Potassium-Dependent Sodium–Calcium Exchange through the Eye of the Fly

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ABSTRACT: In this review, we describe the characterization of a *Drosophila* sodium/calcium-potassium exchanger, *Nckx30C*. Sodium/calcium (-potassium) exchangers (NCX and NCKX) are required for the rapid removal of calcium in excitable cells. The deduced protein topology for NCKX30C is similar to that of mammalian NCKX, with 5 hydrophobic domains in the amino terminus separated from 6 at the carboxy-terminal end by a large intracellular loop. NCKX30C functions as a potassium-dependent sodium–calcium exchanger and is expressed in adult neurons and during ventral nerve cord development in the embryo. *Nckx30C* is expressed in a dorsal/ventral pattern in the eye-antennal disc, suggesting that large fluxes of calcium may be occurring during imaginal disc development in the larvae. NCKX30C may play a critical role in modulating calcium during development as well as in the removal of calcium and maintenance of calcium homeostasis in adults.

KEYWORDS: calcium; development; *Drosophila*; photoreceptor; signal transduction; Na/Ca exchange

INTRODUCTION

Calcium is essential in phototransduction in both *Drosophila* and vertebrates. In both, phototransduction is initiated by the absorption of light by rhodopsin, and the light signal is transduced through G protein-coupled signaling cascades. However, distinct differences exist in the two phototransduction cascades (FIG. 1). In *Drosophila*, phototransduction occurs via a phospholipase C-mediated signaling cascade, and illumination results in the opening of the cation-selective channels and a large rise in intracellular calcium. The photoreceptors depolarize and calcium increases from about 100 nanomolar to as high as tens of micromolar.[1–7] The mechanism for calcium extrusion after illumination is thought to be sodium/calcium exchange. The measurement of sodium/calcium exchange in *Drosophila* and in other invertebrate

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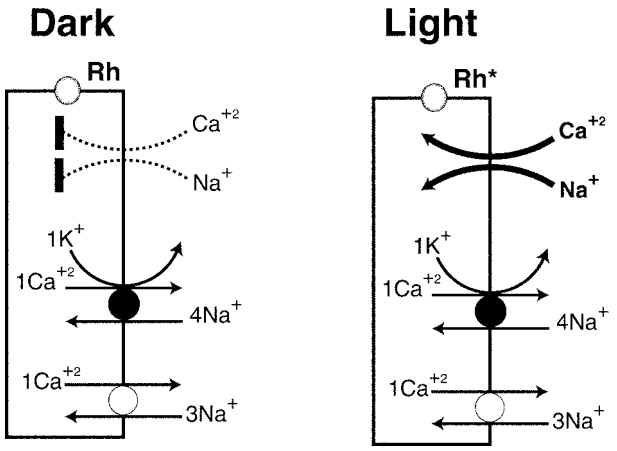

FIGURE 1. Phototransduction cascade in *Drosophila*. Absorption of a photon photoactivates rhodopsin, leading to the opening of the cation-selective channels (Light). Extracellular calcium (Ca^{+2}) and sodium (Na^{+}) enter the cell via the light-activated channels, causing a depolarization of the photoreceptor cells. Calcium levels rise about 100 nanomolar to greater than 10 micromolar upon light stimulation.[5] Calcium entering light-sensitive channels is thought to play a key role in deactivation of the light response and light adaptation. Rapid removal of calcium from the photoreceptor cells is key to the recovery from the light response. Removal of calcium following light stimulation may occur via NCKX (*dark circle*) and NCX (*shaded circle*). NCX uses a stoichiometry of $3Na^{+}$ /$1Ca^{2+}$ to extrude calcium, and NCKX uses both the inward sodium gradient and the outward potassium gradient to extrude calcium at a stoichiometry of $4Na^{+}$/$1Ca^{2+}$,$1K^{+}$. *Dashed arrows* indicate that the cation influx through the light-sensitive channel does not occur in the dark (*Dark*). Rh, rhodopsin molecule; Rh*, photoexcited rhodopsin molecule. This figure is an adaptation of one reproduced in Ref. 18. (Reproduced from J. Cell Biol. 1999. **147:** 659-669, by copyright permission of The Rockefeller University Press.)

photoreceptors has led to the proposal that exchange activity is critical in light adaptation.[2,4,8–12] Therefore, *Drosophila* photoreceptors may use an electrochemical exchanger that couples calcium removal to the inward sodium gradient, which has been demonstrated in vertebrate rod photoreceptors.[13–16] Unlike fly photoreceptor cells, illumination of vertebrate rod photoreceptors results in the closing of cGMP-gated cation channels and in hyperpolarization of the cell.[17,18] Cytosolic calcium falls from 500–600 nM in the dark to less than 50 nM in bright light.[19,20] This process plays a role in light adaptation in both retinal rods and cones.[21,22]

Calcium extrusion or sequestration to intracellular compartments is essential to all excitable cells, and prolonged increases in cytosolic calcium can be toxic, leading to cell death.[23,24] Two classes of plasma membrane proteins are responsible for calcium efflux. They are ATP-driven calcium pumps and sodium/calcium (-potassium) exchangers. The exchangers are essential in excitable cells that experience large fluxes of calcium across their plasma membrane, such as cardiac myocytes, skeletal and smooth muscle, photoreceptors, and other neurons (reviewed in Ref. 25).

Exchangers maintain low levels of intracellular calcium (100 nM or below) by using the transmembrane sodium gradient as an energy source. There are two well-known families of mammalian sodium/calcium exchangers. One family is NCX, and it uses an inward sodium gradient for extrusion of calcium. There are three NCX isoforms and they are expressed in a variety of tissues including heart, kidney, brain, as well as smooth and skeletal muscle.[26,27] The second family is NCKX, and it extrudes calcium using both an inward sodium gradient and an outward potassium gradient.[28,29] There are three NCKX-type exchangers, NCKX1, NCKX2, and NCKX3. Retinal rod NCKX1 exchangers have been cloned from a variety of mammalian species, and potassium-dependent sodium/calcium exchanger activity has been demonstrated.[30–36] NCKX2 has been isolated from human and chicken retinal cone photoreceptors as well as rat brain.[37,38] NCKX3 has been cloned from rat brain.[39] NCKX family members have been detected in both eukaryotic and prokaryotic genomes.[40,41] The NCKX- and NCX-type exchangers are thought to display a similar topology; however, there is little overall amino acid sequence identity. Despite this difference, they are thought to be evolutionarily related.[41,42]

Here we review the cloning and characterization of a *Drosophila* potassium-dependent sodium/calcium exchanger, *Nckx30C.*[43] We have shown that it functions as a potassium-dependent sodium/calcium exchanger and that it is distinct from *Calx*, an NCX-type exchanger in *Drosophila*.[41,44–47] Both *Nckx30C* and *Calx* are expressed in photoreceptor cells, in the adult brain and during embryogenesis and eye development. These exchangers are likely required for the removal of calcium generated during signaling processes in the fly.[43]

MATERIALS AND METHODS

Nckx30C was cloned and sequenced as previously described.[43] The BLAST search and the CLUSTAL W multiple sequence alignment programs were used to assess sequence similarity[48,49] (http://www.ncbi.nlm.nih.gov/BLAST) (http://pbil.ibcp.fr/NPSA/npsa_clustalw.html). The sequence data can be obtained in the DDBJ/EMBL/GenBank databases under accession number AF190455.

The full-length *Nckx30C* cDNA was expressed in High Five insect cells as previoiusly described.[43,50] High Five cells (BTI-TN-5B1-4) are derived from *Trichoplusia ni* egg cell homogenates and were purchased from Invitrogen. We do not detect endogenous exchanger activity in High Five cells. Potassium-dependent sodium/calcium exchange activity was measured as previously described.[43] The sodium-potassium ionophore monensin was used to load the cells with sodium in a medium containing 150 mM NaCl, 80 mM sucrose, 0.05 mM EDTA, and 20 mM Hepes (pH 7.4). This procedure was carried out according to the methods described for rod outer segments.[51] Monensin was removed, and the sodium-loaded cells were washed with and resuspended in 150 mM LiCl, 80 mM sucrose, 0.05 mM EDTA, and 20 mM Hepes (pH 7.4). The cells were resuspended in media containing 80 mM sucrose and 20 mM Hepes (pH 7.4) and either (1) 150 mM KCl, (2) 150 mM NaCl, or (3) 150 mM LiCl. The uptake of ^{45}Ca occurred by adding 35 µM $CaCl_2$ and 1 µCi ^{45}Ca to the media. ^{45}Ca uptake was measured as described previously.[52] Following ^{45}Ca uptake the cells were washed in an ice-cold medium containing 140 mM KCl, 80 mM sucrose, 5 mM $MgCl_2$, 1 mM EGTA, and 20 mM Hepes (pH 7.4).

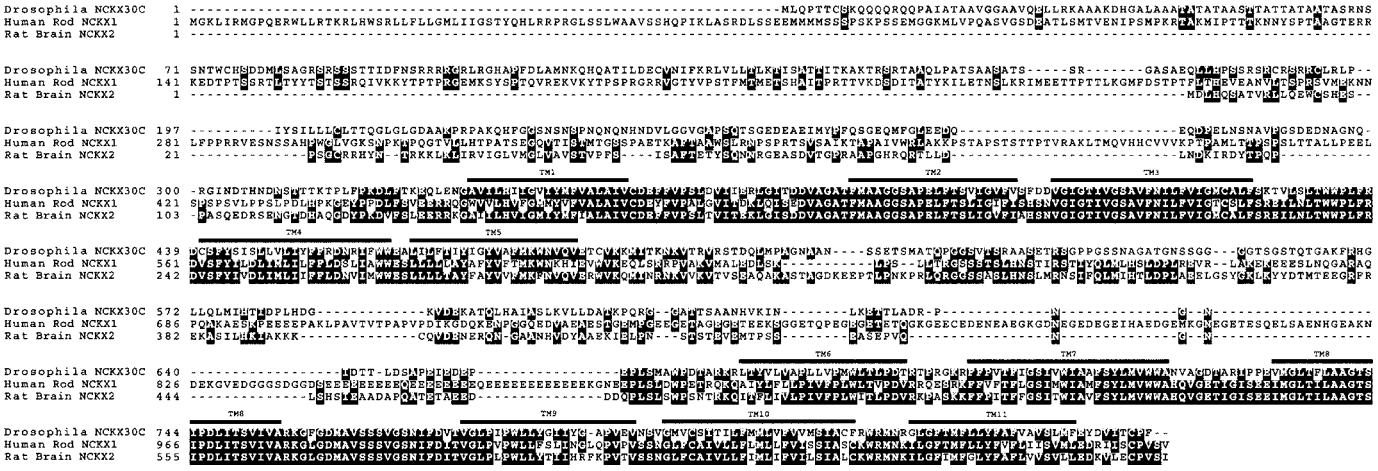

FIGURE 2. *Nckx30C* encodes a novel *Drosophila* NCKX. Clustal/W alignment between *Drosophila* NCKX30C, human rod NCKX1, and rat brain NCKX2.[31,32,56] *Dark shading* indicates identity; *light shading*, similarity. The 11 transmembrame domains of NCKX30C are indicated by lines above each row (Reproduced from Ref. 43, J. Cell. Biol. 1999. **147:** 659-669, by copyright permission of The Rockefeller University Press.)

Heads from wild-type flies (w^{1118}) were embedded in Tissue-Tek OCT Compound (Miles, Inc.), sectioned, and labeled as previously described.[43] Embryo and imaginal disc *in situ* hybridizations were carried out essentially as described by Panganiban *et al*.[53] Digoxigenin-labeled antisense and sense riboprobes were made by *in vitro* transciption, as recommended by the supplier (Boehringer Mannheim Corp., Indianapolis, IN). Five distinct *Nckx30C* cDNA probes, described previously,[43] were used. *Calx* cDNA was generously given to us by E. Schwarz.[41] *Chaoptin* cDNA was sent by D. Van Vactor and S. L. Zipursky.[54]

RESULTS AND DISCUSSION

Molecular and Functional Characterization of a Drosophila *Potassium-Dependent Sodium–Calcium Exchanger*

Potassium-dependent sodium/calcium exchangers (NCKX) are a group of calcium extrusion proteins that use an inward sodium gradient together with an outward potassium gradient to remove calcium from excitable cells. NCKX1 function was originally described in retinal rod photoreceptor cell outer segments (FIG. 1).[28,29] To isolate an NCKX from *Drosophila*, we constructed a cDNA probe from the bovine rod photoreceptor NCKX1 and used this probe to screen the *Drosophila* libraries. The *Drosophila* cDNA was obtained as described previously,[43] and the *Drosophila Nckx* was mapped to 2L at 30C5-7 cytologically by *in situ* hybridization to polytene chromosomes. Based on the chromosomal location we named the gene *Nckx30C.*[43] *Nckx30C* has a single open reading frame that encodes a protein of 856 amino acids. We compared the derived amino acid sequence of *Nckx30C* with the human NCKX1 and rat NCKX2 (FIG. 2). FIGURE 2 shows that there is 66% and 71% identity in two groups of amino acids that correspond to the predicted transmembrane domains of NCKX1 and NCKX2, respectively.

The hydropathy analysis predicts that *Drosophila* NCKX30C protein contains 11 hydrophobic regions that correspond to potential transmembrane (TM) helices predicted in NCXK1 and NCKX2 (FIG. 3A and B). NCKX30C displays a large cytoplasmic loop located between the hydrophobic amino acid clusters. This displays almost no amino acid identity with the intracellular cytoplasmic loops of NCKX1 or NCKX2 (FIG. 2). FIGURE 3B shows the domain structure of the exchangers. Hydropathy analysis reveals that NCKX30C may contain two additional membrane-spanning segments located in the N-terminus, not observed in either NCKX1 or NCKX2.

We examined the ability of NCKX30C to function as an exchanger in High Five cells.[43] Both NCX and NCKX can mediate both calcium efflux (forward exchange) and calcium influx (reverse exchange). The direction of calcium exchange is dictated by the direction of the sodium gradient across the membrane. *In vivo*, the inward sodium gradient removes calcium from the cell (forward exchange). However, *in vitro*, when external sodium is removed, the outward sodium gradient will drive calcium into the cell (reverse exchange). Taking advantage of reverse exchange of NCKX30C, we measured ^{45}Ca uptake in cells loaded with sodium (FIG. 4). We examined NCKX activity by using three different conditions of the cation gradient that are known to inhibit NCKX activity. In a medium containing high sodium, the cells

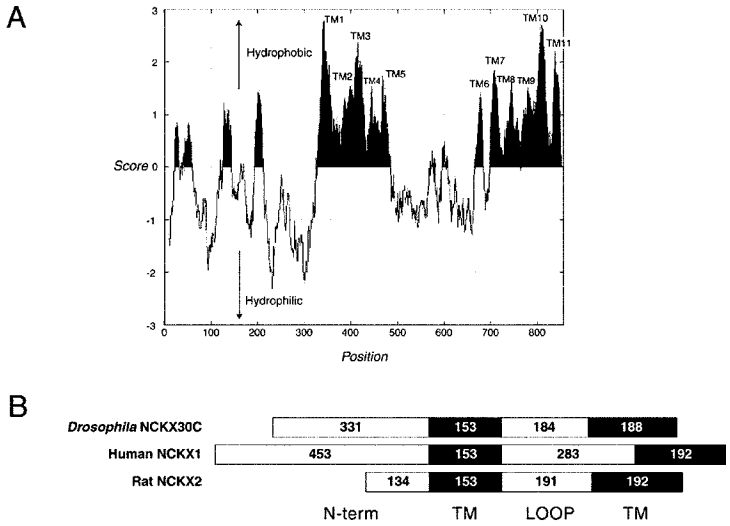

FIGURE 3. Hydropathy plot of the conceptual NCKX30C protein. (**A**) Hydropathy plot of the conceptual NCKX30C protein, analyzed by the Kyte-Doolittle algorithm.[74] Hydrophobic regions are designated in *black*. (**B**) Domain structure of *Drosophila* NCKX30C, human rod photoreceptor NCKX1, and rat brain NCKX2. *Shaded boxes* represent the two clusters of transmembrane segments (TM 1-5 and TM 6-11) that display high identity among the three sequences. Numbers represent the number of amino acids per segment. (Reproduced from Ref. 43, J. Cell Biol. 1999. **147:** 659-669, by copyright permission of The Rockefeller University Press.)

were filled with sodium using the sodium-potassium ionophore monensin.[51] After removal of the ionophore, the sodium-filled cells were washed with and resuspended in low sodium buffer, as described in Ref. 43. The cells were diluted into media containing 80 mM sucrose, 20 mM Hepes (pH 7.4), and either (1) 150 mM KCl, (2) 150 mM NaCl, or (3) 150 mM LiCl. The uptake of ^{45}Ca was initiated by the addition of 35 ∝M $CaCl_2$ and 1 μCi ^{45}Ca. Monensin causes the release of internal sodium and inhibits ^{45}Ca uptake by NCKX30C (FIG. 4B). These data demonstrate that intracellular sodium is required for calcium transport. In both NCK and NCKX, calcium uptake by reverse exchange is inhibited by high external sodium.[55] This property is thought to be due to competition of sodium and calcium for a common binding site.[55.] FIGURE 4C shows that ^{45}Ca uptake by NCKX30C is prevented by high external sodium. These data are consistent with its identity as a sodium/calcium exchanger.[43] NCKX and NCX can be distinguished by employing the property that calcium influx by NCKX requires the presence of potassium, and lithium cannot substitute for potassium.[56] NCKX30C requires potassium for ^{45}Ca uptake (FIG. 4D). demonstrating that NCKX30C functions as a potassium-dependent sodium/calcium exchanger.

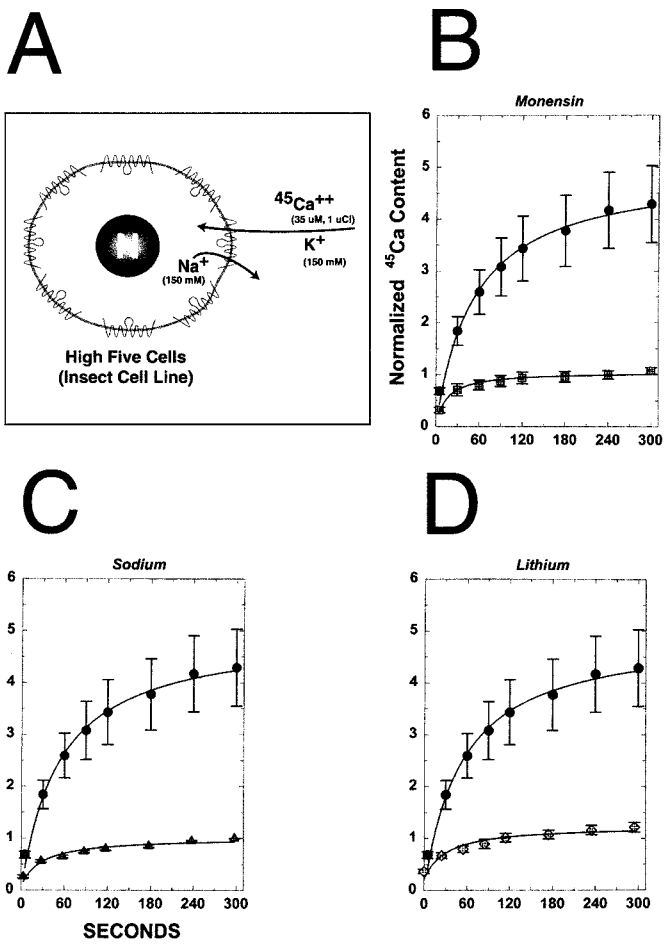

FIGURE 4. *NCKX30C* is a potassium-dependent sodium–calcium exchanger. (**A**) Reverse exchange was measured as Na_{inside} dependent ^{45}Ca uptake in High Five cells transformed with *Nckx30C* in the presence of external KCl (**B**–**D**, *filled circles)*. Mean ± standard error of the mean (SEM). The same results are shown in **B**–**D** (*filled circles*). (**B**) Reverse sodium/calcium exchange requires intracellular sodium. ^{45}Ca uptake was measured in KCl medium with 20 μM monensin present (*shaded squares*) or without monensin (*filled circles*). Monensin was added 30 seconds before addition of ^{45}Ca and causes the release of intracellular sodium to the external medium. Average values ± SEM are shown for 13 experiments conducted in KCl medium and 4 experiments conducted in media containing KCl plus monensin. (**C**) Reverse sodium/calcium exchange is inhibited by extracellular sodium. ^{45}Ca uptake was measured in KCl medium (*filled circles*) or NaCl medium lacking potassium (*triangles*). Average values ± SEM are shown for 13 experiments conducted in KCl medium and 10 experiments conducted in media containing NaCl. (**D**) Reverse sodium/calcium exchange requires extracellular potassium. ^{45}Ca uptake was measured in KCl medium (*filled circles*) or LiCl medium lacking potassium (*diamonds*). Average values ± SEM are shown for 13 experiments conducted in KCl medium and 8 experiments conducted in media containing LiCl. (Reproduced from Ref. 43, J. Cell Biol. 1999. **147:** 659–669, by copyright permission of The Rockefeller University Press.)

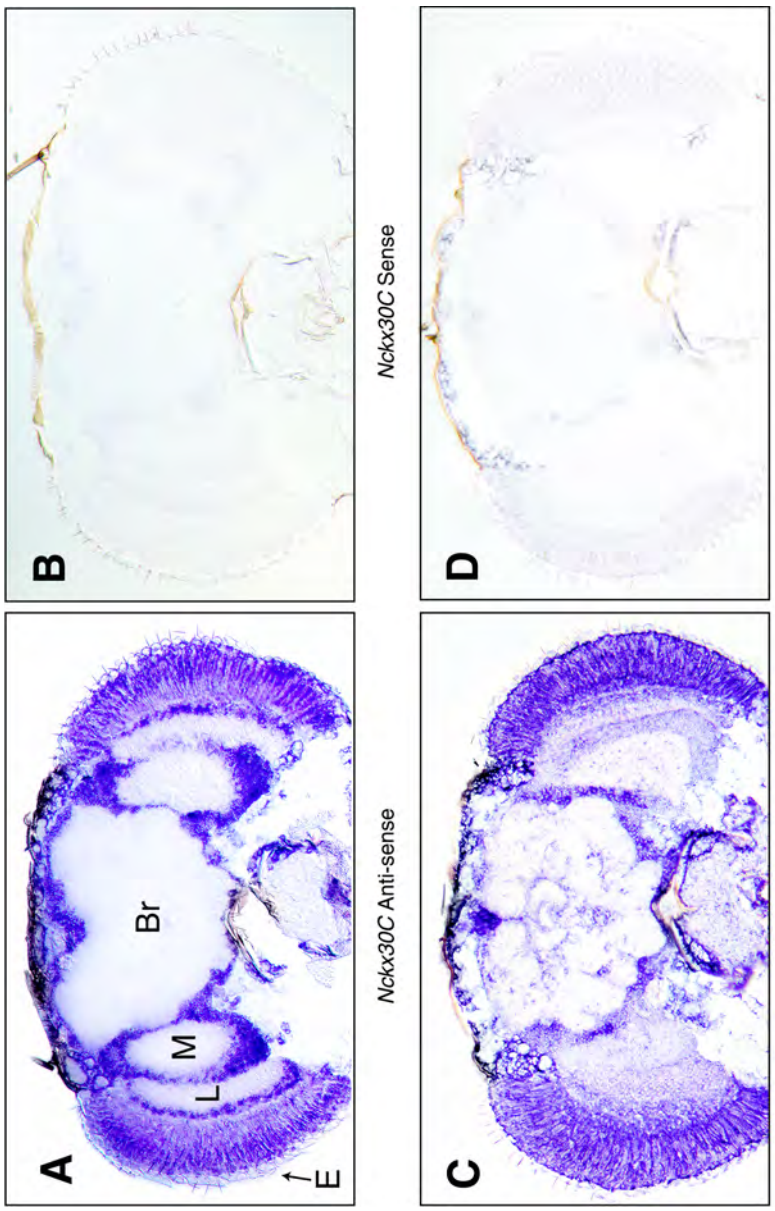

FIGURE 5. *Nckx30C* and *Calx* are expressed in the adult eye and the brain of *Drosophila*. (**A**) Shown are *in situ* hybridizations to 14-µm cryostat sections of adult heads hybridized with digoxigenin-labeled riboprobes. (**A**) antisense riboprobe for *Nckx30C*; (**B**) sense probe for *Nckx30C*; (**C**) antisense riboprobe for *Calx*; (D) sense riboprobe for *Calx*; and (E) eye, (L) lamina, (M) medulla, and (Br) the brain. (Reproduced from J. Cell Biol. 1999. **147:** 659–669, by copyright permission of The Rockefeller University Press.)

Nckx30C and Calx Expression in Drosophila

A *Drosophila* sodium/calcium exchanger, *Calx*, was previously described and shown to be a member of the family of the NCX-type exchangers.[41,44,45] Expression patterns for *Calx* were reported in Refs. 41 and 43. We have shown that *Nckx30C* and *Calx* are expressed in the adult nervous system (FIG. 5). Both *Calx* and *Nckx30C* are expressed in the photoreceptor cells as well as in the lamina, medulla, and optic lobes of the brain (FIG. 5A and C). No signal was detected with the sense probes (FIG. 5B and D).

Both NCX and NCKX are responsible for extruding calcium in cells that are experiencing large calcium fluxes. *Drosophila* photoreceptors as well as other cells in the adult express both types of exchangers, indicating that there may be multiple mechanisms for calcium efflux in these cells. The NCKX exchangers have novel features that make them uniquely suited for calcium extrusion during phototransduction in photoreceptor cells. Light activation of *Drosophila* photoreceptors stimulates the opening of the cation-selective channels and a dramatic increase in intracellular calcium (FIG. 1). In addition, sodium also contributes to the inward current, leading to increased cytosolic sodium.[5,57–59] As the internal sodium concentration increases, the transmembrane sodium gradient is reduced and it possibly collapses during high light stimulation. NCX exchangers, such as *Calx*, likely reverse direction at much lower cytosolic sodium concentrations when compared with potassium-dependent NCKX exchangers.[60] Therefore, potassium-dependent exchangers are more effective for calcium removal during times of high sodium influx (high light intensities). The presence of both exchangers in photoreceptor cells could also be explained if the subcellular distributions of *NCKX30C* and *Calx* are very different, with each fulfilling differing functions. In addition to being present in the adult nervous system, *Nckx30C* and *Calx* are both expressed in the embryonic nervous system. *Calx* expression is present before activation of zygotic transcription, indicating that *Calx* transcripts are probably maternally inherited (FIG. 6B). *Calx* transcripts disappear in the cellular blastoderm (FIG. 6D) and are then detected again during embryonic stage 11 and 12 (FIG. 6F and H). Unlike *Calx*, *Nckx30C* transcripts were not detected in the preblastoderm or blastoderm stage (FIG. 6A and C). *Nckx30C* transcripts were first noted in cells at the ventral midline of the central nervous system during embryonic stage 13–14 (FIG. 6E). By stage 15, *Nckx30C* expression was detected in several neurons within the ventral nerve cord in the embryo (FIG. 6G). *Nckx30C* is expressed in many neurons within the ventral nerve cord and the embryonic brain in stage 16 (FIG. 6I). At similar times during embryogenesis, *Calx* expression is restricted to a smaller subset of neurons in the ventral nerve cord (FIG 6J). *Nckx30C* and *Calx* transcripts were observed in some cells outside the CNS, which may represent parts of the peripheral nervous system (FIG. 6I and J).

In addition to being expressed in the embryo, *Nckx30C* was also detected in larval imaginal discs. By contrast, *Calx* expression was not detected in any of the imaginal discs. *Drosophila* appendages develop from imaginal discs in a series of coordinated events. Photoreceptor differentiation is initiated at the posterior end of the eye disc and proceeds as a wave across the eye disc from posterior to anterior.[61–66] The morphogenetic furrow is a dorsoventral indentation in the eye disc, with the area anterior to the furrow being made up of actively dividing and unpatterned cells and the area posterior to the furrow containing differentiating photoreceptor cells.[66–68] FIGURE

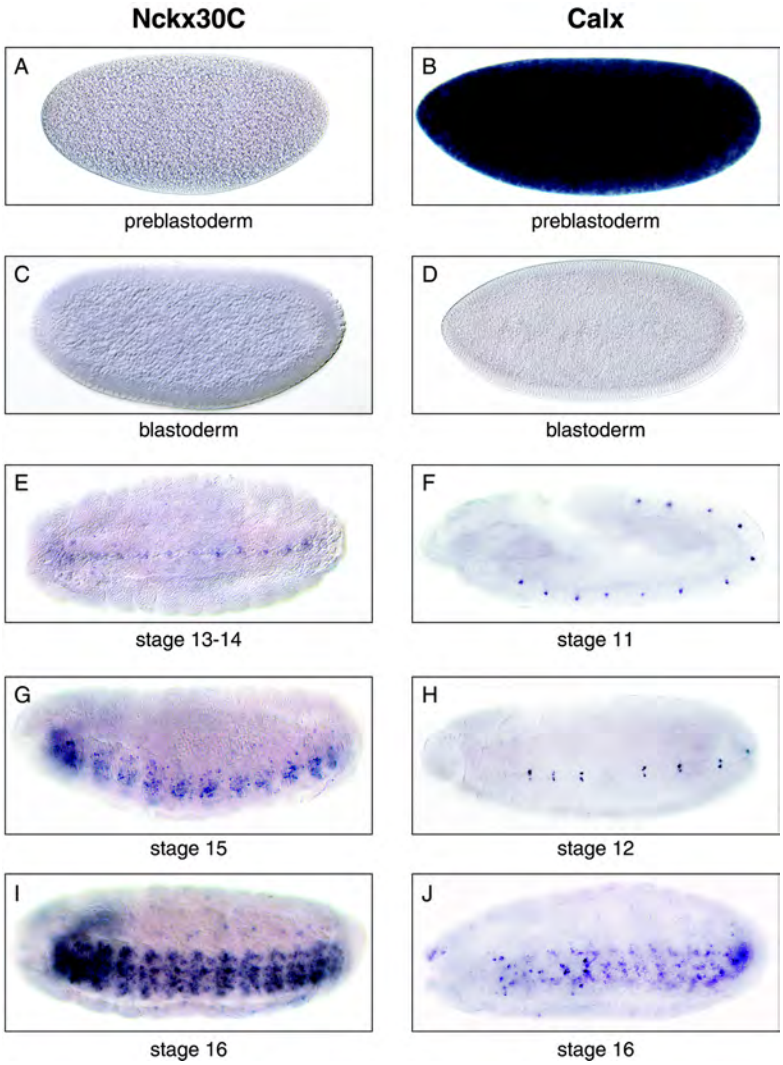

FIGURE 6. *Nckx30C* and *Calx* are expressed in the ventral nerve cord of the *Drosophila* embryo. Shown are *in situ* hybridizations to whole-mount wild-type embryos (Canton S strain) hybridized with digoxigenin-labeled antisense riboprobes for *Nckx30C* and *Calx*. (**A**) Lateral view, preblastoderm, *Nckx30C*; (**B**) lateral view, preblastoderm, *Calx*; (**C**) lateral view, blastoderm, *Nckx30C*; (**D**) lateral view, blastoderm, *Calx*; (E) ventral view, stage 13-14, *Nckx30C*; (**F**) lateral view, stage 11, *Calx*; (**G**) ventrolateral view, stage 15, *Nckx30C*; (**H**) ventral view, stage 12, *Calx*; (**I**) ventrolateral view, stage 16, *Nckx30C*; and (**J**) ventrolateral view, stage 16, *Calx*. Note the labeling of the developing ventral nerve cord. Control hybridizations with sense probes did not produce signals (data not shown). All embryos are oriented anterior to the left. In lateral views, all embryos are oriented dorsal side up. (Reproduced from Ref. 43, J. Cell Biol. 1999. **147:** 659–669, by copyright permission of The Rockefeller University Press.)

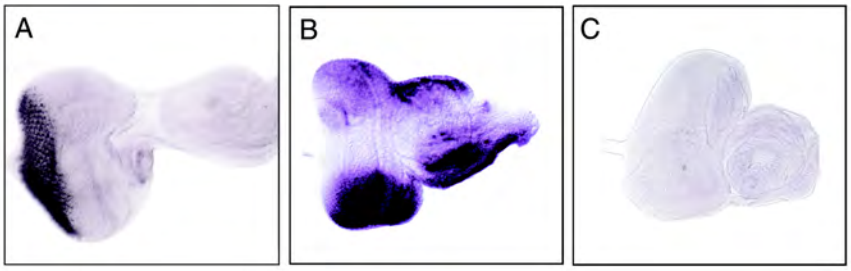

FIGURE 7. *Nckx30C* is expressed in the third instar imaginal discs of larvae. Shown are *in situ* hybridizations to imaginal discs from wild-type larvae with digoxigenin-labeled riboprobes for *Nckx30C* and *chaoptin.* (**A**) Eye-antennal disc, antisense riboprobe for *chaoptin*; (**B**) eye-antennal disc, antisense riboprobe for *Nckx30C*; (**C**) eye-antennal disc, control sense riboprobe for *Nckx30C.* Note that control hybridizations with sense probes did not produce signals. The eye discs are oriented posterior to the left and dorsal up. (Reproduced from Ref. 43, J. Cell Biol. 1999. **147:** 659–669, by copyright permission of The Rockefeller University Press.)

7A shows expression of *chaoptin* in photoreceptor cells located posterior to the furrow. *Chaoptin* expression can be used as a marker for differentiated photoreceptor cells posterior to the morphogenetic furrow. Chaoptin is a photoreceptor cell surface glycoprotein.[54]. *Nckx30C* transcripts are present in a dorsal/ventral pattern, both anterior and posterior to the morphogenetic furrow, with no labeling in the midline (FIG. 7B and C). *Wingless* (*wg*) displays a similar expression pattern, and it is important for dorsal/ventral patterning in eye development.[61–63,69,70] *Nckx30C* expression is wider than that observed for *wingless.* It has been proposed that one of the vertebrate Wingless (Wnt) pathways may use a G-protein–mediated phosphatidylinositol signaling cascade that leads to an increase in intracellular calcium.[71–73] We suggest that NCKX30C may be playing a role in modulating calcium in this or in other patterning pathways.[43] Given what we know about the role of NCKX in extruding large amounts of calcium in rod photoreceptor cells, it is likely that cells that express *Nckx30C* may also be experiencing large and sustained rises in cytosolic of calcium.

Here, we review that both NCX and NCKX-type exchangers not only may function in the removal of calcium and maintenance of calcium homeostasis during signaling in the adult, but they also may play critical roles in signaling events during embryogenesis. In addition, NCKX may play a role in calcium modulation during cell differentiation and patterning in eye development.

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