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Molecular characterization, functional expression and tissue distribution of a second NCKX Na^+/Ca^{2+}-K^+ exchanger from *Drosophila*

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Abstract

The Na^+/Ca^{2+}-K^+ exchanger (NCKX) utilizes the inward Na^+ gradient and the outward K^+ gradient to promote Ca^{2+} extrusion from cells. Here, we have characterized a second NCKX from *Drosophila*. Based on its chromosomal location (X chromosome) we have named it *Ncxk-x*. Three splice variants were isolated with three distinct N-terminal sequences. NCKX-X differs from NCKX proteins described so far in other species by lacking an N-terminal signal peptide. Heterologous expression of the respective cDNA's resulted in NCKX-X protein expression and K^+-dependent Na^+/Ca^{2+} exchange activity for two of the three splice variants. Transcript localization of *Nckx-x* was investigated and compared with that previously described by us for *Nckx30C*.

Keywords: Na^+/Ca^{2+}-K^+ exchanger; NCKX; *Drosophila*; BDGP; Calcium homeostasis; Sodium–calcium exchange

1. Introduction

Changes in intracellular free Ca^{2+} concentration constitute the most ubiquitous cellular signaling system found in all tissues. Intracellular free Ca^{2+} is modulated in a wide range of temporal and spatial patterns by numerous distinct gene families of Ca^{2+} transporting and Ca^{2+} binding proteins [1]. Sustained, elevated intracellular Ca^{2+} is highly toxic to cells and, as a consequence, proteins responsible for maintaining low intracellular Ca^{2+} are essential for cell viability and health. Four distinct gene families of Ca^{2+} extrusion proteins have been widely reported, the SLC8 gene family of NCX-type Na^+/Ca^{2+} exchangers [2], the family of plasma membrane Ca^{2+} pumps (PMCA) [3] and the sarco(endo)plasmic reticulum (SERCA) and Golgi secretory pathway (SPCA) families of intracellular Ca^{2+} pumps [4]. A fifth and perhaps less well-known gene family is the SLC24 family of Na^+/Ca^{2+}-K^+ exchangers (NCKXs). The NCKX gene family contains five human isoforms (NCKX1–NCKX5), several of which have been identified in a wide range of vertebrates [5]. To date, only NCKX1 and NCKX2 have been characterized "in situ", while other NCKX isoforms have been characterized only in heterologous systems. In the vertebrate retina, NCKX1 is expressed in rod photoreceptors, while NCKX2 is found in cone photoreceptors and ganglion cells [6,7]. NCKX2 is also widely expressed in the brain [8]. In rod and cone photoreceptors, the NCKX is located only in the plasma membrane of the outer segment organelle that contains the visual transduction machinery [9]. The physiological role of NCKX is to extrude Ca^{2+} that enters the rod and cone photoreceptor cells via the cGMP-gated (CNG) light-sensitive channels [10,11]. Like the NCKX exchangers, CNG channels are located specifically in the outer segment plasma membrane, and mediate large Ca^{2+} influxes [12]. CNG channels are heteromultimers consisting of three CNGA subunits and one CNGB subunit [13–15]. Both rod NCKX1 and cone NCKX2 have been shown to form homo-oligomers and also form hetero-oligomers with their respective CNGA1 and CNGA3 channel subunits [16–18]. This suggests that

Abbreviations: NCKX, Na^+/Ca^{2+}-K^+ exchanger; NCX, Na^+/Ca^{2+} exchanger; RACE, rapid amplification of cDNA ends; MW, molecular weight; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; TMH, transmembrane spanning helix; VNC, ventral nerve cord; MF, morphogenetic furrow; BDGP, Berkeley *Drosophila* Genome Project

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CNG and NCKX are natural partners in precisely balancing Ca^{2+} influx and efflux in cells. The complete membrane topology has only been determined for the NCKX2 protein [20]. NCKX2 and NCKX1 contain N-terminal hydrophobic signal peptides that are required for correct targeting to the plasma membrane [19]. The N-terminal signal peptide is followed by two clusters of transmembrane spanning helices (TMHs), each thought to contain five membrane spanning alpha helices, that are separated by a large cytosolic loop [20]. Genomic sequencing projects have revealed many NCKX-related proteins in invertebrates that share sequence similarity with the clusters of TMHs of mammalian NCKX. Three invertebrate *Nckx* cDNAs have been cloned to date from *Caenorhabditis elegans* [21], *Drosophila* [22] and sea urchin [23]. All three have been shown to display K^+-dependent Na^+/Ca^{2+} exchange activity when expressed in heterologous systems. *Drosophila Nckx30C* is similar to both NCKX1 and NCKX2 with 66 and 71% sequence identity, respectively, in the two clusters of TMH. *Nckx30C* is expressed in adult neurons as well as during ventral nerve cord development in the embryo and during eye development in the larval imaginal discs [22]. Here, we describe the molecular characterization, functional expression and tissue distribution of a second *Drosophila Ncxk* cDNA, *Nckx-x*, and compare the results with those obtained for *Ncxk30C*.

2. Experimental procedures

2.1. Molecular characterization of three different splice variants of Nckx-x

The *Nckx-x* cDNA clone, LD13015, was generated by the Berkeley *Drosophila* Genome Project (BDGP)[2] [24] and purchased from ResGen Invitrogen Corporation. The Accession # is AABU01002702. This clone contained all of the *Nckx-x* coding sequence except for the 5′ end. 5′ rapid amplification of cDNA ends (RACE) on first-strand *Drosophila* cDNA was used to obtain the upstream sequence. Six RACE products were sequenced and three distinct 5′ ends were obtained. The *Eco*RI/*Cla*I fragment at the 5′ end of LD13015 was excised from pBS-SK^+-containing *Nckx-x* (pBS-*Nckx-x*), the sequence coding for the human Myc tag was added, and the fragment was reinserted yielding pBS-*Nckx-x*-Myc. *Spe*I and *Eco*RI sites were added at the 5′ and 3′ ends, respectively, and the resulting fragments were cloned into pBS-SK^+ and sequenced. The three *Spe*I/*Eco*RI fragments with different 5′ ends were isolated and cloned in pBS-*Nckx-x*-Myc. The three full-length clones were subsequently subcloned into the expression vector pIE1/153A [21] and expressed in insect High Five cells. The resulting clones were verified for the correct reading frame by sequencing.

2.2. Functional analysis of Ncxk-x

The human c-Myc tag (EQKLISEEDL) was inserted just upstream of the *Cla*I site in all three splice variants of *Nckx-x*. The constructs were expressed by transient or stable transfection in a lepidopteran expression system of BTI-TN-5B1-4 insect cells (High Five cells, Invitrogen) as described before [21]. Protein expression levels for the three *Ncxk-x* splice variants were assessed by Western blotting with the monoclonal Myc antibody (Roche) after SDS–polyacrylamide gel electrophoresis (PAGE). Function was assayed by measuring ^{45}Ca uptake in Na^+-loaded cells representing reverse Na^+/Ca^{2+}-K^+ exchange essentially as described before [25]. Briefly, High Five cells were loaded with high Na^+ with the use of the ionophore monensin; monensin was subsequently removed by washing with NaCl–bovine serum albumin (BSA)-containing solutions, followed by washing in choline chloride solutions. ^{45}Ca uptake was measured in high KCl medium and compared with that observed in high NaCl medium in which ^{45}Ca uptake via reverse Na^+/Ca^{2+}-K^+ exchange is completely inhibited. ^{45}Ca taken up by cells was separated from that still present in the medium by a rapid filtration technique using borosilicate glass fiber filters.

2.3. In situ hybridization of Nckx-x

Embryo and imaginal disc in situ hybridizations were carried out by a modification of Panganiban et. al. [26], as described in Haug-Collet et al. [22]. The *Canton S* strain of embryos were collected, dechorionated, fixed, and processed for in situ hybridization and then hybridized at 55 °C for 24–30 h using probe concentrations of 90 ng/300 μl. Heads from the *w1118* strain of flies were embedded in Tissue-Tek OCT Compound (Miles, Inc.) and frozen on dry ice. Fourteen micrometer head sections of adult flies were cut and hybridized as described previously [22]. The larval imaginal discs were dissected from crawling third instar larvae, and left attached to the cuticle. The discs were fixed, permeabilized with Proteinase K at 25 μg/ml for 3 min at room temperature, and post-fixed. Probe concentrations were 120 ng/300 μl. Digoxigenin-labeled sense and antisense riboprobes were generated by in vitro transcription of five DNA templates as recommended by the digoxigenin/UTP supplier (Boehringer Mannheim Corp.). The antisense probe was generated by linearizing the *Nckx-x* cDNA (LD13015) with *Xba*I and the sense probe was generated with *Xho*I. After each transcription reaction, the mixture was treated with DNase and the riboprobes were subsequently hydrolyzed in carbonate buffer for 2 min at 80 °C. All riboprobes were quantified by analysis in denaturing 0.8% agarose gels and by dot blot

[2] The Berkeley *Drosophila* Genome Project (BDGP) is a consortium of the *Drosophila* Genome Center (funded by the National Human Genome Research Institute, National Cancer Institute, and the Department of Energy), and the Howard Hughes Medical Institute (through its support of work in the Gerald Rubin and Allan Spradling Laboratories).

analysis. All detection steps were as described by Tautz and Pfeifle [27] and no signal was detected with the sense probes.

3. Results and discussion

We have characterized a novel NCKX found in *Drosophila* that is located on the X chromosome (cDNA clone LD131015 [28]). Based on its chromosomal location, we have named it *Nckx-x*.

3.1. 5′ RACE yields three distinct splice variants of Nckx-x

The partial *Nckx-x* cDNA clone LD13015 [28] purchased from Research Genetics contained most of the NCKX-X coding sequence, but lacked the 5′ end. Therefore, we carried out a 5′ RACE which yielded three distinct transcripts representing three different 5′ ends of *Nckx-x*. The three different 5′ ends we obtained encoded the following three N-terminal amino acid sequences: MEDYWGLSSTTDIN (NCKX-X-SA), MYIYIN (NCKX-X-SB) and MMRIRNMKSSISHILLFCYIN (NCKX-X-SC). The full sequence of the NCKX-X splice variants is shown in Fig. 1. The *Drosophila* genome project has identified CG2893 as the gene containing our *Nckx-x* sequence. Information for *Nckx-x* can be found at http://www.flybase.org/.data/docs/refman/refman-I.html#I.2. Annotation of the CG2893 gene predicts three different splice variants of *Nckx-x*, two of which are identical with those obtained here by 5′ RACE (CG2893-RB corresponds to our SA splice variant, while CG2893-RC corresponds to our SC splice variant), but one is distinct (our SB splice variant). We did not find the CG2893-RA transcript. A full-length clone of the CG2893-RB splice variant is available (RE34149).

3.2. Analysis of NCKX-X sequence and comparison with other NCKX

Fig. 2 shows a sequence alignment of the NCKX-X-SA splice variant with a number of other NCKX proteins representing different NCKX isoforms from various species. For the sake of clarity, most of the sequence representing the two large hydrophilic domains, one at the N-terminus and one in the middle of the NCKX proteins, is not included here. These large hydrophilic domains within NCKX-X do not contain any sequence similarity with similarly placed domains of any of the other NCKX sequences currently in the database. Each NCKX protein listed contains 11 hydrophobic segments that show sequence similarity with similarly placed segments in all other NCKX isoforms, and these are numbered H1–H11. In addition, NCKX proteins from species other than *Drosophila* all contained a hydrophobic segment within the first 50 residues from the N-terminus that has the hallmarks of a signal peptide as judged by the SignalP Server (http://www.cbs.dtu.dk/services/SignalP/). Thus, the first distinguishing feature of NCKX-X is that it has a short (<50 residues) hydrophilic N-terminal sequence and no signal peptide. This holds true for all three splice variants. In the case of the mammalian NCKX1 and NCKX2 proteins, it has been shown that a single hydrophobic segment within the first 50 residues acts as a cleaved signal peptide that is

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                  1        5        10
NCKX-X-SA         MEDYWGLSSTTDIN
NCKX-X-SB         MYIYIN
NCKX-X-SC         MMRIRNMKSSISHILLFCYIN

15
CTQPAIDAFP RDLFSEAQRQ  SGAVVLHVIA  SLYLFVALAV  VCDEYEVPAV
65
EKICAALNMS  NDVAGATFMA  AATSAPELFV  NVIGTFITEG  DIGVGTIVGS
115
AVFNILAVAA  CCGIGAGMTI  PLDWWPLTRD  SIAYGVTVAI  LICVMHDERV
165
EWYEALILVS  LYAVYLAVMY  FDKTFQKCAK  GGVKQARSRS  RSSNCSIHTK
215
NSNEKEPELV  ENRICQNMAN  IQLNGGLNKE  QAKATSTSGA  GTTAISITTS
265
TMTTTLSTTH  SAGGGGGAVG  LCSGPGAIVQ  MAPLDDAEAES  HVAVAAADEG
315
RKEEGYSLLS  YPKDKSCFAQ  FTWLIIWPIH  LLFRIAIPDC  KKAKNNKIFP
365
LTFIMCIVWI  GSLSYVVAWM  ITIIGDTLKI  PDSVMGITFL  AAGTSVPEAV
415
SSVIVAKRGHG SMGICNSIGS  NTFDILLCLG  VPWLIKVVFF  PIQPGQNYVA
465
INSAGLEYSA ITLLSTLFLL  YLTFSTNKFK  LDKKVGTACL  VMYLVFMVFA
515
SLIELNVFFR  VNLPTCGRS533
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Fig. 1. Amino acid sequences of the three splice variants of NCKX-X. Amino acid sequence of the three different 5′ ends of NCKX-X, followed by the invariant part of the sequence.

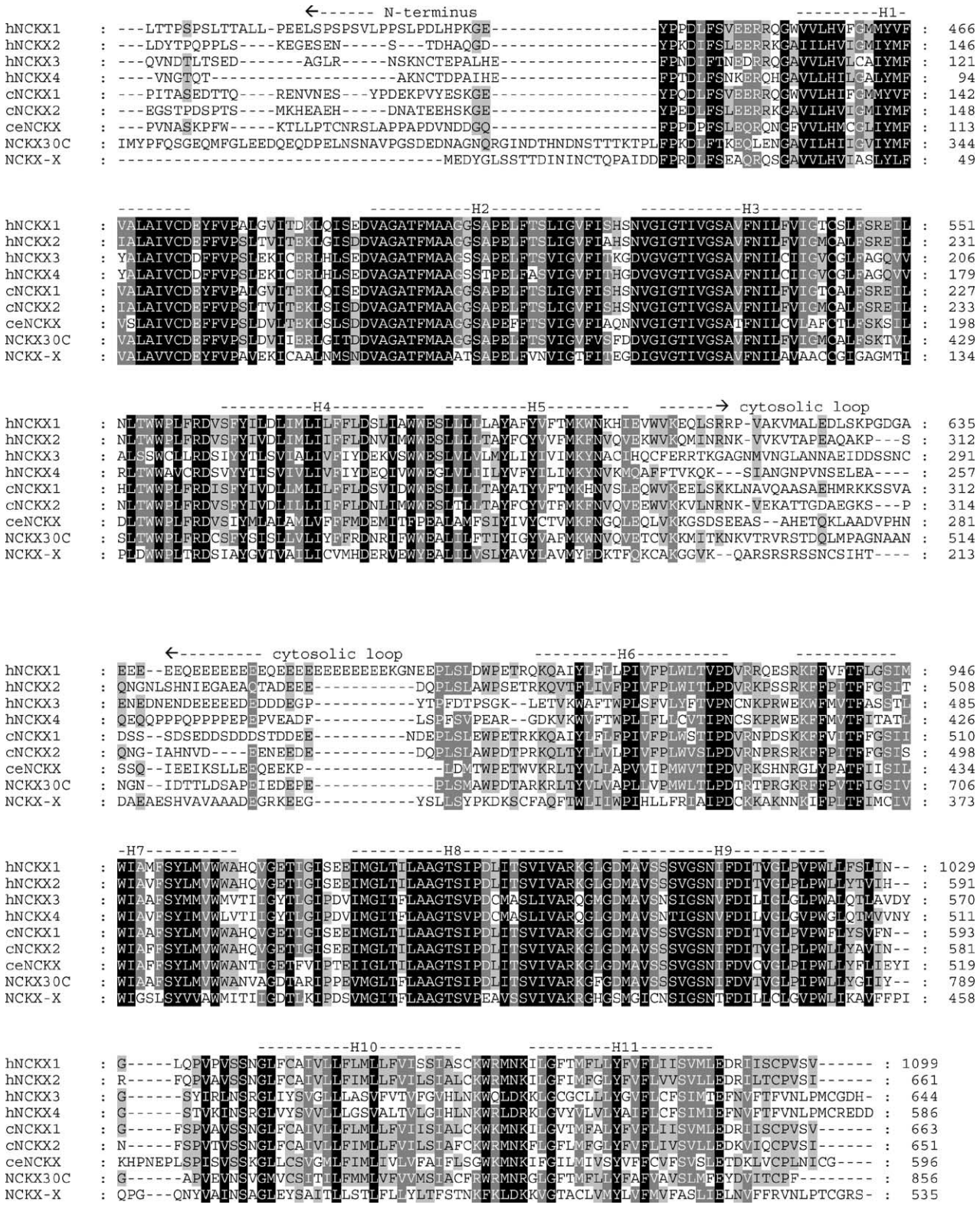

Fig. 2. Comparison of NCXK-X sequence with other NCKX sequences. Clustal W (http://www.ebi.ac.uk/clustalw/) was used to generate the alignment of multiple NCKX sequences in which NCKX-X was compared with human hNCKX1 through hNCKX4, chicken cNCKX1 and cNCKX2, NCKX30C, and with ceNCKX from *Caenorhabditis elegans*. The hydrophobic segments H1–H11, discussed in the text, are underlined.

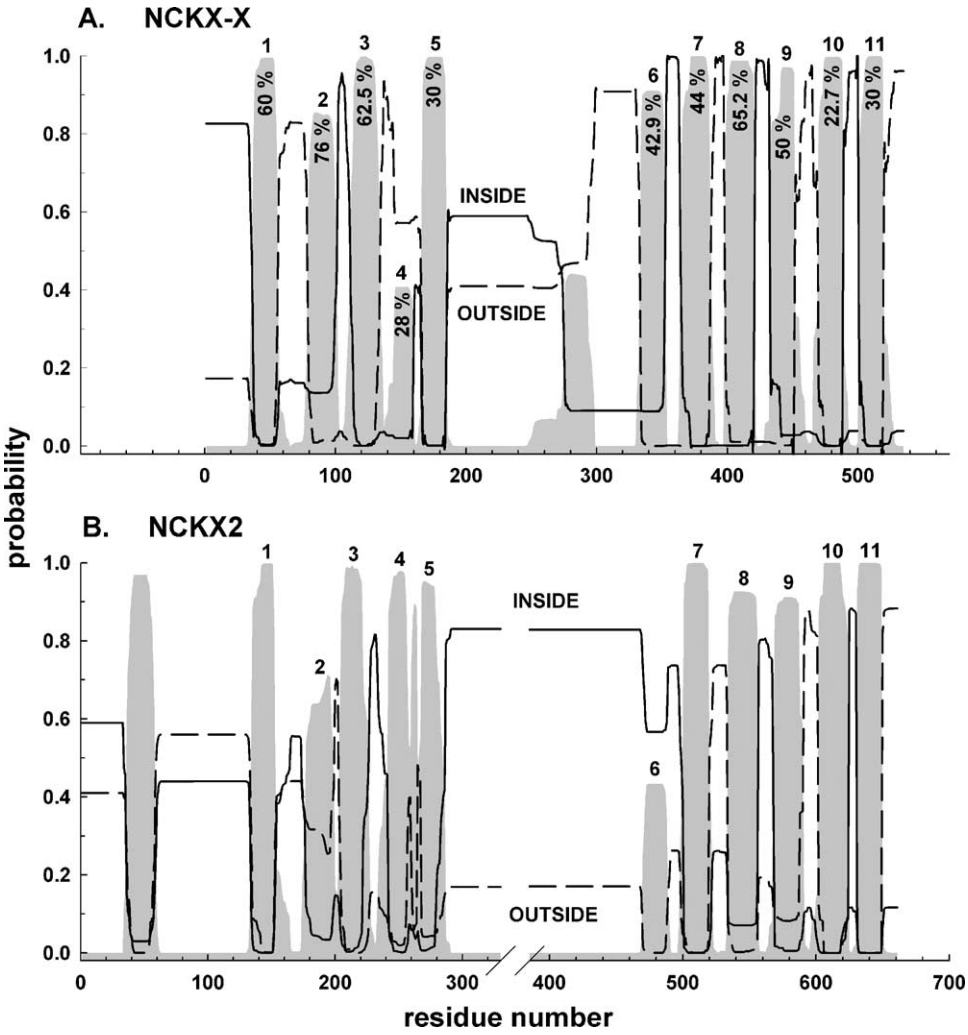

Fig. 3. Prediction of transmembrane helices in NCKX-X. The TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict TMHs in NCKX-X, and compare them with those predicted and validated for NCKX2 [20]. The solid line indicates the probability of an extracellular localization, while the broken line indicates the probability of an intracellular localization. The shaded bars represent the hydrophobic segments 1–11, and the height of the bar indicates the probability that these represent transmembrane spanning helices. A probability of >0.5 is thought to indicate a TMH. The percentage in each of the shaded bars indicates the percent sequence identity between NCKX-X and human NCKX2 for that particular hydrophobic segment.

critical for correct plasma membrane targeting of the NCKX protein in heterologous systems [19].

Next, we used the TMHMM Server for the prediction of TMHs in proteins (http://www.cbs.dtu.dk/services/TMHMM/) to predict the NCKX-X topology (Fig. 3). The TMHMM algorithm (version 1) is based on a hidden Markov model in which hydrophobicity, helix length and charge bias (i.e. the presence of clusters of positively charged residues in short connecting loops) are combined [29]. The gray bars represent hydrophobic segments with the height of the bar indicating the probability that it is a true membrane spanning alpha helix. The solid and dashed lines indicate the probable localization of the connecting hydrophilic stretches to the intracellular or extracellular space, respectively. It is not uncommon for the predicted orientation in the membrane to be inverted. The membrane topology of NCKX-X as judged by the pattern of predicted TMHs is remarkably different from the topology predicted by TMHMM for NCKX1, NCKX2 and NCKX3, respectively (illustrated here for NCKX2). Instead of the two clusters of five TMHs predicted for NCKX2, NCKX-X is predicted to have two clusters of four and six TMHs, respectively. The two clusters of TMHs are separated by a large hydrophilic loop as for other NCKX proteins. Different from other NCKX proteins, this hydrophilic loop of NCKX-X is predicted to contain an additional TMH. Very little work has yet been carried out to experimentally address NCKX topology. The localization of all the extracellular connecting loops and domains of NCKX2 was recently shown to be consistent with the predicted TMHMM topology shown here [20]. Furthermore, most of the two large hydrophilic loops could be removed from NCKX1 without affecting NCKX transport function [21]. This NCKX1 deletion mutant consists only of the 11 numbered hydrophobic segments and the very short

hydrophilic loops that connect them. Function observed with this deletion mutant suggests that the deleted domains do not contain membrane spanning helices that contribute to the NCKX topology. Significant sequence identity exists between NCKX-X and NCKX1 or NCKX2 in each of these eleven hydrophobic segments as indicated in Fig. 2. Sequence identity is most pronounced in two areas, H1–H3 and H8–H9, areas shown to contain residues essential for cation transport [25]. In view of the above considerations, we suggest that NCKX-X and NCKX2 (and other NCKX isoforms) adopt the same membrane topology despite the different topologies predicted by TMHMM or other computational methods. On the basis of available experimental evidence the NCKX topology appears best represented by that predicted by TMHMM for NCKX2 [20].

In contrast to the TMH, the large hydrophilic loops share very limited or no sequence similarity when comparing NCKX-X with other NCKX isoforms. Therefore, these two large hydrophilic loops are unlikely to be important for NCKX transport function per se. Putative regulatory features imposed on NCKX function remain to be uncovered for any of NCKX proteins described so far.

3.3. Heterologous expression of the three NCKX-X splice variants

The human Myc tag was inserted into the N-terminus of each the three different splice variants of *Nckx-x*, the constructs were cloned into the pIE1/153A expression vector and expressed in High Five insect cells as described before [21]. In comparison with most other NCKX cDNAs, including NCKX2 and NCKX30C, NCKX-X protein levels were rather modest when High Five cells were transiently transfected with the *Nckx-x* cDNA's representing the three

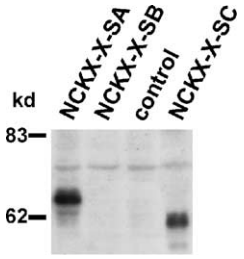

Fig. 4. Protein expression of the three NCKX-X splice variants. The indicated Myc-tagged NCKX-X splice variants were expressed in High Five cells, separated by SDS–PAGE, and analyzed by Western blotting with the Myc monoclonal antibody. Results are shown typical for at least three experiments.

different splice variants (data not shown). Therefore, we developed clonal High Five cell lines that showed higher and stable NCKX-X protein expression levels for subsequent functional analysis. The NCKX-X-SA and SC splice variants yielded Myc-tagged NCKX protein expression, while the SB splice variant did not show any protein expression as detected by Western blotting with the monoclonal Myc antibody (Fig. 4). The reason for the lack of expression of the SB splice variant is unclear. The SA splice showed an apparent MW of ~66 kDa (calculated MW 57.5 kDa), while the SC splice variant showed an apparent MW of ~60 kDa (calculated MW 58.4 kDa). The significant difference in apparent MW may be explained by the presence of a high probability *O*-glycosylation site (Thr17) in the SA splice variant. Also, the presence of three additional acidic residues lowers the pI of the SA splice variant to 5.59 compared to 6.70 for the SC splice variant, and this may contribute to the observed lower mobility on SDS–PAGE. Most NCKX1 proteins contain a long stretch of acidic residues in the large cytosolic domain and the expressed

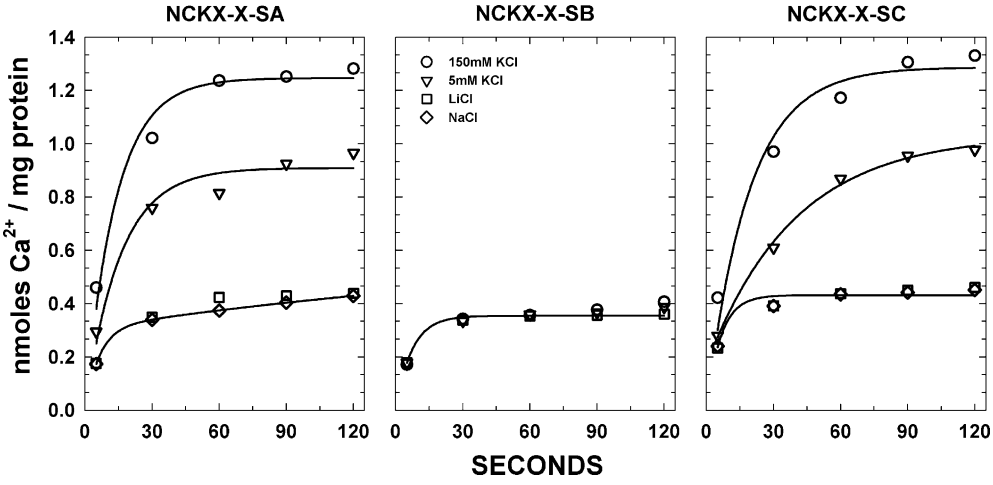

Fig. 5. Functional analysis of the three NCKX-X splice variants. The three NCKX-X splice variants were expressed in High Five cells and NCKX-X function was analyzed by the difference in ^{45}Ca uptake observed in KCl medium vs. NaCl or LiCl medium as described under Section 2. Uptake medium contained in all cases 80 mM sucrose, 20 mM HEPES (pH 7.4), 6 μM EDTA, and either 150 mM KCl (circles), or 145 mM LiCl + 5 mM KCl (inverted triangles), or 150 mM LiCl (squares), or 150 mM NaCl (diamonds). Uptake was initiated by addition of 1 μCi ^{45}Ca 36 μM $CaCl_2$ at time zero. Results of a single experiment are shown typical for at least four additional experiments. Temperature: 25 °C.

NCKX1 proteins show an anomalously high MW when compared with the expressed NCKX2 proteins [19].

3.4. NCKX-X is a K^+-dependent Na^+/Ca^{2+} exchanger

NCKX function was tested by measuring ^{45}Ca uptake in Na^+-loaded High Five cells after stable transfection with the different NCKX-X splice variants. This assay takes advantage of the well documented property that NCKX proteins can carry out not only forward Na^+/Ca^{2+}-K^+ exchange (Na^+_{out}-dependent Ca^{2+} release from cells), but can also reverse and carry Ca^{2+} into the cell via so-called reverse Na^+/Ca^{2+}-K^+ exchange; the latter occurs when the transmembrane Na^+ gradient is reversed by removal of extracellular Na^+ (Na^+_{in}-dependent Ca^{2+} uptake into cells) [21,30]. In the experiment illustrated in Fig. 5, we compared ^{45}Ca uptake in four different external media. As a control, ^{45}Ca uptake in NaCl medium was very similar to that observed in control cells since high external Na^+ inhibits ^{45}Ca uptake via the exchanger (not illustrated). A significant increase in ^{45}Ca uptake was observed in KCl medium compared with NaCl medium for cells expressing the NCXK-X-SA and SC splice variants while no additional ^{45}Ca uptake was observed for the SB splice variant. The latter is consistent with the lack of protein expression observed in Fig. 4. ^{45}Ca uptake observed for

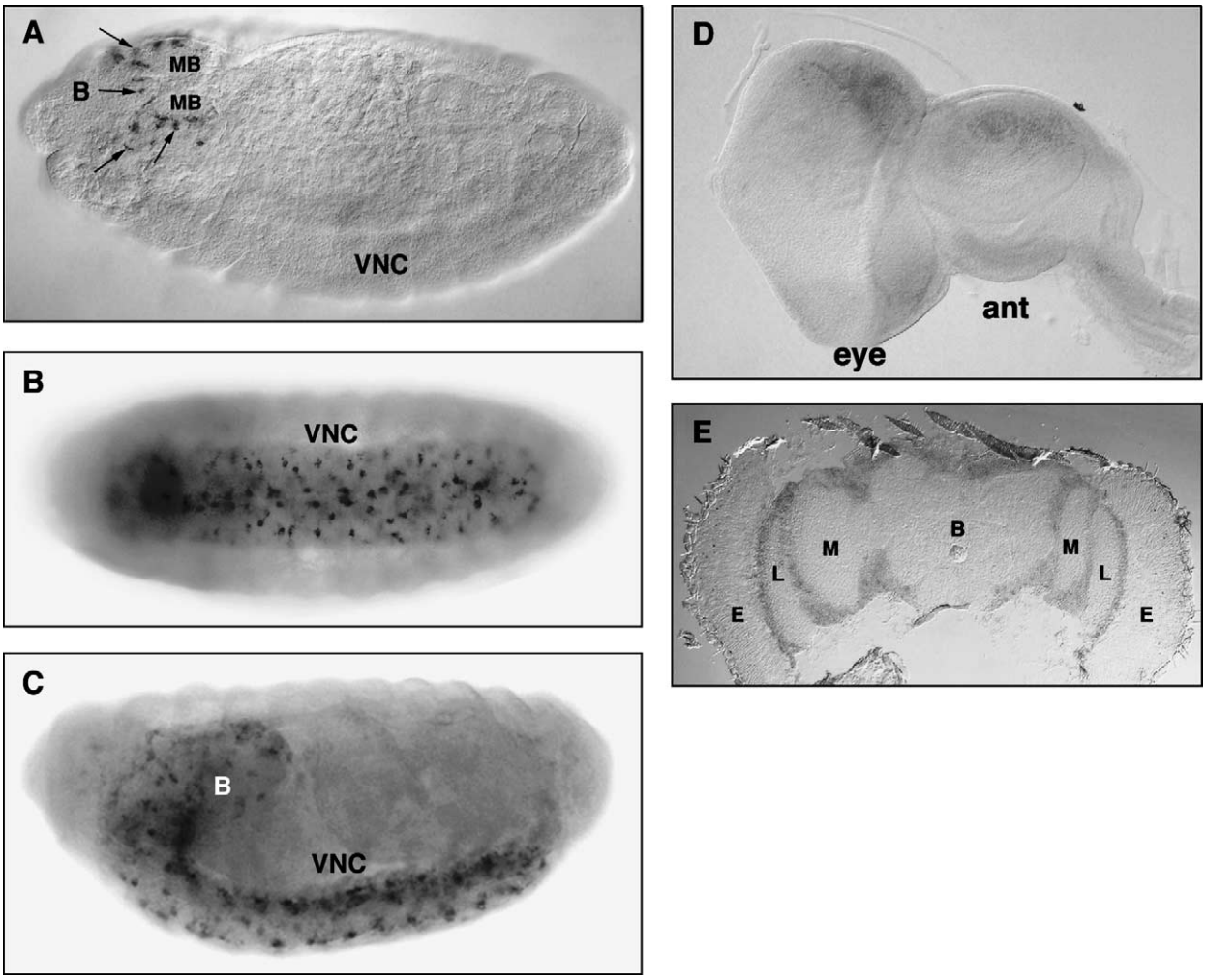

Fig. 6. *Nckx-x* is expressed in the embryonic nervous system, the eye-antennal disc and the adult nervous system in *Drosophila*. Shown are in situ hybridizations to whole mount wild-type embryos, and a third instar larval eye-antennal disc as well as a 14 μm cryostat section of an adult head hybridized with digoxigenin-labeled antisense riboprobes for *Nckx-x*. (A–C) Whole mount wild-type embryos (*Canton S* strain). Embryonic expression of *Nckx-x* is restricted to the CNS. All panels are shown with anterior to the left. (A) A dorsal view of the embryonic brain at stage 13. Expression of *Nckx-x* (arrows) begins in the embryonic brain (B) at stage 13 in several dorsal cells near the developing mushroom body (MB), but not in the ventral nerve cord (VNC). (B) A ventral view of the developing nerve cord at stage 15. *Nckx-x* is expressed in a specific subset of cells, reminiscent of the pattern which has been described for glia [32]. (C) A lateral view of a stage 16 embryo shows *Nckx-x* expression in the embryonic brain and ventral nerve cord. (D) Eye-antennal disc isolated from third instar larva, displaying *Nckx-x* transcript in the dorsal and ventral margins with no labeling in the midline. Eye (eye) is on the left and antennal portion (ant) of the disc is on the right. Posterior region of the eye disc is to the left. (E) Cryostat section of an adult head showing *Nckx-x* expression in the brain (B), lamina (L), and medulla (M), with no detectable labeling in the eye (E).

the SB splice variant was indistinguishable from ^{45}Ca uptake observed in control cells not expressing NCKX (not illustrated). The K^+ dependence of Na^+/Ca^{2+}exchange transport was tested by comparing ^{45}Ca uptake in high external LiCl medium with that observed in high KCl medium as Li^+ cannot replace K^+ in cotransport with Ca^{2+} via NCKX. Clearly, no reverse Na^+/Ca^{2+}exchange was carried out via the NCKX-X-SA and SC splice variants in high LiCl medium (compare squares with circles in Fig. 5). The inability of NCKX proteins to carry out reverse Na^+/Ca^{2+}exchange in high LiCl medium marks an important distinction with members of the NCX and *Calx* Na^+/Ca^{2+}exchanger gene families, in which case ^{45}Ca uptake via reverse exchange is not dependent on external K^+, and works equally well in media containing external Li^+ or K^+ [21]. Finally, we looked at the sensitivity towards external K^+ by replacing only 5 of the 150 mM LiCl by KCl, which resulted in about half-maximal ^{45}Ca uptake when compared to uptake observed in the 150 mM KCl medium (Fig. 5, inverted triangles). These results are very similar to those previously described for a number of other heterologously expressed NCKX proteins [6,21,22]. From our results it is clear that the functional characteristics of NCKX-X are consistent with K^+-dependent Na^+/Ca^{2+}exchange transport observed previously for other NCKX proteins.

3.5. *Nckx-x is expressed in the embryonic nervous system*

Nckx-x transcripts were not detected in the syncytial blastoderm, cellular blastoderm or germ band elongated stages (data not shown). However, at embryonic stage 13, *Nckx-x* transcripts were detected in a discrete pattern of cells in the brain (Fig. 6A). At stage 14/15, *Nckx-x* expression is seen in the ventral nerve cord (VNC) in a discrete pattern of cells (Fig. 6B). In the late embryo at stage 16, *Nckx-x* remains restricted to the nervous system, with expression seen throughout the brain and ventral nerve cord (Fig. 6C). Interestingly, the embryonic expression of *Nckx-x* differs significantly from the expression of another NCKX, *Nckx30C* and an Na^+/Ca^{2+} exchanger (NCX gene family), *Calx* [22,31]. Both *Calx* and *Nckx30C* are expressed earlier in the brain and VNC, and are more pan-neural, whereas *Nckx-x* is expressed in a highly restricted pattern which resembles what has been reported for glia [32].

3.6. *Nckx-x is expressed in the eye-antennal disc, but not in the adult eye*

Drosophila appendages develop from imaginal discs in a highly orchestrated series of events that divide the discs into distinct subregions during patterning. Retinal differentiation begins at the posterior end of the eye disc which coincides with the dorsal/ventral midline [33–35] and proceeds as a wave across the eye disc from posterior to anterior [36–38]. The morphogenetic furrow (MF) is a dorsoventral indentation in the eye disc, with the region anterior to the furrow comprising actively dividing and unpatterned cells and the region posterior to the furrow containing differentiating photoreceptor cells assembling into ommatida [36,37,39]. Previous work has shown that *Nckx30C* is expressed in the eye disc in both anterior and posterior to the MF in a dorsal/ventral pattern with no labeling in the midline [22,31]. We observe a similar pattern of expression for *Nckx-x* in the eye disc (Fig. 6D).

Since *Nckx30C* is expressed in the eye disc, and in the adult photoreceptors and brain, one might expect *Nckx-x* to also have a similar adult expression pattern. While eye disc expression of the two exchangers is identical, the adult expression is not. Although *Nckx-x* is not detected in the adult eye, it is expressed in the lamina and the medulla. The R1–6 photoreceptor axons synapse in the lamina and the R7 and R8 photoreceptors synapse in the medulla. Therefore, like *Nckx30C* and *Calx*, *Nckx-x* is expressed in the optical regions of the brain [22]. In addition, all three exchangers *Nckx30C*, *Nckx-x* and *Calx* are expressed in the brain (Fig. 6) [22]. Our data suggest that in some cells, where more than one exchanger is expressed, there may be multiple mechanisms for Ca^{2+} efflux.

3.7. *Conclusions*

We have characterized a novel K^+-dependent Na^+/Ca^{2+}-exchanger from *Drosophila* that is located on the X chromosome. Based on its location, we have named it *Nckx-x*. The NCKX-X protein lacks an N-terminal signal peptide found in NCKX isoforms from other species. Two of the three NCKX-X splice variants described here showed K^+-dependent Na^+/Ca^{2+} exchange transport when expressed in cell lines. The K^+ sensitivity was very similar to that described for other NCKX isoforms. Surprisingly, the predicted NCKX-X topology is quite different from that predicted for other NCKX proteins, but this may reflect a limitation of topology prediction methods rather than present a real difference. We have shown that *Nckx-x* transcripts are found in the adult nervous system and also during development in the embryo and the imaginal discs. A puzzling finding is that while the photoreceptors in *Drosophila* express both *Nckx30C* and *Calx* exchangers, *Nckx-x* expression is not detected in the adult eye. However, all three exchangers are expressed in the optical portions of the brain, the lamina and medulla, as well as in the central brain. These results highlight the similarities and differences in expression between two similar NCKXs and an NCX. The isolation of *Nckx-x* in *Drosophila* permits further genetic analysis of the in vivo role of Ca^{2+} in modulating signaling pathways in *Drosophila*. *Nckx-x* appears most abundant in the ventral nerve cord of the embryo, pointing to exciting possibilities for a role for the NCKXs in Ca^{2+} modulation during embryogenesis and development.

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