Review

Na⁺/Ca²⁺-K⁺ Exchangers (NCKX)

Functional Properties and Physiological Roles

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ABBREVIATIONS
NCKX Na⁺/Ca²⁺-K⁺ exchanger
NCX Na⁺/Ca²⁺ exchanger
TMS transmembrane segment
RT PCR reverse transcriptase polymerase chain reaction
PMCA plasma membrane Ca²⁺ ATPase
SERCA sarco/endoendoplasmic reticulum Ca²⁺ ATPase
NMDA N-methyl-D-Aspartate
fEPSP excitatory postsynaptic field potentials
LTD long term depression

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ABSTRACT

The most numerous Ca²⁺ extrusion protein family, in terms of distinct genes, is the SLC24 gene family of Na⁺/Ca²⁺-K⁺ exchangers (NCKX). Five distinct gene products have been identified, mostly from specific animal excitable tissues such as neurons and smooth muscle, but also in places like skin pigment epithelium, signifying that NCKX proteins may play very specific roles, related to Ca²⁺ homeostasis, in these tissues. However, progress in elucidating the specific physiological roles of NCKX proteins has been slow in coming, largely because of challenges relating to isolating the activity of these proteins in their native tissues. Herein, we provide an overview of NCKX protein functional characteristics, highlighting properties that are unique and useful as distinguishing features over other Ca²⁺ handling mechanisms. We also present the first comprehensive review of the literature concerning physiological roles of NCKX proteins.

INTRODUCTION

The process of Na⁺/Ca²⁺ exchange was first described in the late 1960s through measurements of Na⁺-dependent ⁴⁵Ca fluxes in various tissues (reviewed in ref. 1, Na⁺/Ca²⁺ exchange, including a historical perspective). In the late 1970s and 1980s cardiac sarcolemmal vesicles and isolated retinal rod outer segments became popular preparations for biochemical and functional characterization of Na⁺/Ca²⁺ exchange as these preparations appeared to contain high concentrations of the putative Na⁺/Ca²⁺ exchange protein. Although Na⁺/Ca²⁺ exchange in various tissues displayed many common features including the competitive interaction between multiple Na⁺ ions and a single Ca²⁺ ion, the sigmoidal dependence on external [Na⁺], and the occurrence of Ca²⁺-Ca²⁺ self-exchange fluxes, Na⁺/Ca²⁺ exchange in retinal rod outer segments displayed some unique features, all concerning the effects of K⁺ on Na⁺/Ca²⁺ exchange.² In the late 1980s these observations culminated in the demonstration that Na⁺/Ca²⁺ exchange in retinal rod outer segments required and transported K⁺ in addition to Na⁺ and Ca²⁺ with a stoichiometry of 4Na⁺:1(Ca²⁺ + 1K⁺).³,⁵ distinct from a stoichiometry of 3Na⁺:1Ca²⁺ previously established in cardiac sarcolemmal vesicles.⁶ The cardiac Na⁺/Ca²⁺ exchanger was first cloned from dog heart² and subsequent cloning of the retinal rod Na⁺/Ca²⁺-K⁺ exchanger from bovine retina⁸ revealed that these two exchanger cDNAs represented the first members of two rather distinct gene families, the SLC8 gene family of Na⁺/Ca²⁺ exchangers (NCX) containing three members and the SLC24 gene family of Na⁺/Ca²⁺-K⁺ exchangers (NCKX). Six different mouse or human NCKX gene products have been described in the literature which would make it the most numerous gene family of Ca²⁺ extrusion proteins. A more careful analysis of the six putative NCKX proteins reveals that NCKX6 may be part of a distinct gene family and this is illustrated by the sequence alignment of the alpha 1 and alpha 2 repeats shown in Figure 1. The alpha 1 and 2 repeats are thought to have arisen from an ancient gene duplication event, they contain many residues critical for transport function and they also contain the only sequence elements conserved between NCX and NCKX proteins. The 19 shaded residues have been shown to be important for Na⁺/Ca²⁺-K⁺ exchange transport⁹,¹² and are conserved between the NCKX1-5 isoforms, with only a single conservative substitution (Asp to Glu in the alpha 2 repeat). In contrast, 10 of the 19 shaded residues are not conserved between NCKX1-5 and NCKX6, in many cases through non-conservative substitutions. As a matter of fact, a more significant number of these residues (15 out of 19) are conserved between NCX1 and NCKX1-5. Therefore, we will limit this review to the NCKX1-5 isoforms. Analysis of the many genomic sequencing projects has revealed that NCKX proteins are widely...
expressed throughout the animal kingdom, but are not found in plants and fungi. In addition to many NCKX cDNAs cloned from a variety of vertebrate species (mammals, birds, fish), NCKX cDNAs have been cloned from *C. elegans* and *Drosophila* and shown to encode K+-dependent Na+/Ca2+ exchangers.

**OBJECTIVES OF THIS REVIEW**

Although the SLC24 gene family of Na+/Ca2+-K+ exchangers is the most numerous gene family of Ca2+ extrusion proteins, rather limited insight into the physiological role of NCKX proteins in various tissues that contain NCKX transcripts has been gained to date. Most of our detailed understanding of in situ NCKX physiology has come from studies exploring the role and properties of NCKX1 in vertebrate retinal rod photoreceptors, while functional characterization and structure-function studies of NCKX clones expressed in cell lines has mostly been carried out on the NCKX2 isoform. The objectives of this review are:

1. To detail the functional characteristics of NCKX proteins with a particular focus on identifying NCKX activity in cells and tissues, and contrast it with properties of the NCX proteins that are often expressed in the same cells/tissues.
2. To review the literature on NCKX physiology with special emphasis on recent studies suggesting exciting new roles of NCKX2 in hippocampal neurons and NCKX5 as a genetic determinant of skin pigmentation in humans.

As mentioned above, most NCKX structure-function studies have been carried out on the NCKX2 isoform, and we have recently reviewed this work elsewhere. As the hydropathy plots of the NCKX1-5 isoforms are very similar, and residues shown to be important for NCKX2 cation binding and transport are conserved among the NCKX1-5 isoforms (Fig. 1), we surmise that our insights into NCKX2 structure-function relationships are equally valid for the other four NCKX isoforms. The current topological model of NCKX2 is illustrated in Figure 2; it consists of cleavable signal peptide at the N-terminus and two sets of transmembrane segments (TMS) separated by a large hydrophilic loop located in the cytosol. Sequence similarity among all five human NCKX isoforms, and among all the various NCKX sequences found in lower organisms is limited to the two sets of TMS.

**FUNCTIONAL CHARACTERISTICS**

**Modes of transport: Ca2+ influx and Ca2+ extrusion.** Both NCX and NCKX proteins belong to the large group of secondary transport proteins, which mediate multiple transport pathways.  

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**Figure 1.** Sequence alignments of the alpha 1 and alpha 2 repeats of NCKX1-6 and NCX1.
transporters many of which utilize the energy stored in the transmembrane Na\(^+\) gradient for uphill transport of other substrates, in this case Ca\(^{2+}\). Secondary transporters, including NCX and NCKX proteins, are generally thought to operate via the so-called alternating access model in which a single set of substrate or ion binding sites change their accessibility from the extracellular environment to the cytosol and vice versa. Unlike ion channels, each ion translocation event of the NCX and NCKX proteins is thought to be accompanied by a major conformational change, and these proteins are therefore thought to exist in at least two, and possibly more distinct conformational states. Perhaps the best evidence for the alternating access model in the case of the NCX and NCKX proteins is the well-established occurrence of self-exchange fluxes in both NCX, (Ca\(^{2+}\)/K\(^+\)):Ca\(^{2+}\),18,19 and NCKX, Ca\(^{2+}\)/Ca\(^{2+}\),20 at rates comparable to Na\(^+\)/Ca\(^{2+}\) (or K\(^+\)/Ca\(^{2+}\)) exchange fluxes. Another important physiological consequence of the alternating access model is that NCX and NCKX proteins are expected to be bidirectional. NCX and NCKX proteins can mediate both Ca\(^{2+}\) extrusion as well as Ca\(^{2+}\) import, dependent on the transmembrane Na\(^+\) gradient and to a lesser degree on membrane potential and on the transmembrane K\(^+\) gradient (NCKX only).5,21 Specifically, maneuvers that remove or significantly lower extracellular Na\(^+\) or increase intracellular Na\(^+\) are expected to shift the transport mode from Ca\(^{2+}\) extrusion (forward exchange) to Ca\(^{2+}\) influx (reverse exchange) and may rapidly lead to Ca\(^{2+}\) overload in cells.

**Ion selectivity and apparent dissociation constants.** Most functional data published on NCKX proteins concern the NCKX1 and 2 isoforms measured in retinal rod outer segments or expressed in cell lines, respectively. A recent study reported very similar ion dependencies for the NCKX3 and 4 isoforms as previously observed for NCKX1 and 2,22 whereas no data have been reported for NCKX5. The key feature of both NCX and NCKX proteins is the absolute selectivity for Na\(^+\) ions over all other alkali cations to initiate Ca\(^{2+}\) transport2 and this includes Li\(^+\) which can replace Na\(^+\) in many other Na\(^+\) channels and transporters. Most studies on NCX and NCKX proteins employ three different methods to assess function: \(^{45}\)Ca and \(^{86}\)Rb fluxes (example in refs. 19 and 23), patch-clamp measurements (both NCX and NCKX are electrogenic) (example in refs. 24 and 25) and measurement of changes in intracellular free Ca\(^{2+}\) using fluorescent dyes of the fluo, fura or indo variety (example in refs. 22, 26 and 27). In addition, a few studies have used atomic absorption measurements\(^3,23,26\) and metallochromic dyes (arsenazo III).2,5

The external Na\(^+\) dependence of forward exchange has been measured in rod outer segments (NCKX1) or in cell lines expressing NCKX1 or NCKX2 cDNAs; The data reveal a sigmoidal dependence on the external [Na\(^+\)] with reported Hill coefficients between 2 and 3 and apparent Na\(^+\) dissociation constants of 30–50 mM.24,25,28 Similarly, Ca\(^{2+}\) influx via reverse exchange is absolutely dependent on internal Na\(^+\) (again no other alkali cation including Li\(^+\) can replace Na\(^+\)) with a Hill coefficient of 2.6 and an apparent Na\(^+\) dissociation constant of 50 mM.26

A critical difference between NCX and NCKX proteins is the requirement for Ca\(^{2+}\) and K\(^+\) cotransport for NCKX proteins, whereas no such requirement exists for NCX proteins. As most functional assays to test for NCKX presence or function rely on measurements of reverse exchange, either in situ or in cell lines expressing NCKX cDNAs, it is important to keep in mind that a measurable K\(^+\)-independent component of Ca\(^{2+}\) transport has been observed for both NCKX1 and NCKX2 under certain conditions.29,30 This is demonstrated most easily when Ca\(^{2+}\) influx via reverse exchange is measured in extracellular solutions in which Na\(^+\) is replaced by choline chloride, tertramethylammonium chloride or sucrose as the main osmotic component. Replacement of extracellular Na\(^+\) with Li\(^+\) suppresses the K\(^+\)-independent component of reverse exchange, and for this reason reverse exchange via NCKX proteins is most conveniently measured in an extracellular solution in which Li\(^+\) is the major cation. Rb\(^+\) and NH\(_4\)^+ can replace K\(^+\) in activating forward or reverse exchange mediated by NCKX proteins, Cs\(^+\) is a very poor substitute, while Li\(^+\) and Na\(^+\) cannot replace K\(^+\).31,32 Determination of the apparent K\(^+\) dissociation constants, both is situ and in heterologous systems have yielded a range of values between 1 and 10 mM dependent on the specific experimental conditions, e.g., values in choline or sucrose containing solutions are ~5-fold lower than values observed in lithium medium.2,24,26,28,29,33,34 Much higher values of >30 mM have been reported in some studies for NCKX2 and the reason for this discrepancy remains to be resolved.22,25

Ca\(^{2+}\) influx into cells can be mediated by a large number of different Ca\(^{2+}\) permeable channels present in the plasma membrane as well as by NCX proteins found in many cell types. A useful distinguishing feature of NCKX1-4 proteins is the relatively high affinity for extracellular Ca\(^{2+}\) (1–3 μM)22,24,28 in Na\(^+\)-free media, a
considerably higher affinity than values of 0.2–0.8 mM reported for NCX proteins.\textsuperscript{1} We are not aware of any Ca\textsuperscript{2+} permeable channels or transporters with external Ca\textsuperscript{2+} affinities close to those reported for NCKX. Like in most Ca\textsuperscript{2+} permeable channels or transporters, only Sr\textsuperscript{2+} can replace Ca\textsuperscript{2+} while most other divalent or trivalent cations including Mg\textsuperscript{2+} appear to compete with Ca\textsuperscript{2+} but are not transported themselves.\textsuperscript{18,35}

Pharmacology of NCXX. Very little is known about the pharmacology of NCKX proteins. Earlier work on NCKX1 in retinal rod outer segments showed that 3',5'-dichlorobenzamil,\textsuperscript{36} tetrodamine and L-cis diltiazem\textsuperscript{3} could block forward exchange at relatively high concentrations, while the same compounds were much more effective in inhibiting cGMP-gated channels present in this preparation. This implies that these compounds are unlikely to serve as useful diagnostics to detect NCKX activity. More recently, three studies have suggested that KB-R7943 is a blocker of NCKX proteins found in platelets,\textsuperscript{37} sea urchin sperm\textsuperscript{38} and starfish sperm\textsuperscript{39} whereas no inhibitory action on NCKX2 was found.\textsuperscript{40} It is important to resolve these apparently conflicting results as several recent studies\textsuperscript{41-43} that address the presence of NCX and NCKX in various types of neurons in the brain have used KB-R7943 to selectively eliminate NCX activity and reveal the contribution of putative NCKX isoforms to Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} fluxes observed in these neurons.

**CLONING AND TISSUE DISTRIBUTION OF THE FIVE NCXX ISOFORMS**

Shortly after the first descriptions of the unique Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange process in vertebrate retina that requires and transports K\textsuperscript{+}, the rod NCKX1 cDNA was cloned from bovine retina and transcripts appeared to be highly restricted to the retina and were not found in any other tissues suggesting that NCKX could be a photoreceptor-specific protein.\textsuperscript{8} Dahan et al.\textsuperscript{44} sought to investigate whether K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange was evident in other excitable tissue. In rat brain synaptic plasma membrane vesicle preparations, they deduced that K\textsuperscript{+} specifically stimulated both Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} efflux from, and Ca\textsuperscript{2+} influx into brain synaptic plasma membrane vesicles. Moreover, they showed that 86Rb\textsuperscript{+} was cotransported with Ca\textsuperscript{2+}, and assuming a stoichiometry of 1 Ca\textsuperscript{2+} and 1 K\textsuperscript{+} exchange process (as shown in retina), 10–20% of the transporters in their preparation mediated Na\textsuperscript{+}/Ca\textsuperscript{2+}-K\textsuperscript{+} exchange. Interestingly, another report by Kimura et al.\textsuperscript{45} also documented K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in human platelets, and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange process resulted in cotransport of 86Rb\textsuperscript{+} along with Ca\textsuperscript{2+} at a ratio of 0.9 when [Rb\textsuperscript{+}] was 1.3 mM. These studies indicated that Na\textsuperscript{+}/Ca\textsuperscript{2+}-K\textsuperscript{+} exchange is likely to be found in other tissues than the retina.

A second NCKX cDNA was cloned by Tsoi et al.\textsuperscript{46} who found widespread distribution of transcripts of NCKX2 in rat brain. Using in situ hybridization, the following regions of adult rat brain were reported to be particularly enriched in transcripts of NCKX2: the deeper layers of the cortex (V and VI), in the CA3 pyramidal neurons of the hippocampus, in thalamic medial geniculate nucleus, in the pontine nuclei and the reticulotegmental nuclei of pons, and within the molecular layer of the cerebellum. The next isoform to be cloned was NCKX3,\textsuperscript{47} which proved to also be particularly abundant within the adult brain. It was reported to be particularly prominent in hippocampal CA1 pyramidal cells, within thalamic nuclei, and molecular layer of the cerebellum; transcripts of NCKX3 displayed a selectively laminar distribution within cortex, with higher expression in large neurons of layer IV. Additionally, transcripts of NCKX3 were reported in abundance in various other excitable body tissues rich in smooth muscle, particularly aorta, intestine, lung, and uterus; notably transcripts of NCKX3 were not detected in heart—otherwise known to be highly enriched in the K\textsuperscript{+}-independent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger NCX1 of the SLC8 gene family. NCKX4 was subsequently cloned and its transcripts reported to be uniformly expressed in adult brain, with prominent expression in CA1, CA3, and dentate gyrus of hippocampus, in the granular layer of cerebellum, the anterodorsal nucleus of thalamus, and granule cell layer of olfactory bulb.\textsuperscript{48} In addition, transcripts of NCKX4 were abundantly expressed in aorta, lung, and thymus, and at lower levels in a variety of other tissues such as heart, stomach, small intestine, spleen, lymph node, skeletal muscle, kidney and adrenal gland. The tissue distribution of NCKX5 will be discussed below.

**PHYSIOLOGY OF NCXX PROTEINS**

**Retina.** Of all NCXX proteins, only NCKX1 has been ascribed a unique physiological role. This is because of its privileged location in retinal rod photoreceptor outer segments where, along with the light-sensitive cyclic nucleotide gated channels, they make up the only ion transporting proteins in the plasma membrane.\textsuperscript{19,49} Cyclic nucleotide gated channels are closed by exposure to light, hence leaving retinal rod NCKX1 as the only ion transporter in rod outer segments. Since rod outer segments can be readily isolated, with sealed plasma membranes and in large quantities, they have proven very useful in elucidating the biochemical and biophysical properties of Na\textsuperscript{+}/Ca\textsuperscript{2+}-K\textsuperscript{+} exchangers.\textsuperscript{4,50}

In the dark, a current (~30 pA carried by Na\textsuperscript{+} and Ca\textsuperscript{2+} making up 80% and 20% of the total current respectively) enters rod outer segments through cyclic nucleotide gated channels;\textsuperscript{51,52} the Na\textsuperscript{+} is cleared by Na\textsuperscript{+}/K\textsuperscript{+} ATPase in the inner segment of rod photoreceptors, while Ca\textsuperscript{2+} (along with K\textsuperscript{+}) is transported back across the plasma membrane by NCKX1. Due to this so called dark current, dark-adapted retinal rod photoreceptor outer segments typically have a relatively depolarized resting plasma membrane potential of ~40 mV, and a relatively elevated cytosolic [Ca\textsuperscript{2+}] of ~0.5 μM.\textsuperscript{53,54} Thus, an exchanger such as NCKX1 which not only uses the inwardly directed Na\textsuperscript{+} gradient to extrude Ca\textsuperscript{2+}, but also the outwardly directed K\textsuperscript{+} gradient seems ideally suited for the purpose of clearing intracellular Ca\textsuperscript{2+}; an exchanger such as the cardiac type NCX1 with an exchange stoichiometry of 3 Na\textsuperscript{+} for 1 Ca\textsuperscript{2+} could possibly reverse and promote Ca\textsuperscript{2+} influx rather than clearance under the ionic and voltage conditions of the rod outer segment.\textsuperscript{55}

With a stoichiometry of exchange of 4 Na\textsuperscript{+} ions for 1 Ca\textsuperscript{2+} ion + 1 K\textsuperscript{+} ion, NCKX1 is expected to lower free cytosolic [Ca\textsuperscript{2+}] in retinal rod outer segments to values below 1 nM; however, such low values have not been observed in isolated bovine rod outer segments\textsuperscript{53,55} nor in the rod outer segments of intact photoreceptors.\textsuperscript{53,54} A more detailed analysis of Ca\textsuperscript{2+} fluxes in isolated rod outer segments revealed that a process of inactivation of Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} extrusion after a brief burst of high capacity operation mediated by NCKX1 is probably responsible for the above observations.\textsuperscript{55} As regulation of intracellular Ca\textsuperscript{2+} in rod outer segments is involved in several processes related to phototransduction and light adaptation. Hence, inactivation of NCKX1 may prevent lowering of rod intracellular Ca\textsuperscript{2+} to unfavourably low levels when rod cells are saturated for the duration of bright daylight illumination.

NCKX1 in the retina is restricted to rod outer segments, and is not found in the rest of the rod photoreceptor or in cone outer
Na+/Ca2+ exchange had been previously documented in cone outer segments, but the molecular nature of the exchanger in cone outer segments, and whether it was also K+-dependent was unknown. A second retinal NCKX cDNA was cloned from chicken and human retina which proved to be NCKX2, and with use of in situ hybridization, localized NCKX2 transcripts to cone inner segments (as well as ganglion cells). Curiously, the NCKX2 knockout mouse did not show any obvious retinal dysfunction which raises a question concerning the localization of NCKX2 to cone outer segments in this preparation. It was only recently that Paillard et al. have definitively shown K+-dependent Na+/Ca2+ exchange currents in isolated cones, in striped bass. These currents were measured under ionic conditions where NCKX is expected to operate in its reverse mode, and was accompanied by rise in cytosolic free Ca2+. Using single-cell RT-PCR, the NCKX isoform in isolated cone outer segments was identified as NCKX2, while NCKX1 mRNA was not detected. Hence, it is apparent that vertebrate rods and cones express different NCKX isoforms—NCKX1 being the exchanger of rod outer segments, while NCKX2 carries out the function of Ca2+ extrusion in cone outer segments.

Brain. With other isoforms of NCKX cloned and transcripts found in abundance in the brain, and in various other animal tissues, it has become evident that these proteins potentially participate in many physiological processes. However, ascribing distinct roles to the other isoforms is not as clear cut as is the case for NCKX1 in rod outer segments. That is because in other cell types NCKX is only but one class of Ca2+ transporter, and shares this function with many other proteins including, but not limited to, K+-independent Na+/Ca2+ exchangers (NCX) and plasma membrane Ca2+ ATPases (PMCA) which also extrude Ca2+ from the cytosol to the extracellular milieu, sarco/endothelial reticulum Ca2+ ATPase (SERCA), and mitochondrial Ca2+ transporters which sequester cytoplasmic Ca2+. There are two approaches to investigating the contribution of Na+/Ca2+ exchange in a given cell type. One way is to run the exchanger in its reverse (Ca2+ import) mode, which is typically accomplished by equimolar substitution of extracellular Na+. In the case of NCKX, the reverse mode has an absolute dependence on extracellular K+ (at least when the extracellular substitute for Na+ ions is Li+), and with a low apparent affinity for extracellular K+ on the order of 1–5 mM, the contribution of NCX can be evaluated without major effects on membrane potential induced by high K+ depolarization (and the possibility of resultant Ca2+ influx through voltage gated Ca2+ channels). The other method of investigating Na+/Ca2+ exchange physiologically is by measuring cytosolic Ca2+ clearance rates (after imposing cytosolic Ca2+ loads) in the absence of extracellular Na+. In both these approaches, data must be evaluated with prudence, since as aforementioned, other cellular Ca2+ handling mechanisms can influence and obscure the contributions of NCKX (or NCX) under investigation.

Following the indication of K+-dependent Na+/Ca2+ exchange in brain by Dahan et al., the next study that illustrated such activity in neurons is that of Boyer et al. In vestibular sensory hair cells of guinea pig, brief (5–10 s) substitution of extracellular Na+ resulted in elevation of cytosolic free Ca2+, and this elevation was completely dependent on extracellular K+. In that study, Boyer et al. also characterized the ion selectivity of the binding site of the exchanger mediating reverse exchange, and found that only K+, Rb+, and NH4+ supported Ca2+ influx, but not Li+, nor Cs+ (similar to ion selectivity of K-site of NCKX1 and NCKX2). Interestingly, a subsequent study investigating the contribution of Na+/Ca2+ exchange in the semicircular canal hair cells of frog found no evidence for Na+/Ca2+ exchange altogether. Aside from the difference in species used by the two studies, the latter study of Martini et al. used solutions of different ionic compositions from those used by Boyer et al. for their fluorescence and whole-cell current recordings.

The definitive characterization of K+-dependent Na+/Ca2+ exchange in brain neurons came from the studies of Kiedrowski et al., where they demonstrated extracellular K+-dependent Ca2+ accumulation in Na+-loaded cerebellar granule cells cultured from rat for 9–11 days in vitro. In a subsequent study, the same group demonstrated that inhibition of NCX or NCKX independently failed to limit NMDA-induced Ca2+ accumulation in depolarized and glucose-deprived cerebellar granule cells in vitro (to mimic brain ischaemic conditions). But, when both NCX and NCKX were inhibited simultaneously by application of 10 µM KB-R7943 and removal of extracellular K+ respectively, NMDA-induced Ca2+ accumulation and resultant excitotoxicity were limited significantly. A later study from the same lab compared the contributions of NCX and NCKX to Na+/Ca2+ exchange-mediated Ca2+ influx between neurons of rat cerebellum and forebrain in vitro culture. In forebrain neurons, inhibiting NCX with 10 µM KB-R7943 resulted in ~85% decrease in the cytosolic free Ca2+ elevation rate, while removing extracellular K+ to inhibit NCKX (when NCX was operational) did not influence the rate of cytosolic free Ca2+ elevation. In cerebellar granule cells, however, inhibiting NCKX decreased the rate of cytosolic free Ca2+ elevation by ~65%. This shows that NCX and NCKX have differential contributions in different brain neurons; although northern blot analysis of forebrain neurons and cerebellar granule cells did not reveal pronounced differences in the expression of different NCKX isoforms. These studies all implicate NCKX (as well as NCX) as important transporters in physiological regulation of neuronal cytosolic Ca2+, and potentially as important therapeutic targets under pathophysiological conditions such as stroke and brain ischaemia.

While all the aforementioned studies relied primarily on elevation of cytosolic Ca2+ (reverse exchange) to demonstrate the activity of NCKX, Lee et al. have demonstrated operational K+-dependent Na+/Ca2+ exchange in clearance of cytosolic Ca2+ loads in isolated nerve terminals of rat neurohypophysis. In this preparation, NCKX was demonstrated to specifically contribute to Ca2+ clearance in the neurohypophysial nerve terminal compartment, but not in the cell bodies of the same neurons in brain slices of hypothalamus containing the somata of supraoptic magnocellular neurosecretory cells. Although, these functional studies do not conclusively indicate which isoform of NCKX is in these nerve terminals, mRNA transcript localization, and immunohistochemical evidence suggest it is NCKX2. NCKX was later shown, by the same group, to be an important Ca2+ clearance mechanism in another neuronal axon terminal, the Calyx of Held, in the medullary trapezoid body medial nucleus. In these mammalian large presynaptic terminals, NCKX was deduced to contribute ~42% of the cytosolic Ca2+ clearance (for cytosolic Ca2+ loads up to 2.5 µM), while NCX and PMCA each contributed 26% and 23% respectively; mitochondria became an important contributor to cytosolic Ca2+ clearance once cytosolic [Ca2+] exceeded 3 µM. It is not yet known which isoform(s) of NCKX (or NCX) are present in the presynaptic axon terminals of the Calyx of Held.

A defined neuronal physiological role for an NCKX isoform was obtained recently by Li et al. who developed an embryonic NCKX2 knockout mouse. Analysis of isolated and cultured forebrain
cortical neurons from NCKX2 knockout mice revealed a decrease in the NCKX-mediated reverse exchange Ca\(^{2+}\) import by -50%; the remainder of the K\(^{+}\)-dependent component presumably attributed to NCKX3 and NCKX4. Since NCKX expression is particularly prominent in the hippocampus both at the mRNA level,\(^{46}\) as well as functionally,\(^{62}\) the NCKX2 knockout mice were examined for alterations in hippocampal synaptic plasticity, and behavioural measures of learning and memory. Long term potentiation was induced in hippocampal Schaffer collateral/CA1 synapses in brain slices by two 0.5 s bouts of tetanic (100 Hz) stimulation, and then excitatory postsynaptic field potentials (fEPSPs) were elicited at baseline stimulation (0.1 Hz). At 30 min post tetanus, the slopes of fEPSPs (calculated from the rising phase of the peak response) measured in hippocampal slices from wild-type mice were increased to 131% of baseline, whereas in NCKX2 knockout littermates, it was significantly reduced to 88%. On the other hand, long term depression (LTD) was elicited by 15 min of stimulation at 1 Hz; comparison of fEPSP responses in wild-type hippocampal slices revealed that the fEPSP slopes were reduced to 75% of baseline 30 min after the induction of LTD, whereas it was 50% in NCKX2 knockout littermates. Interestingly, these changes in hippocampal synaptic plasticity observed in NCKX2 knockout mice were not accompanied by changes in synaptic transmission, as revealed by the relation of fEPSP slope to stimulus amplitude, nor in presynaptic facilitation, as revealed by a paired-pulse protocol. The aforementioned effects of NCKX2 loss on hippocampal synaptic plasticity were not accompanied by differences in postsynaptic CA1 neuronal responses as revealed by comparisons of various measures of neuronal excitability, such as resting membrane potential, input resistance, nor in action potential spike firing frequency (and associated spike parameters). Behavioural studies of NCKX2 knockout mice displayed normal performance on a rotating rod task (a test of motor learning) with respect to their wild-type littermates, however, NCKX2 knockout mice failed to improve their performance on this task with subsequent trials, while their wild-type littermates were able to do so. In another behavioural test of spatial learning, a moving platform version of the Morris water maze task, wild-type mice showed improvement in their ability to locate a hidden platform when it was in the same location as the previous day, compared with when it was in a novel location; NCKX2 knockout littermates, however, did not show such improvement.

**Other vertebrate tissue.** Other than the brain, very little is known of the role of NCKX. As aforementioned, a report published by Kimura et al.\(^{45}\) suggested the existence of a K\(^{+}\)-dependent Na\(^{+}/Ca\(^{2+}\) exchanger in human platelets. The same group later demonstrated in the human megakaryocytic cell line CHRF-288 the expression of a 1.45 kb mRNA transcript fragment identical in sequence to that of human retinal rod NCKX1.\(^{65}\) Indeed, the existence of such an exchanger in platelets seems puzzling, considering that most NCKX isoforms (\(slc24a5\) being a notable exception) are found in excitable tissue where they are presumed to serve the special need for handling large and dynamic Ca\(^{2+}\) fluxes.

Thus far, only one study has addressed the contribution of NCKX in smooth muscle.\(^{64}\) Isolated aortic smooth muscle cells of 6-week-old rats displayed an increase in cytosolic free Ca\(^{2+}\) when extracellular Na\(^{+}\) was replaced with Li\(^{+}\). Approximately half of this Ca\(^{2+}\) influx was abolished by removal of extracellular K\(^{+}\), while the remainder of the signal can be abolished by inhibiting NCX with application of 10 \(\mu\)M KB-R7943. They also showed by RT-PCR and northern blot analysis the expression of NCKX3 and NCKX4; NCKX3 mRNA was readily detected in both 6-week- and 20-week-old rat aorta, while NCKX4 mRNA levels were low in 6-week-old rat aorta, and barely detectable in 20-week-old rat aorta. Antibody labelling also revealed a plasma membrane pattern of staining for both NCKX3 and NCKX4 in the isolated rat mesenteric arterial myocytes. The authors further demonstrate that the phenylephrine-induced contractions in segments of rat aorta are partially mediated by Ca\(^{2+}\) influx resulting from the reverse mode of exchange of both NCX and NCKX; although these experiments were carried out in the presence of ouabain to inhibit Na\(^{+}/K^{+}\) ATPase, and hence elevate cytosolic Na\(^{+}\).

An exciting new finding was the identification of \(slc24a5\) as one of the key genes involved in pigmentaution in humans, through zebrafish genetics.\(^{65}\) The gene was identified initially from the golden phenotype in zebrafish, which results in delayed and reduced development of pigmentation. Cloning of the human gene proved that it has a conserved role in pigmentation, as the golden phenotype in zebrafish embryos can be rescued by injection of full-length \(slc24a5\) RNA, and alternatively, morpholino targeting of the translational start site of zebrafish \(slc24a5\) in wild-type embryos produces hypopigmentation. A single nucleotide polymorphism in the human \(slc24a5\) gene was shown to be the major genetic determinant of light skin in Europeans and East Asians.\(^{65,66}\) It remains to be determined if the gene product of \(slc24a5\) mediates K\(^{+}\)-dependent Na\(^{+}/Ca\(^{2+}\) exchange; what is intriguing is that when heterologously expressed, the protein is expressed intracellularly rather than in the plasma membrane and it is suggested that NCKX5 might function as a Ca\(^{2+}\) regulator in intracellular compartments—melanosomes or their precursors.\(^{65}\)

**Invertebrate.** Several NCKX sequences have been identified, and cloned from invertebrate species. These include \(Nckx30c\) and \(Nckx-x\) from *Drosophila*,\(^{13,14}\) both of which were localized to embryonic nervous system. \(Nckx-x\) expression remains restricted to the brain in adult *Drosophila*, but \(Nckx-30c\), on the other hand, is expressed in third instar imaginal discs, and in adult neurons, including photoreceptors. This suggests that aside from neuronal Ca\(^{2+}\) handling, these NCKX isoforms may be involved in signalling in developmental processes.

Two studies have reported the cloning of NCKX isoforms from sperm—from sea urchin *Strongylocentrotus purpuratus*,\(^{38}\) and from starfish *Asterias amurensis*.\(^{39}\) Cytosolic Ca\(^{2+}\) influences axonemal microtubule dynamics, and hence sperm flagellar swimming; hence, Su and Vacquier have suggested that sea urchin NCKX may be required as a Ca\(^{2+}\) extrusion mechanism for this purpose. It is worth noting that while Na\(^{+}/Ca\(^{2+}\) exchange has been documented in mouse sperm, K\(^{+}\) has been found to have no effect on Ca\(^{2+}\) fluxes.\(^{67}\)

**Concluding Remarks**

In recent years exciting new roles have emerged for NCKX proteins beyond the well-established role of NCKX1 in vertebrate rod phototransduction. NCKX proteins have been shown to participate in significant ways in Ca\(^{2+}\) homeostasis in many neurons in the brain and NCKX5 appears to play a key role in melanocytes and skin pigmentation. We anticipate that new and exciting roles for NCKX proteins will emerge in the immediate future as more new tools are developed to study NCKX physiology.

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