potassium for the cation-binding pockets of NCKX2. Asp575 establishes potassium dependence of NCKX2 (Kang et al., 2005b). The amino acids Glu188, Asp548, and Asp575 are found in close proximity to each other in the folded NCKX2 protein (Kang and Hilgemann, 2004); these residues, Glu188, Asp548, and Asp575, have been implicated as the key residues of the NCKX cation-binding pocket. NCKX3 and NCKX4 display much higher potassium affinity than NCKX2 (Visser et al., 2007). Affinity for potassium increases in NCKX2 to similar levels of NCKX4 if the residue Thr551 (located on the same helical side as Asp575) is mutated to alanine, the corresponding residue found in NCKX4 (Visser et al., 2007).

### Topology

While a complete crystal structure has not been proposed for the eukaryotic NCX, CCX, or NCKX proteins, recently the crystal structure for the yeast Vcx1 calcium antiporter, which belongs to the calcium/proton (CAX) family of exchangers, was resolved (Waight et al., 2013). Commonly found in fungi and plants, CAX antiporters exchange calcium for protons and have structural and physiological properties similar to NCX exchangers (Waight et al., 2013). The CAX proteins are low affinity/high capacity exchangers and are made up of 10 transmembrane helices, which are divided symmetrically in two halves (TM1-TM5 and TM6-TM10). Two α-repeat sequences and a cytosolic acidic motif are found within the transmembrane domains. Vcx1 is “wedge” shaped when viewed perpendicular to the membrane plane (Waight et al., 2013). The α-repeats are located within an hourglass-shaped central four-helix core composed of TM2-TM3 and TM7-TM8. TM2 and TM7 are kinked in the middle and change directions forming TM2a/TM2b and TM7a/TM7b (Waight et al., 2015) and the conserved GNXXE motif necessary for calcium binding and transport is located within this membrane kink. TM3 and TM8, constituting the rest of the central core, are also tilted and line the interior of the central four-helix core. TM4-TM5 and TM9-TM10 form the outer parts of the protein and flank the central core. The two symmetrical halves are connected by a 20-residue intracellular acidic motif located between TM5 and TM6. The acidic helix is more flexible when not bound by calcium ions but takes on a more rigid conformation upon binding (Waight et al., 2013). In the NCX exchangers of eukaryotes, the intracellular loop is much larger and contains two distinct calcium-binding domains. The active transport site of Vcx1 is occupied by a centrally located calcium ion. Residues Glu502 on TM7b and Ser525 on TM8 coordinate the calcium ion in the active transport site (Waight et al., 2013). The crystal structure of the inward-facing conformation of the *Archeaboglus fulgidus* Ca$^{2+}$/H$^{+}$ CAX exchanger (CAX_Af) was recently resolved to 2.3 Å resolution (Nishizawa et al., 2013). CAX_Af contains 12 TM helices, with core domains (TM2 to TM5 and TM7 to TM10) tightly packed together and two α-repeat domains located between TM2 and TM3, and TM6 and TM7. The inward-facing conformation of CAX_Af arises from the interactions of TM1 and TM6 with the core domain TM1. TM6 adopts a bent conformation and bundling with TM2 and TM7 is facilitated by hydrophobic interactions of Phe227, Leu231, and Leu232 situated in TM2 and TM7. The inward-facing conformation of CAX_Af is formed by kinks in TM2 and TM7 and a gap forms between TM2 and TM7 that fixes the gating bundle in the inward-facing conformation. Binding of calcium or protons closes the gap and enables the gating bundles to slide.

Recently, the outward-facing crystal structure of the *Archaea Methanococcus jannaschii* NCX (NCX_Mj) was determined at 1.9 Å (Liao et al., 2012). The crystal form of NCX_Mj exists as a monomer, has 10 transmembrane domains, two α-repeats, and a small intracellular
motif dividing the 10 transmembrane domains in half (Liao et al., 2012). Both the N and C termini are on the extracellular side. TM2–5 and TM7–10 form a tightly packed core that is embedded perpendicularly into the membrane. TM1 and TM6 are longer helices that are loosely packed around the tight core and slightly bent at 45° angles. The α repeats are located between TM2 and TM3 (α1) and TM7 and TM8 (α2). The α repeats are bent and closely bundled in the center of the protein. NCX_Mj has four ion-binding sites located within the α repeats (Liao et al., 2012); these sites are oriented in a diamond shape and located in close proximity to each other. Two sites face the intracellular (Sint) and extracellular (Sext) site and their ion-ligand binding properties are indicative of sodium binding. Two sites, S_mid and S_Ca, are located in the middle (Liao et al., 2012) and calcium binding is inferred for S_Ca. Two deep cavities that sit on the extracellular side of the protein penetrate into the protein core and form the sodium- and calcium-binding pockets. The pocket formed by the external halves of TM3, TM7, TM9, and TM10 connects the sodium binding S_ext site with the extracellular environment. The pocket formed by external halves of TM2, TM6, and TM7 provide calcium access to the extracellular environment from the S_Ca binding pocket. Rapid interchange to an inward-facing conformation is modeled to involve a sliding motion of loosely packed helices formed by TM1, TM2A, TM6, and TM7 (Fig. 2). When bound by calcium in the outward facing conformation, the exchange begins when sodium ions enter from the extracellular side, and binding of two sodium ions decreases the calcium affinity at S_Ca. During high external sodium concentration, the entry of a third sodium ion increases sodium occupancy at S_mid, which further reduces the calcium affinity and results in calcium ion release to the extracellular side. Upon conformational change to an inward-facing state, the three bound sodium ions are exposed to the low intracellular sodium environment. The S_mid sodium ion is likely released first along with the S_ext sodium ion. This release of the bound sodium ions restores high-affinity calcium binding at S_Ca, which in turn leads to the release of the third sodium ion from S_Ca. The conformational change reverting NCX_Mj to the calcium bound outward-facing state completes the cycle. This figure was reproduced with permission from (Liao et al., 2012).

FIG. 2. Cartoon representation of the Na⁺/Ca²⁺ exchange cycle of NCX in the extrusion mode. When bound by calcium in the outward facing conformation, the exchange begins when sodium ions enter from the extracellular side, and binding of two sodium ions decreases the calcium affinity at S_Ca. During high external sodium concentration, the entry of a third sodium ion increases sodium occupancy at S_mid, which further reduces the calcium affinity and results in calcium ion release to the extracellular side. Upon conformational change to an inward-facing state, the three bound sodium ions are exposed to the low intracellular sodium environment. The S_mid sodium ion is likely released first along with the S_ext sodium ion. This release of the bound sodium ions restores high-affinity calcium binding at S_Ca, which in turn leads to the release of the third sodium ion from S_Ca. The conformational change reverting NCX_Mj to the calcium bound outward-facing state completes the cycle (see Fig. 2 for cartoon).

PHYLOGENY

Cai and Lytton (2004a) performed phylogenetic analysis of the Cation/Ca²⁺ exchange superfamily using 147 sequences from archaea, bacteria, and eukaryotes. Phylogenetic analysis orders the Cation/Ca²⁺ superfamily into five primary groups: YRBG, CAX, NCX, NCKX, and CCX. The YRBG family is specific to prokaryotes and does not catalyze Na⁺/Ca²⁺ exchange, and the likely transport mechanism is H⁺/Ca²⁺ exchange (Nishizawa et al., 2013). Interestingly, the YRBG is more closely related to the NCX and NCKX than the CCX and CAX exchangers. The NCX and NCKX exchangers share
sequence similarity in the α-repeats: G(S/G)SAPE within the α₁ repeat, and GTS(I/V)PD within the α₂ repeat. The NCKX branch arose due to a sequential gene duplication event; one branch gave rise to NCKX1 and NCKX2, the other gave rise to NCKX3, NCKX4, and NCKX5. This gene duplication event likely preceded the divergence of invertebrates and vertebrates (Cai and Lytton, 2004a). The position of exon boundaries in the NCX family in mammals is conserved and the only notable difference is the splitting of the large exon number two of NCX1 and NCX3 into three different exons in NCX2. Within the NCKX of mammals, NCKX1 shares exon boundaries with NCKX2, and NCKX3 shares exon boundaries with NCKX4. The CCX exchanger has a unique conserved sequence within the α-repeats: GNG(A/S)PD in α₁ and (G/S)(N/D)SxGD in α₂. Only two CCX sequences from mammals, one in humans and the
other in mice, have been identified. Eleven CCX have been identified in invertebrates. The only human CCX exchanger, NCKX6, is expressed ubiquitously in all tissues examined (Cai and Lytton, 2004b). Caenorhabditis elegans and Drosophila melanogaster each have multiple CCX exchangers suggesting a more diverse role for the CCX in invertebrates (Fig. 3A). Residues and motifs necessary for ion binding and transport are highly conserved in the Na\(^{+}/Ca^{2+}\) family of exchangers (Cai and Lytton, 2004a). Using the C. elegans NCX-1 as a reference point reveals robust conservation of the \(\alpha_1\) and \(\alpha_2\) repeats containing the conserved “GSSAPE” and “GTSXPD” motifs (Fig. 3B). Through mutational analysis, these residues have been implicated as being important for ion binding and translocation properties of the \(\alpha\)-repeat domains (Winkfein et al., 2003), as serine and threonine residues in the \(\alpha\)-repeats provide oxygen side chains that create a hydrophilic environment for calcium binding (Winkfein et al., 2003).

**MITOCHONDRIAL NCX**

Mitochondria function as intracellular calcium stores and can release or sequester calcium ions based on
changes in cytosolic calcium levels. Thus, mitochondria function as key regulators of calcium-mediated signaling pathways. Calcium also plays a significant role in modulating mitochondrial respiratory activity and influencing the bioenergetic properties of cells. Early evidence reporting Na+/Ca2+ exchange in excitable cells was observed in isolated heart mitochondria where calcium release was observed in isolated heart mitochondria incubated in 20–50 mM sodium (Carafoli et al., 1974). Investigation into the cellular distribution of mitochondrial Na+/Ca2+ exchange revealed expression in various tissues such as the central nervous system (CNS), adrenal cortex, parotid gland, and skeletal muscle (Crompton et al., 1978). The reported stoichiometry of NCXmito mediated exchange is three sodium ions for one calcium ion (Jung et al., 1995), and the mode of transport for NCXmito can be reversed as a consequence of increasing cytosolic sodium concentration (Kim and Matsuoka, 2008). The molecular identity of NCXmito has been the source of much controversy. A mitochondrial gene does not encode NCXmito and a nuclear gene was not initially identified. Antibodies against cardiac, liver, and kidney NCXmito did not cross-react with plasma membrane NCX, suggesting the existence of two distinct proteins (Li et al., 1992). The identity of an NCXmito was recently resolved by the identification of NCLX (NCX6), a Na+/Li+/Ca2+ exchanger, belonging to the CCX branch of Na+/Ca2+ exchangers (Palty et al., 2010). NCLX is the primary Na+/Ca2+ exchanger in the mitochondria. Targeted blocking of this exchanger using siRNA or CGP-37157, a selective blocker of NCLX activity, blocked mitochondrial Na+/Ca2+ exchange (Palty et al., 2010). This was further validated in another study, which implicated NCLX in mitochondrial Na+/Ca2+ exchange in B-lymphocytes (Kim et al., 2012). Blocking NCLX activity in B-lymphocytes abolished mitochondrial Na+/Ca2+ exchange. Together, these studies strongly implicate NCLX as the NCXmito. Growing data on the function of NCLX highlights the important regulatory role in various cellular processes. NCLX functions in controlling cytosolic calcium levels in response to action potentials, neurotransmitter release, synaptic plasticity, neuronal bioenergetics, and mitochondrial nitric oxide and free radical production (Castaldo et al., 2009; Marks et al., 2005; Raiteri et al., 2002, 2007; Tang and Zucker, 1997). NCLX is also implicated in the refilling of calcium stores in the endoplasmic reticulum (ER) (Arnaudeau et al., 2001). NCLX alters neurotransmitter release in pathological conditions when extracellular potassium levels are higher than they are in normal physiological conditions (Raiteri et al., 2002, 2007). It has been theorized that high potassium concentrations lead to an increase in cellular concentrations of sodium, which cause the expulsion of calcium from the mitochondria via NCLX. This mechanism might be prevalent in hyperglycemia, a disease characterized by high levels of glucose in the blood plasma. Hyperglycemic patients experience epileptic symptoms, which may be explained by the reduced expression of the Na+/K+ ATPase in hippocampal neurons (Candy and Szatkowski, 2000). Reduction in expression of the Na+/K+ ATPase causes buildup of extracellular potassium and intracellular sodium and may trigger neurotransmitter release. NCLX also contributes to post-tetanic potentiation in neuromuscular junctions of mice by maintaining high cytosolic calcium concentration through the release of calcium from mitochondrial stores in the presynaptic terminal (Garcia-Chacon et al., 2006). Mitochondrial localization of plasma membrane NCX has been observed in brain tissue samples (Gobbi et al., 2007). NCX1, NCX2, and NCX3 are differently expressed in mitochondria of glia and neurons in the hippocampus (Gobbi et al., 2007); however, the extent of the contribution by Na+/Ca2+ exchange in mitochondria via plasma membrane NCX is still unclear. More recently, it has been demonstrated that the nuclear-encoded NCX3 localizes to the outer mitochondrial membrane (OMM) of neurons where it handles calcium regulation with protein kinase A anchoring proteins (Scorziello et al., 2013).

**DISTRIBUTION OF SODIUM CALCIUM ANTIPORTERS**

NCX expression is highest in cardiac muscle, skeletal muscle, and brain tissue and has also been reported in vascular smooth muscle and urinary bladder smooth muscle (see Table 1) (Murata et al., 2010). NCX1 localizes at the presynaptic and postsynaptic sites and in the endoplasmic reticulum membrane of neurons (Caniotto et al., 2002), and high expression was also reported in axons, dendrites, and growth cones (Luther et al., 1992). NCX1 also localizes to the inner membrane of the nuclear envelope and complexes with GM1 (Ledeen and Wu, 2007). GM1 is a ganglioside and its expression in the nucleus has been functionally linked to maintenance of calcium homeostasis during neurite growth (Wu et al., 1995). NCX expression in the nucleus was surprising as calcium exchange was previously considered to occur through simple diffusion across the nuclear pores (Ledeen and Wu, 2007). NCX1 expression is increased in microglial cells following brain ischemia (Boscia et al., 2009). NCX2 and NCX3 are highly expressed in brain tissue and skeletal muscles (Li et al., 1994; Nicoll et al., 1996), and all three NCX proteins are widely expressed throughout the rat CNS (Caniotto et al., 2002). Calx, a homolog of the mammalian NCX, in D. melanogaster is highly expressed in photoreceptor cells and mediates Na+/Ca2+ exchange (Wang et al., 2005). NCXK are widely expressed in various cells in mammals: NCXK1 is expressed in rod photoreceptors and platelets (Kang et al., 2012).
and Schnetkamp, 2003; Roberts et al., 2012); NCKX2 is expressed in the brain, cone photoreceptors, and retinal ganglion cells (Kang and Schnetkamp, 2003; Kinjo et al., 2003; Li et al., 2006); NCKX3 is expressed in the vascular smooth muscles, uterus, brain, and intestines (Kraev et al., 2001; Lytton et al., 2002; Yang et al., 2006). NCKX4 is expressed in the brain, vascular smooth muscles, lungs, and thymus (Li et al., 2002; Lytton et al., 2002); and NCKX5 localizes to the retina and the trans-Golgi network of epidermal cells (Lamason et al., 2005). The CCX exchanger, NCKX6, is expressed in many different tissues including the brain, thymus, heart, skeletal muscles, lungs, kidneys, intestines, and testis (Cai and Lytton, 2004b). Emerging data suggest that NCX and NCKX are targeted to membranes by different mechanisms; NCKX1 and NCKX2 contain a plasma membrane targeting signal peptide (Kang and Schnetkamp, 2003), whereas the cardiac NCX1 does not require a signal peptide for membrane localization (Sahin-Toth et al., 1995). The kinesin motor KIF21a facilitates the cellular trafficking of NCKX2 to the axonal terminals of CNS neurons in rats with the large intracellular loop of NCKX2 interacting with the putative cargo-binding domain of KIF21a (Lee et al., 2012).

**REGULATION OF NCX EXPRESSION**

NCX1 is the main Na\(^{+}\)/Ca\(^{2+}\) exchanger in cardiac tissue and important transcriptional regulators of cardiac NCX1 are serum response factor (SRF) and GATA-4 (Xu et al., 2006). SRF, a transcription factor primarily involved in early embryonic development, turns on the expression of NCX1 and a microRNA called mir-1; mir-1 further regulates its expression in cardiac tissue and reduces NCX1 translation (Tritsch et al., 2013). GATA-4 regulates the expression of genes involved in embryogenesis and myocardial differentiation and modulates NCX activity in both neonatal and isolated adult cardiomyocytes (Xu et al., 2006). The GATA-binding element on the NCX1 promoter is necessary for the cardiac specific expression of NCX1. The cardiac minimal promoter of NCX1 contains binding sites for several transcriptional factors including GATA, CArG, MEF-2, and E-box (Menick et al., 2007). Alternative splicing also plays an important function in determining NCX1 localization. The mammalian NCX1 gene has six exons: A–F (Kofuji et al., 1994). Localization of NCX1 transcripts is determined by the expression of either exon A or B (Quednau et al., 1997; Schulze et al., 2002). Transcripts with exon A are found predominantly in excitable cells like muscles and neurons (Schulze et al., 2002). Transcripts with exon B are found mostly in non-excitable cells like astrocytes and liver cells (Schulze et al., 2002). The strength of ionic currents generated by transcripts with exon A is significantly higher (Schulze et al., 2002). NCX1 expression can be driven by \(\alpha\)-adrenergic stimulation via a cardiac minimal promoter (Cheng et al., 1999). \(\beta\)-adrenergic stimulation also upregulates NCX1 via the transcription factor AP-1 that binds the AP-1 elements of the NCX1 promoter.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression Pattern</th>
<th>Localization</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCX1</td>
<td>Brain, Heart, Bladder</td>
<td>Plasma Membrane, Nucleus, Endoplasmic Reticulum, Mitochondria</td>
<td>Canitano et al., 2002; Gobbi et al., 2007; Ledeen and Wu, 2007; Murata et al., 2010</td>
</tr>
<tr>
<td>NCX2</td>
<td>Bain, Skeletal Muscle</td>
<td>Plasma Membrane, Mitochondria</td>
<td>Gobbi et al., 2007; Li et al., 1994</td>
</tr>
<tr>
<td>NCX3</td>
<td>Brain, Skeletal Muscle</td>
<td>Plasma Membrane, Outer Mitochondrial Membrane</td>
<td>Gobbi et al., 2007; Nicoll et al., 1996; Scorziello et al., 2013</td>
</tr>
<tr>
<td>NCKX1</td>
<td>Rod Photoreceptors, Platelets</td>
<td>Plasma Membrane</td>
<td>Kang and Schnetkamp, 2003; Roberts et al., 2012</td>
</tr>
<tr>
<td>NCKX2</td>
<td>Brain, Cone Photoreceptors, Retinal Ganglion Cells</td>
<td>Plasma Membrane</td>
<td>Kang and Schnetkamp, 2003; Kinjo et al., 2003; Li et al., 2006</td>
</tr>
<tr>
<td>NCKX3</td>
<td>Brain, Vascular Smooth Muscles, Uterus, Intestines</td>
<td>Plasma Membrane</td>
<td>Kraev et al., 2001; Lytton et al., 2002; Yang et al., 2010</td>
</tr>
<tr>
<td>NCKX4</td>
<td>Brain, Vascular Smooth Muscles, Lungs and Thymus</td>
<td>Plasma Membrane</td>
<td>Li et al., 2002; Lytton et al., 2002</td>
</tr>
<tr>
<td>NCKX5</td>
<td>Retina, Epidermis</td>
<td>Plasma Membrane, Melanosome, Golgi Apparatus</td>
<td>Ginger et al., 2008</td>
</tr>
<tr>
<td>NCLX</td>
<td>Brain, Thymus, Heart, Skeletal Muscles, Lungs, Kidneys, Intestines and Testis</td>
<td>Mitochondria</td>
<td>Cai and Lytton, 2004b; Palty et al., 2010</td>
</tr>
</tbody>
</table>
(Mani et al., 2010). Prolonged β-adrenergic receptor activation leads to an increase in heart rate and contractility but chronic stimulation leads to changes in gene expression that can cause heart failure (Mani et al., 2010). Work in PC12 neuronal cell lines has shown that NCX is regulated by extracellular-signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK), and p38 mitogen-activated protein kinases (MAPKs) (Sirabella et al., 2012). Blocking JNK or MEK1, which is an upstream activator of ERK1/2, down-regulates NCX1 and NCX3 (Sirabella et al., 2012). Neural growth factor (NGF) stimulated CREB1 and SP1 binding to the NCX1 promoter, and CREB1 binding to the NCX2 promoter (Sirabella et al., 2012). SP1 is a transcription factor involved in early development, suggesting a developmental role for NCX1 in neuronal tissue. During anoxic conditions in neurons, NCX1 is up-regulated by the transcription factor nuclear factor kappa B (NF-kB) leading to an increase in endoplasmic reticulum (ER) calcium levels, thus preventing ER stress (Sirabella et al., 2009). Similarly, in hypoxic conditions, NCX1 transcription is up-regulated by hypoxia-inducible factor-1 (HIF-1) (Valsechci et al., 2011), thus suggesting a role for NCX1 during stroke. Hormones and metabolites also regulate NCX and NCKX function (Herchuelz et al., 2007; Yang et al., 2010, 2011; Chen et al., 2011). In beta cells, which are located in the pancreas and function in release and storage of insulin, exposure to glucose switches the cells to NCX-mediated extrusion of intracellular calcium instead of PMCA (Herchuelz et al., 2007). NCKX3 and NCX1 are highly expressed in the endometrium luminal and glandular epithelial cells, and the expression of NCKX3 is temporally up-regulated by the hormones 17β-estradiol (E2) and progesterone (P4) throughout the menstrual cycle (Yang et al., 2011). E2 and P4 also up-regulate NCKX3 expression in the uterus of rats during the estrous cycle (Yang et al., 2010). E2 has also been shown to regulate NCX1 expression in the female rabbit heart (Chen et al., 2011).

**PHYSIOLOGY**

The NCX exchanger has a stoichiometry of three sodium ions for one calcium ion (Reeves and Hale, 1984). Under physiological conditions, the exchanger can function in the forward direction by coupling the extrusion of calcium with the influx of sodium ions (Hilgemann et al., 1991) and also in the reverse direction by coupling the influx of calcium ions with the extrusion of sodium ions (Kang and Hilgemann, 2004). Based on the NCX_MJ structure, the forward direction ion exchange is initiated by the binding of sodium ions from the extracellular environment (Liao et al., 2012), which reduces the binding affinity of the calcium ion and result in its release to the extracellular side (see Fig. 2 for overview of the extrusion cycle). The forward and reverse capacity of Na⁺/Ca²⁺ exchangers has been studied in the case of the cardiac Na⁺/Ca²⁺ exchanger NCX1, which can function as a bidirectional exchanger depending on the membrane potential and may function in reverse in the absence of cytoplasmic calcium (Kang and Hilgemann, 2004). In rat cortical astrocytes, influx of calcium through reverse-mode NCX is the primary effector of glutamatergic glial transmission (Reyes et al., 2012). During reverse-mode NCX function, NKA, an ATP pump that extrudes three sodium ions and allows influx of two potassium ions, alongside PMCA pumps, mediates calcium extrusion. In Purkinje neurons, high intracellular sodium triggers the reverse mode activity of NCX (Roome et al., 2013) and boosts presynaptic transmission and neurotransmitter release (Roome et al., 2013); this influx of calcium through NCX enhances neurotransmitter release for 400 ms and contributes significantly to postsynaptic events by increasing presynaptic activity (Roome et al., 2013). Under normal physiological conditions, bovine rod NCKX2 functions in forward mode, although it may function in reverse mode as well (Dong et al., 2001) with the reversal requiring the presence of extracellular potassium to allow calcium to enter the cell (Dong et al., 2001).

**DEVELOPMENT AND PATHOPHYSIOLOGY**

Calcium signaling is essential at various stages of development for different developmental events. Expression levels of NCX1 and NCX3 are highly modulated in oligodendrocyte progenitor cells (OPC) (Boscia et al., 2012). NCX3 is expressed in both the white and gray matter of the spinal cord (Fig. 4A), and interestingly in ncx3/- null mice the isolated spinal cords are smaller than in wildtype animals (Fig. 4B). At the protein level, it was revealed that myelin markers (CNPase and MBP) were reduced in ncx3/- null mice compared with wildtype animals (Fig. 4C, left) and that a marker of oligodendrocyte progenitor cells (NG2) was up-regulated in ncx3/- mice (Fig. 4C, right). These findings were also confirmed using immunofluorescence analysis on spinal cord white matter (Fig. 4D). These findings clearly demonstrated a critical role for NCX3 in the progression of oligodendrocyte progenitor cells into mature oligodendrocytes and also in myelination within the white matter of the spinal cord (Boscia et al., 2012, 2013). Up-regulation of NCX1 and NCX3 was observed during short sub-lethal brain ischemia (ischemic pre-conditioning) prior to a prolonged harmful ischemic episode (Pignataro et al., 2012), and up-regulation of NCX3 was observed during ischemic post-conditioning subsequent to a prolonged harmful ischemic episode (Pignataro et al., 2011). Up-regulation of the NCX genes during ischemic pre-conditioning and post-conditioning events confers
neuroprotective roles, and consequently the knockdown of NCX1 and NCX3 via siRNA partially reverses the neuroprotective effects of both events (Pignataro et al., 2011, 2012).

Outside the nervous system, maintenance of calcium homeostasis is extremely important during pregnancy and labor where it plays a significant role in uterine smooth muscle contraction and fetal implantation (Daston and Naciff, 2005; Dong et al., 2006; Salamonsen et al., 2001). The role of NCX and NCKX in the menstrual cycle and during pregnancy in female reproductive organs is not well understood but expression pattern analysis suggests a role for these proteins (Yang et al., 2010, 2011). NCKX3 mRNA expression increases during the early- and mid-proliferative phases and the early secretory phase of the menstrual cycle whereas the expression levels of NCX1 does not change. Developmental processes like fetal bone mineralization are dependent on the placental transfer of calcium and phosphorous ions from maternal blood to fetal blood and calcium exchange is high during late gestation to meet the developing needs of the mineralizing fetal skeleton. Varied expression of the NCX1 and NCKX3 transporters is observed in the placenta, but their exact function still remains unknown (Yang et al., 2011). NCX1 mRNA expression is altered during the second and third trimester and a rapid increase is observed at the 40 week stage (Yang et al., 2011). NCKX3 mRNA expression is highest on the maternal section of the human placenta in comparison to the fetal or central section, and altered expression of NCKX3 and NCX1 mRNA is observed in preeclamptic placenta. During preterm labor, NCKX3 and NCX1 are overexpressed in preeclamptic placenta in comparison to normal placenta.

FIG. 4. Expression of OPC, oligodendrocyte, astrocyte, and axonal markers in the spinal cord of ncx3−/− and ncx3−/− mice. (A) Distribution of NeuN (a), NCX3 (b), and MBP (c) immunoreactivities in the spinal cord of ncx3−/− mice. Colocalization of NCX3 with NeuN in the gray matter (d–f), or with MBP in the white matter (g–i), of ncx3−/− spinal cord sections. (j–l) High-magnification images of the frame depicted in panels (g–i) illustrating co-expression of MBP with NCX3 immunosignals around a single axon (asterisk). Scale bars in A; d–i: 50 μm; j–l: 2.5 μm. (B) Representative images of 4-month ncx3−/− (left) and ncx3−/− (middle) whole-brain and spinal cord samples. The red boxes indicate the reduction in spinal cord size between ncx3−/− and ncx3−/− mice. Transverse measurements of the cervical spinal cord diameter in ncx3−/− and ncx3−/− mice. (C) Western blotting of MBP, CNPase, NG2, GFAP, and NF200 in ncx3−/− and ncx3−/− mice. (D) Distribution of MBP immunoreactivity (a–b) and quantification of its fluorescence intensity (c) in the white matter of the spinal cord from ncx3−/− and ncx3−/− mice. (D) Distribution of NF-200 immunoreactivity (d–e) and quantification of its fluorescence intensity (f) in the white matter of the spinal cord from ncx3−/− and ncx3−/− mice. (D) Distribution of NG2 immunoreactivity (g–h) and quantification of NG2-positive cells (i) in the white matter of the spinal cord from ncx3−/− and ncx3−/− mice. Scale bars in D: a, b: 20 μm; d, e, 50 μm; g, h: 50 μm. This figure was reproduced with permission from Boscia et al., (2012).
to normal placenta (Yang et al., 2011). However, during term labor both NCX1 and NCKX3 expression are downregulated in all sections of preeclamptic placenta relative to normal placenta (Yang et al., 2011). These expression patterns during preeclampsia suggest a role for the NCX and NCKX in maternofetal calcium absorption and proper fetal development.

A role for NCX in learning and memory has been shown via the generation of NCX knockout mice (Jeon et al., 2003; Molinaro et al., 2008). NCX2 is the primary calcium clearing protein at presynaptic sites of hippocampal CA1 pyramidal neurons (Jeon et al., 2003). In ncx2\(^{-/-}\) knockout mice, there is a significant delay in intracellular calcium clearance following neuronal depolarization at the presynaptic sites of hippocampal CA1 pyramidal neurons (Jeon et al., 2003), which prolongs the clearance of intracellular calcium resulting in a decreased threshold for long-term potentiation (LTP) and suppression of long-term depression (LTD). Prolonging the clearance time of intracellular calcium also enhances short-term plasticity, and this decreased threshold for LTP and enhanced short-term plasticity results in greater functionality of ncx2\(^{-/-}\) knockout mice in learning and memory tasks including the water maze (spatial learning and memory), context fear conditioning, and object recognition memory (Jeon et al., 2003). NCX3 is expressed in the CA1 region (Molinaro et al., 2008), and ncx3\(^{-/-}\) knockout mice display elevated basal levels of intracellular calcium and exhibit slow declines in presynaptic intracellular calcium levels after depolarization (Molinaro et al., 2008). There is, however, no change in presynaptic peak intracellular calcium levels after neuron depolarization. A moderate elevation of postsynaptic intracellular calcium occurs and leads to activation of a phosphatase cascade rather than a kinase cascade, and consequently Ca\(^{2+}\)-calmodulin kinase II\(\alpha\) (CaMKII\(\alpha\)) is not phosphorylated resulting in reduced activity (Molinaro et al., 2008). Six-month-old ncx3\(^{-/-}\) knockout mice also show reduced motor activity, weakness of forelimb muscles, and fatigability (Molinaro et al., 2008). NCX2 and NCX3 vary in their contribution to memory and learning since NCX2 primarily exerts its influence at the presynaptic level whereas NCX3 exerts its effect postsynaptically (Jeon et al., 2003; Molinaro et al., 2011). More evidence for Na\(^{+}/Ca^{2+}\) exchange is central to development, and dysfunction may result in numerous pathologies, some of which we detail above.

**NA\(^{+}/Ca^{2+}\) EXCHANGERS IN THE NEMATODE CAENORHABDITIS ELEGANS**

Here we highlight recent data characterizing the family of sodium calcium exchangers in the model system, *Caenorhabditis elegans*, and propose that *C. elegans* may be an ideal model to complement other systems and help fill gaps in our knowledge of sodium calcium exchange biology: Phylogenetic analysis of the protein sequence of NCX, NCKX, and CCX exchangers from human, *D. melanogaster*, and *C. elegans* reveal significant sequence conservation across all three species (Fig. 3A). Human NCX1, NCX2, and NCX3 are closely related to *C. elegans* NCX transporters NCX-1, NCX-2,
and NCX-3 and also the *Drosophila melanogaster* NCX transporter, Calx. The human NCKX1–5 are most closely related phylogenetically to the *C. elegans* NCX-4 and NCX-5 transporters and also the *D. melanogaster* Nckx-30C and Nckx-X-SC. The human NCKX6 groups with the divergent *C. elegans* CCX exchangers: NCX-6, NCX-7, NCX-8, NCX-9, and NCX-10. The human NCKX6 lacks a highly conserved aspartic acid residue located in TM9, which confers potassium dependence in the other NCKX transporters (Kang et al., 2005b). The invertebrate CCX clade is expanded in comparison to vertebrates. *D. melanogaster* and *C. elegans* each contain five copies of CCX exchangers. The two α-repeats (located between TM2 and TM3, TM7, and TM8), the intracellular loop, and the transmembrane domains in the NCX protein sequences from *C. elegans*, *D. melanogaster*, and humans are also highly conserved (Fig. 3B). Furthermore, key aspartic acid and glutamic acid residues in the calcium-binding domains are also conserved (Fig. 3B); these residues have been implicated in stabilization and coordination of calcium to the binding sites of the calcium-binding domains in other studies (Waight et al., 2013). Interestingly, the XIP region is conserved in humans and *D. melanogaster* but no clear XIP motif is present in *C. elegans* exchangers.

Within *C. elegans* the Na*/Ca*<sup>2+</sup> exchangers are expressed in many cell and tissue types including primary sensory neurons, interneurons, motor neurons, pharyngeal muscle, body wall muscle, vulval muscle,
CONCLUDING PERSPECTIVE

Sodium calcium exchangers regulate calcium levels both during development and also in the adult. They are an incredibly influential family of proteins whose contributions and implications are rapidly expanding as research into their biology increases. The general properties of these transporters are now well understood through decades of impressive experimentation into their physiology. However, functionality of these transporters have not been fully defined. The recent characterization of this family of transporters within Caenorhabditis elegans (Sharma et al., 2013) raises the possibility of obtaining more mechanistic insight into the features of control and activation at single cell resolution in live animals. By employing cell biological reporters at the transcriptional and translational levels and integrating these tools with optogenetics and genetic encoding calcium reporters in C. elegans, we may be able to take an unprecedented look at cell membrane, nuclear, and mitochondrial Na⁺/Ca²⁺ exchange, as well as isoform specific trafficking in live animals during development and later life. Understanding this family of proteins means also understanding differences within this family—the C. elegans genome encodes multiple genes for each branch of the sodium calcium exchanger superfamily (e.g., humans only encode one CCX member), and a molecular entry point into providing mechanism will be understanding how these functional specializations map onto different behavioral programs. Thus, by encoding a diverse family of Na⁺/Ca²⁺ exchanger, that often present distinct expression patterns, C. elegans may aid in our understanding of specificity within this family. In short, it is an exciting time to study this family of exchangers as we can learn much about their biology and instructive capacity through the use of tools developed in recent years to build on decades of extraordinary research.

ACKNOWLEDGEMENTS

We are very grateful to Youxing Jiang for providing us with a high resolution file of Figure 2 and for comments on the manuscript and to Lucio Annunziato for his kind permission to reproduce Figure 4. We are also grateful to Anthony LaMantia and Sally Moody for advice. We thank The George Washington University Department of Biological Sciences for funding to V.S. and The George Washington University Department of Biological Sciences and Columbia College of Arts and Sciences for funding to D.M.O'H.

LITERATURE CITED


Boscia F, D’Avanzo C, Pannaccione A, Secondo A, Casamassa A, et al. 2012. Silencing or knocking out the na(+)−/cat(2+)− exchanger-3 (NCX3) impairs...
sharma and o’halloran


Cai X, Lytton J. 2004a. The cation/ca(2+)


Reyes RC, Verkhovsky A, Parpura V. 2012. Plasmalemmal Na+/Ca2+ exchanger modulates Ca2+-dependent exocytotic release of glutamate from rat cortical astrocytes. ASN Neuro 4:.


Sheng JZ, Prinsen CF, Clark RB, Giles WR, Schnetkamp PP. 2000. Na(+)-Ca(2+) currents measured in insect cells transfected with the retinal cone or rod Na(+)-Ca(2+)-K(+) exchanger cDNA. Biophys J 79: 1945–1953.


