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A novel family of transmembrane proteins interacting with β subunits of the Na,K-ATPase

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We characterized a family consisting of four mammalian proteins of unknown function (NKAIN1, 2, 3 and 4) and a single *Drosophila* ortholog dNKAIN. Aside from highly conserved transmembrane domains, NKAIN proteins contain no characterized functional domains. Striking amino acid conservation in the first two transmembrane domains suggests that these proteins are likely to function within the membrane bilayer. NKAIN family members are neuronally expressed in multiple regions of the mouse brain, although their expression is not ubiquitous. We demonstrate that mouse NKAIN1 interacts with the β1 subunit of the Na,K-ATPase, whereas *Drosophila* ortholog dNKAIN interacts with Nrv2.2, a *Drosophila* homolog of the Na,K-ATPase β subunits. We also show that NKAIN1 can form a complex with another β subunit-binding protein, MONaKA, when binding to the β1 subunit of the Na,K-ATPase. Our results suggest that a complex between mammalian NKAIN1 and MONaKA is required for NKAIN function, which is carried out by a single protein, dNKAIN, in *Drosophila*. This hypothesis is supported by the fact that dNKAIN, but not NKAIN1, induces voltage-independent amiloride-insensitive Na^+-specific conductance that can be blocked by lanthanum. *Drosophila* mutants with decreased dNKAIN expression due to a P-element insertion in the *dNKAIN* gene exhibit temperature-sensitive paralysis, a phenotype also caused by mutations in the Na,K-ATPase α subunit and several ion channels. The neuronal expression of NKAIN proteins, their membrane localization and the temperature-sensitive paralysis of NKAIN *Drosophila* mutants strongly suggest that this novel protein family may be critical for neuronal function.

INTRODUCTION

It is estimated that human or mouse genomes contain approximately 20 000–25 000 genes (1). More than half of these genes are expressed in the central nervous system (CNS) (2). Relative to the total number of proteins expressed in the CNS, only a small percentage of genes with known CNS-specific function have been identified (3). In order to find previously uncharacterized genes that are likely to be involved in brain development and function, we selected a number of brain-specific novel genes and gene families using a bioinformatic approach. Of these novel proteins, one protein family consisting of four mammalian proteins of unknown function was selected for further biochemical analysis. We named these proteins NKAIN (Na,K-Atpase INteracting) as they were found to interact with the β1 subunit of the Na,K-ATPase.

Na,K-ATPase is a plasma membrane enzyme that is responsible for maintaining an electrochemical gradient across cellular membranes by transporting two K^+ ions inside cells in exchange for three Na^+ ions using an ATP-dependent mechanism. Na,K-ATPase requires association of α and β subunits in order to form functionally active enzymes. The α subunit has 10 membrane spanning regions and is responsible for catalytic functions of the enzyme, whereas the β subunit contains a single transmembrane domain and helps to deliver and stabilize the α subunit in the plasma membrane as well as influence its transport properties (4,5). Nrv1 and Nrv2 are the most well-studied *Drosophila* homologs of the β subunits of the Na,K-ATPase (6,7). Nrv1 is expressed in multiple tissues, whereas two Nrv2 splice variants, Nrv2.1 and Nrv2.2, are thought to be the neuronal isoforms (8,9).

Point mutations in the *ATPalpha* gene, encoding the *Drosophila* Na,K-ATPase α subunit, are associated with a

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temperature-sensitive paralysis phenotype (10), whereas P-element insertions in the *ATPalpha* gene lead to 'bang sensitivity', that is, paralysis after a short vortex stimulus (11,12). The temperature-sensitive *Drosophila* phenotype is also caused by mutations in ion channels or proteins regulating channel function (13–16).

In this report, we describe a family of evolutionary conserved transmembrane proteins (NKAINs) that localize to neurons and interact with the Na,K-ATPase β1 subunit. A *Drosophila* mutant with a P-element insertion in the *Drosophila NKAIN* ortholog gene (*dNKAIN*) exhibits temperature-sensitive paralysis. Remarkably high evolutionary amino acid conservation of NKAIN transmembrane domains and dNKAIN-induced Na^+-specific conductance in *Xenopus* oocytes suggest that NKAINs might function as subunits of pore or channel structures in neurons or affect the function of other membrane proteins.

RESULTS

NKAINs, a family of four evolutionary conserved novel membrane proteins

As part of a bioinformatics screen for novel proteins expressed in the mammalian CNS, we identified a family of four proteins—NKAIN1, 2, 3 and 4. The sequences of NKAIN family members and their splice variants were determined from extensive expression sequence tag (EST) data available from GenBank. mRNA sequence of *NKAIN* genes was then confirmed by reverse transcription–polymerase chain reaction (RT–PCR) and sequencing. There are three splice variants of *NKAIN2, 3* and *4*, whereas only a single form of *NKAIN1* was found. Accession numbers, chromosomal locations and corresponding UniGene clusters of *NKAIN* genes are shown in Figure 1A.

Seven-exon splice forms with conserved exon–intron boundaries exist for *NKAIN1*, *NKAIN2* and *NKAIN4*. Exon 6 is skipped in *NKAIN3v.1* and *NKAIN4v.2*, whereas an extra exon 6 is included in *NKAIN2v.2* (Figs 1B and 3A). In addition to these splice forms, several additional variants can also be detected, although they are expressed at much lower levels. Alternatively spliced variants terminating exon 4 are detected for *NKAIN2v.3*, *NKAIN3v.3* and *NKAIN4v.3*, but not for *NKAIN1*. Finally, an alternative exon 5 is included in *NKAIN3v.2* (Fig. 1B). As the first four exons of *NKAIN* genes are not alternatively spliced, NKAIN protein variants differ only in their C-terminal domains (Fig. 1C).

NKAIN proteins show no similarity to any protein with known function and contain no characterized domains, except for three putative transmembrane domains and a predicted cleavable signal peptide (Fig. 2A). We developed two polyclonal antibodies (anti-NKAIN1 and anti-NKAIN1/2) using peptides corresponding to two regions of NKAIN1. Using immunohistochemistry on HEK293T cells expressing NKAIN proteins, we determined that anti-NKAIN1 antibody is specific to NKAIN1, whereas anti-NKAIN1/2 detects both NKAIN1 and NKAIN2 (Fig. 2B). No immunolabeling was observed when pre-immune sera of these antibodies were used (data not shown). Cells expressing NKAIN4 did not stain with either anti-NKAIN1 or anti-NKAIN1/2 antibodies (Fig. 2B). As seen from this experiment, NKAIN proteins localize to cell membranes of transfected HEK293T cells. Both polyclonal antibodies labeled NKAIN1-transfected cells in non-permealizing conditions, suggesting that epitopes recognized by these antibodies are extracellular. Since anti-NKAIN1 recognizes the region between the second and third putative transmembrane domains of NKAIN1, while anti-NKAIN1/2 is specific to the C-terminal tail, the third putative membrane domain could represent a re-entrant loop or could be completely extracellular. The third putative membrane domain was also absent from alternatively spliced forms of NKAIN2, 3 and 4 (Fig. 1C). There are two conserved cysteine residues in the putative extracellular region, suggesting a possible disulfide bridge formation (asterisks, Fig. 2A).

There are four family members in vertebrates, one in *Drosophila melanogaster* and one in *Caenorhabditis elegans*. As seen from the multiple alignment of NKAIN proteins from several organisms, these proteins are highly conserved (Fig. 2A). There is 81–96% amino acid similarity between mouse, chicken and frog orthologs. The *Drosophila* ortholog dNKAIN shares 59–60% amino acid similarity with mouse NKAINs in the first 200 amino acids of the protein and has an additional 458 amino acid C-terminal tail that is absent from vertebrate NKAINs. Amino acid similarity between NKAIN proteins is highest in the first two putative transmembrane domains in the N-terminal part and is almost identical in the first transmembrane region (Fig. 2A). Remarkably high evolutionary conservation of the transmembrane domains suggests that the function of NKAIN proteins is confined to membranes, such as forming channels or pore structures, or affecting function of other membrane proteins.

NKAIN proteins are expressed by neurons in the mouse brain

As most of the ESTs corresponding to *NKAIN1, 2* and *3* derive from brain cDNA libraries, we predicted that expression of these family members is restricted to the CNS. *NKAIN4* is likely to be expressed ubiquitously, because ESTs corresponding to *NKAIN4* originate from a number of different tissues in addition to CNS. RT–PCR was used to determine the expression of *NKAIN* genes. As predicted from EST data, all four family members are highly expressed in mouse brain (Fig. 3A). Except for testis, *NKAIN1, 2* and *3* are not detectable outside the CNS (Fig. 3A). Interestingly, the six-exon splice variant of *NKAIN4* (*NKAIN4v.2*) is brain- and testis-specific, whereas the seven-exon splice variant *NKAIN4v.1* is expressed ubiquitously.

Brain expression patterns of mouse *NKAIN1, 3* and *4*, obtained by radioactive *in situ* hybridization, are available from the Brain Gene Expression Map (BGEM) (17). Expression data for *NKAIN2* and *3* are also available from the Gene Expression Nervous System Atlas (GENSAT), which was created using bacterial artificial chromosome (BAC) technology (18). In these BAC transgenic mice, green fluorescent protein (GFP) is expressed under the regulatory elements of *NKAIN2* or *NKAIN3*, filling the NKAIN2 or NKAIN3 protein expressing cells with GFP. NKAIN proteins are expressed in multiple regions of mouse brain, such as cerebral cortex, thalamus, cerebellum, hippocampus, olfactory

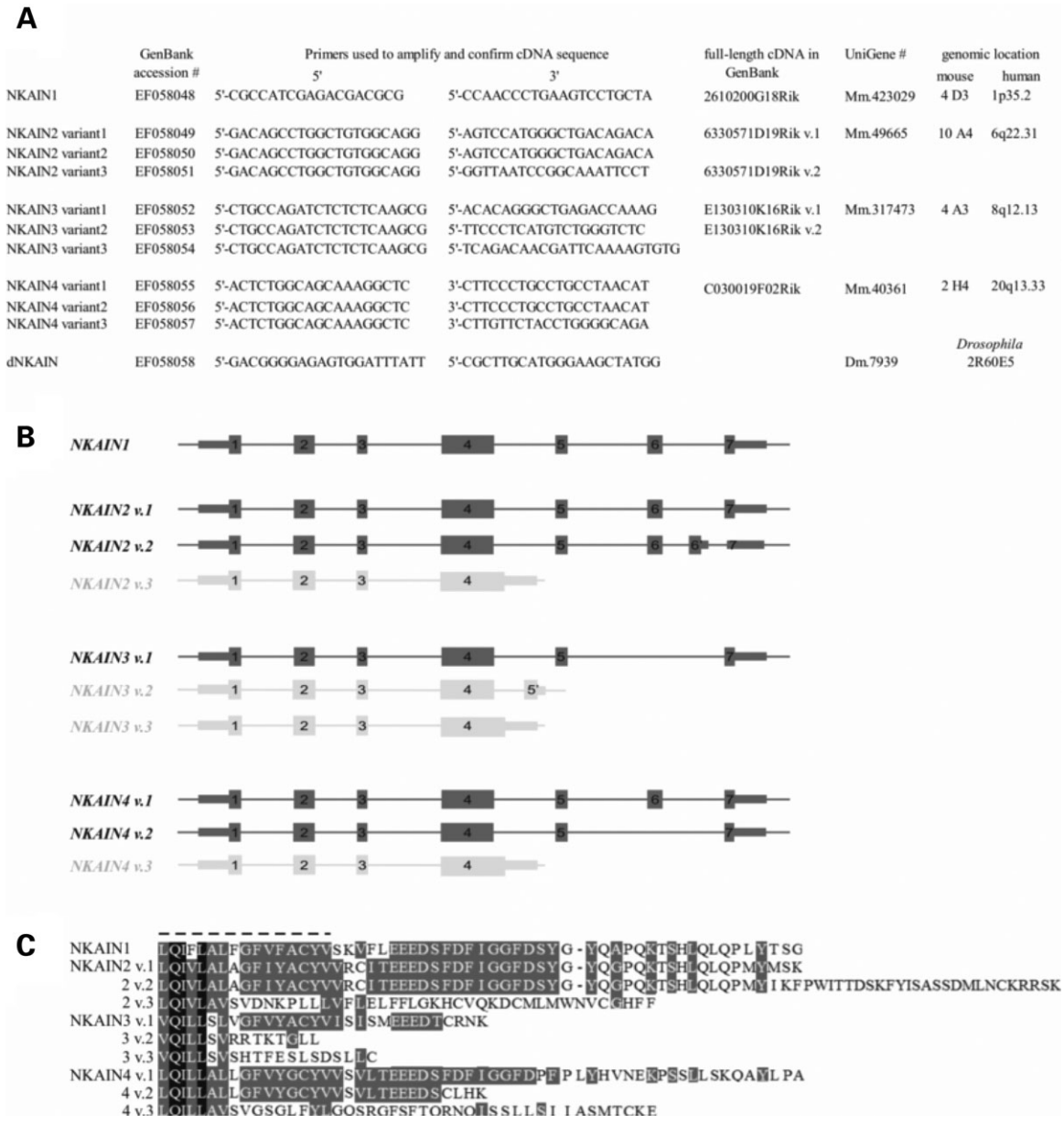

Figure 1. *NKAIN* genes are alternatively spliced. (**A**) GenBank accession numbers, corresponding full-length cDNAs and UniGene clusters, genomic locations of mouse and *Drosophila NKAIN* genes. (**B**) Exon–intron structure of mouse *NKAIN* genes. The splice variants that are expressed at a lower level compared with the major splice forms are shown in light gray. (**C**) Multiple alignment of the alternatively spliced NKAIN C-terminal domain. The putative third transmembrane domain is marked with a dashed line.

bulb and brainstem (Fig. 3B). However, their expression is not ubiquitous. It is also distinct between different family members. For example, NKAIN2 is not expressed in the hippocampus, whereas NKAIN1, 3 and 4 are strongly expressed in this region. Similarly, NKAIN1 is highly expressed in cerebellar granular cell layer, compared with lower expression of NKAIN3 and NKAIN4, and absent expression of NKAIN2 in this region (Fig. 3B).

Taking advantage of the fact that GFP protein is distributed in the cytoplasm of NKAIN2- or NKAIN3-positive cells in BAC-transgenic mice, it is possible to visualize the morphology of these cells. As seen in Figure 3C, multiple cortical neurons are positive for NKAIN2 or NKAIN3. We then determined expression of NKAIN1 and NKAIN2 in mouse brain by immunohistochemistry using double labeling with NKAIN1/2-specific antibody and a neuron-specific marker NeuN. As shown in Figure 3D, cortical and hippocampal cells expressing NKAIN1 or NKAIN2 (red) are also stained with NeuN (green), demonstrating that NKAIN1 and NKAIN2 are neuron-specific. No labeling was observed when pre-immune sera for anti-NKAIN1/2 was used for immunohistochemistry (data not shown).

We then tested whether highly homologous *Drosophila* ortholog dNKAIN was also expressed in nervous tissues.

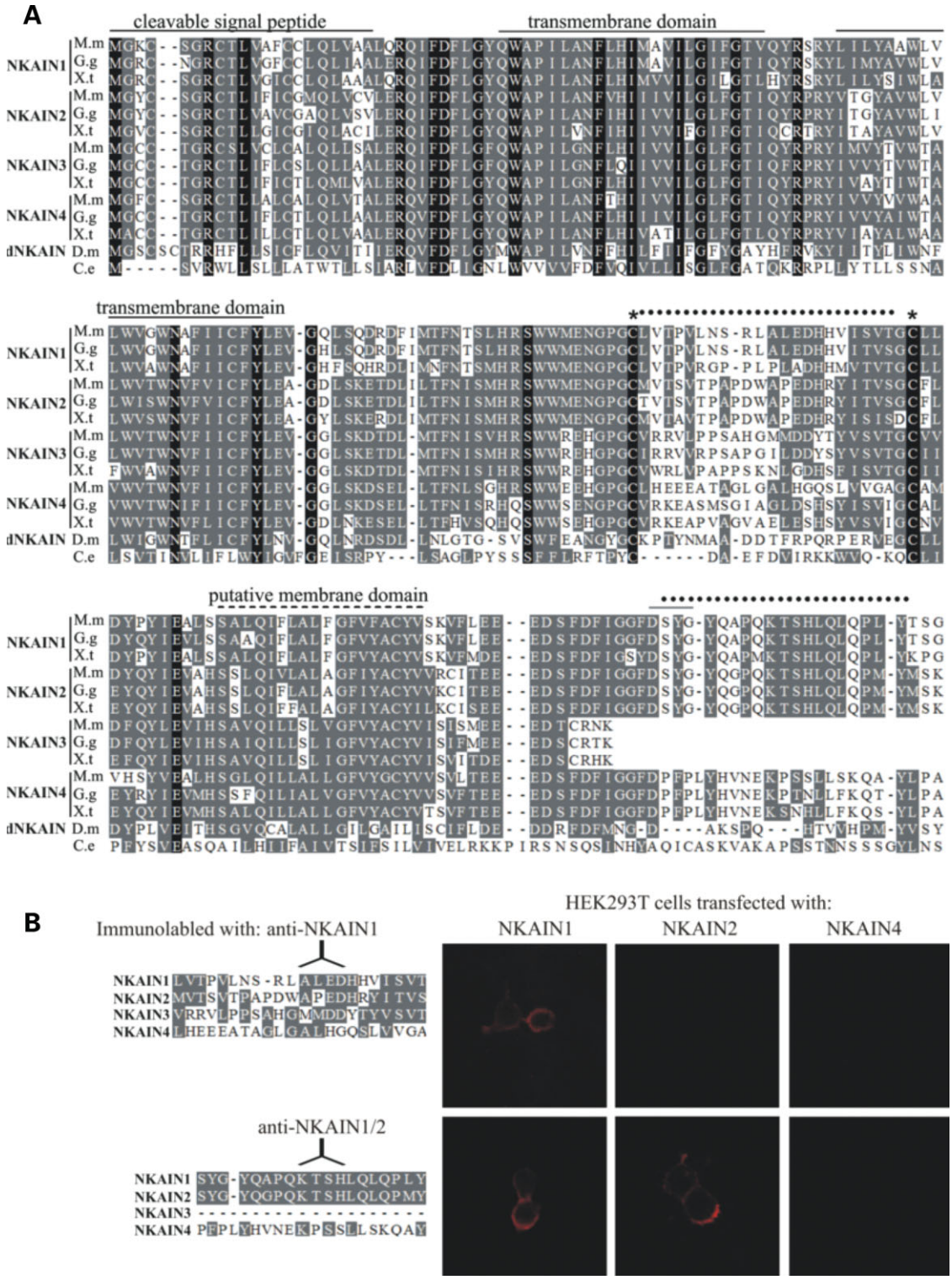

Figure 2. Multiple alignment of the NKAIN protein family. (**A**) NKAIN proteins from *Mus musculus* (M.m), *G. galus* (G.g), *X. tropicalis* (X.t), *D. melanogaster* (D.m) and *C. elegans* (C.e) are included in the multiple protein alignment. The following mouse NKAIN proteins were used in the alignment: NKAIN1 (EF058048), NKAIN2 variant 1 (EF058049), NKAIN3 variant 1 (EF058052), NKAIN4 variant 1 (EF058055). For dNKAIN (EF058058) and *C. elegans* NKAIN ortholog T13H5.6 (NP_495801), the first 200aa and 199aa, respectively, are included in the alignment. Putative transmembrane domains are labeled with solid lines and the putative re-entrant membrane domain is marked with a dashed line. Regions that were used for peptide antibody production are labeled with dotted lines. Conserved cysteine residues are marked with asterisks. The DSYG peptide, identical to the motif present in M7–M8 loop of the Na,K-ATPase α subunit, is labeled with a gray line. (**B**) Anti-NKAIN1 antibody labels cell membranes of HEK293T cells transfected with NKAIN1, but not NKAIN2 or NKAIN4 constructs. Anti-NKAIN1/2 antibody detects both NKAIN1 and NKAIN2. No staining was observed on untransfected cells or when pre-immune serum was used for the immunohistochemistry.

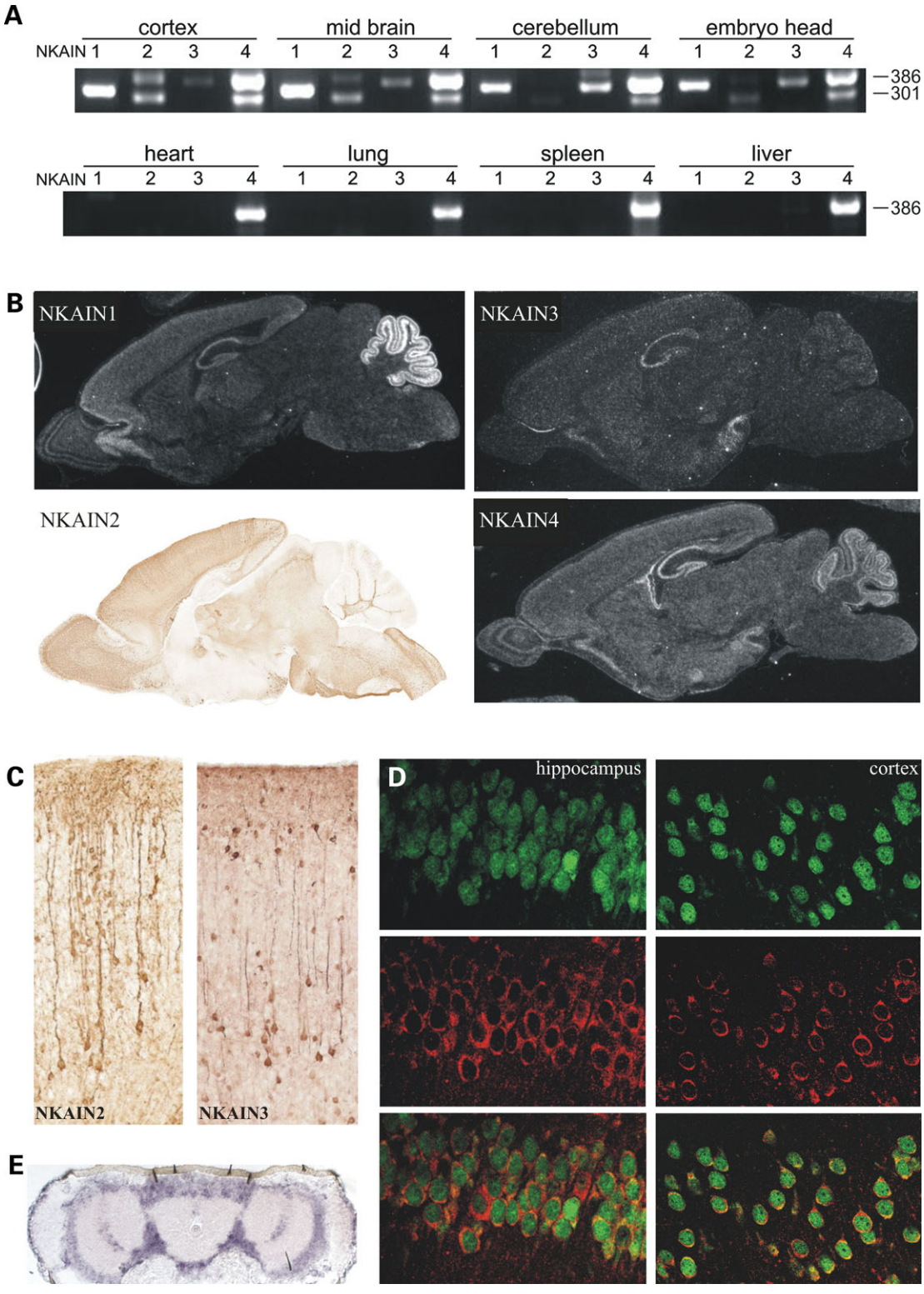

Figure 3. NKAINs are expressed in brain, specifically in neurons. (**A**) RT–PCR using primers located in the fourth exon and 3′-UTR was performed on RNA from different tissues. *NKAIN1*, *2*, *3* and *NKAIN4* splice variant (*NKAIN4v.2*) are expressed in several brain regions but not in other tissues. Splice form *NKAIN4v.1* is expressed ubiquitously. Multiple bands corresponding to *NKAIN2* and *4* are splice variants *NKAIN2.1*, *NKAIN2v.2* and *NKAIN4v.1*, *NKAIN4v.2*. (**B**) Mouse *NKAIN1*, *3* and *4* mRNA *in situ* hybridization expression data obtained from BGEM database. Mouse NKAIN2 expression data are from GENSAT database. (**C**) Expression of NKAIN2 and NKAIN3 in mouse cerebral cortex obtained from GENSAT database. (**D**) NKAIN1 and NKAIN2 are specific to neurons. NKAIN1 and 2 labeling with anti-NKAIN1/2 antibody (red) is present on the cells positive for the neuron-specific marker NeuN (green). A section of cerebral cortex and of hippocampus are shown. Similar staining was observed in the other areas of mouse brain. (**E**) Expression of *dNKAIN* mRNA by *in situ* hybridization in *Drosophila* adult brains. Level of *dNKAIN* expression is higher in some cells (arrowheads).

Using *in situ* hybridization, we show that *dNKAIN* is expressed in most regions of adult *Drosophila* brain (Fig. 3E). The expression is not uniform, as some cells seem to have a much higher level of *dNKAIN* expression (Fig. 3E, arrowheads). Adult *Drosophila* brain sections were not labeled when a sense probe was used as a control (data not shown). Taken together, these results indicate that NKAIN family members localize to neurons and have distinct and overlapping expression profiles.

C-terminal domain of NKAIN proteins interacts with the β1 subunit of the Na,K-ATPase

As NKAINs neither resemble any proteins with known function nor contain any characterized domains, their function cannot be predicted based on the protein sequence alone. In order to find proteins interacting with NKAIN family members, we screened a mouse brain cDNA library using the C-terminal tail of NKAIN1 as a bait in the yeast-two-hybrid system (Fig. 4A). Of 170 000 screened transformants, six independent interacting clones corresponded to the Na,K-ATPase β1 subunit. All six clones encoded different C-terminal fragments of β1 subunit, starting from positions 181, 189, 190, 193, 194 and 249 (Fig. 4B). The shortest interacting clone of β1 subunit consisted of the 56 most C-terminal amino acids of this 304 amino acid protein. The interacting constructs were then re-tested by co-transformation with NKAIN1 bait in yeast cells. We then questioned whether other NKAIN family members also interacted with the β1 subunit of the Na,K-ATPase. Using the C-terminal domains of NKAIN2v.1 or NKAIN4v.1 together with β1 constructs in the yeast-two-hybrid assay, we found that these spice variants of NKAIN2 and NKAIN4 interacted with the β1 subunit. NKAIN3 and the alternatively spliced form of NKAIN4v.2 were not tested as they do not contain the C-terminal bait region.

To confirm the results of the yeast-two-hybrid screen, we expressed full-length β1 subunit and full-length flag-tagged NKAIN1 in HEK293T cells. Anti-flag antibody was used to immunoprecipitate NKAIN1–β1 complexes. As shown in Figure 4C, β1 subunit co-immunoprecipitated with NKAIN1. No immunoprecipitation was observed with anti-flag antibody when untagged NKAIN1 was co-expressed with the β1 subunit. In addition, β1 did not immunoprecipitate when anti-glutamate receptor δ2 antibody was used for immunoprecipitation as a control antibody (Fig. 4C).

As NKAIN proteins are evolutionarily conserved, we tested if NKAIN *Drosophila* ortholog, dNKAIN, would interact with Nrv2.2 protein, one of the *Drosophila* homologs of the Na,K-ATPase β subunit. HEK293T cells were co-transfected with Nrv2.2 and flag-tagged dNKAIN. Immunoprecipitation with anti-flag antibody pulled down Nrv2.2 protein along with flag-tagged dNKAIN (Fig. 4D). When Nrv2.2 was transfected together with untagged dNKAIN, no Nrv2.2 was present in the immunoprecipitate fraction. These results demonstrate that mouse NKAIN1 protein interacts with the β1 subunit of the Na,K-ATPase, whereas *Drosophila* dNKAIN interacts with Nrv2.2, a *Drosophila* homolog of the Na,K-ATPase.

MONaKA, NKAIN1 and the Na,K-ATPase β1 subunit form a complex in transfected cells

In contrast to mammalian NKAINs, which contain 181–233 amino acids, *Drosophila* ortholog dNKAIN is 658 amino acid long. Only the N-terminal 200 amino acids of dNKAIN proteins are highly homologous to mammalian NKAIN family members. The existence of an additional 458aa C-terminal domain in dNKAIN protein sequence raised a possibility that the function of this region is performed by another protein in vertebrates. We noted that MONaKA, a recently discovered mammalian protein that binds to the β1 subunit of the Na,K-ATPase (19), contained regions that shared up to 33% amino acid identity with dNKAIN when aligned using an algorithm DIALIGN, designed for finding amino acid similarity between distantly related proteins (20) (Supplementary Material, Fig. S1). Similar to the C-terminal part of dNKAIN, MONaKA is also proline-rich (19). However, amino acid identity between dNKAIN and MONaKA is mostly due to amino acids other than proline.

On the basis of our yeast-two-hybrid results, NKAIN1-interacting domain lies within the 56 most C-terminal amino acids of the β1 subunit of the Na,K-ATPase, whereas the MONaKA-interacting region is located in the central part of the β subunit (Fig. 5A) (19). Thus, it is possible that the β1 subunit interacts and forms a complex with MONaKA and NKAIN1 using domains located in distinct regions of the ectodomain. To test this hypothesis, we transfected HEK293T cells with myc-tagged MONaKA, NKAIN1 and the β1 subunit of the Na,K-ATPase. Using anti-myc antibody, both β1 and NKAIN1 were immunoprecipitated with MONaKA (Fig. 5B). When MONaKA and NKAIN1 were transfected without β1, almost no NKAIN1 was detected in the immunoprecipitate, suggesting that β1 is needed to form a stable complex between MONaKA, NKAIN1 and β1. It also seems that MONaKA-myc precipitates with a lower efficiency in the absence of β1. This effect was seen in several repeated experiments. No β1 or NKAIN1 were immunoprecipitated by anti-myc antibody in the absence of MONaKA-myc. These results demonstrate that NKAIN1, MONaKA and β1 form a complex in transfected cells. As MONaKA shares amino acid similarity with the C-terminal tail of dNKAIN, we propose that MONaKA and mouse NKAIN1 bind the β1 subunit of the Na,K-ATPase together to form a complex functionally equivalent to the one formed by *Drosophila* ortholog dNKAIN and Nrv2.2 (Fig. 5C).

Drosophila mutants expressing lower level of dNKAIN are temperature-sensitive

In order to determine the function of NKAIN proteins, we analyzed the *Drosophila* mutant *EP(2)0560* (21), which contains a P-element inserted in the first exon of *dNKAIN* gene. After outcrossing *EP(2)0560* flies to *yw* wild-type flies to eliminate an unrelated lethal mutation present in the background strain, we analyzed homozygous $dNKAIN^{EP560}$ flies. At room temperature, $dNKAIN^{EP560}$ homozygous flies exhibited increased wing beating and excessive grooming compared with wild-type flies. When placed at 38°C, homozygous $dNKAIN^{EP60}$ flies became severely uncoordinated, exhibited seizure-like

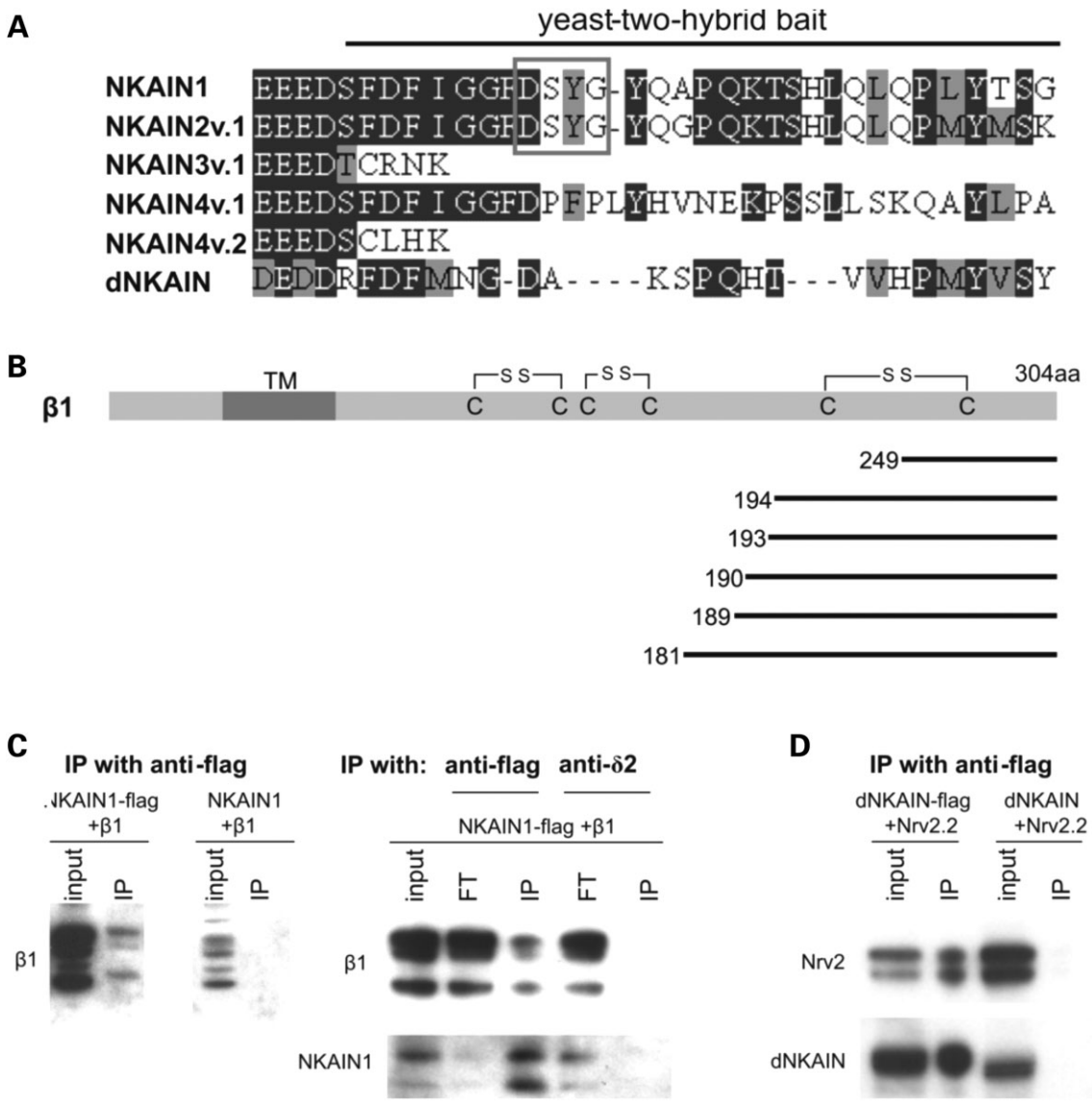

Figure 4. NKAIN1 interacts with the Na,K-ATPase β1 subunit. (**A**) Multiple alignment of the C-terminal regions of mouse NKAINs. The C-terminal tail (177–207aa) of NKAIN1, used as a bait to screen a mouse brain library, is shown. DSYG peptide, identical to the motif present in M7–M8 loop of the Na,K-ATPase α subunit, is boxed. (**B**) Six independent clones interacting with NKAIN1 bait contained C-terminal fragments of the β1 subunit of the Na,K-ATPase. The C-terminal β1 truncations identified in the screen are shown with amino acid positions marked. The shortest interacting construct contained the last 56 amino acids of the β1 subunit. Three cysteine bridges are shown. (**C**) When the β1 subunit and flag-tagged NKAIN1 are expressed in HEK293T cells, β1 can be immunoprecipitated with anti-flag antibody. No immunoprecipitation was observed when non-tagged NKAIN1 is expressed with β1 in transfected cells. β1 immunoprecipitated together with NKAIN1 flag when anti-flag antibody was used for the immunoprecipitation, but not when anti-glutamate receptor δ2 antibody is used as control. Cell extracts, antibody coupling and washing procedures were identical for both antibodies. (**D**) dNKAIN co-immunoprecipitates with Nrv2.2. HEK293T cells were co-transfected with Nrv2.2 and either dNKAIN or flag-tagged dNKAIN. Anti-flag antibody was used for immunoprecipitation and anti-Nrv as well as anti-dNKAIN for western blotting. Nrv2.2–dNKAIN interaction is specific, as Nrv2.2 immunoprecipitates with the flag-tagged dNKAIN but not with the untagged dNKAIN. FT, flowthrough; IP, immunoprecipitate.

movements and became paralyzed within 10 min (Fig. 6A). In contrast, *dNKAIN*^{0EP560}*/CyO* or wild-type flies did not show such changes in behavior. When shifted back to room temperature, homozygous flies return to normal within 10–15 min.

To investigate if the temperature-sensitive phenotype was due to the P-element insertion, we crossed *dNKAIN*^{EP560} flies to *Df(2R)ED4071*, a mutant strain with a deletion of the chromosomal region containing the *dNKAIN* gene (22). *dNKAIN*^{EP560}*/Df(2R)ED4071* flies were not viable, however there was a very small number of escapers that died shortly after eclosion. These escapers exhibited temperature-sensitive paralysis when tested at 38°C. Flies carrying the deficiency chromosome *Df(2R)ED4071* and *dNKAIN*-null allele generated by imprecise excision of the P-element (*dNKAIN*^{EP560rv9}) were not viable. Thus, complete deletion of *dNKAIN* leads to

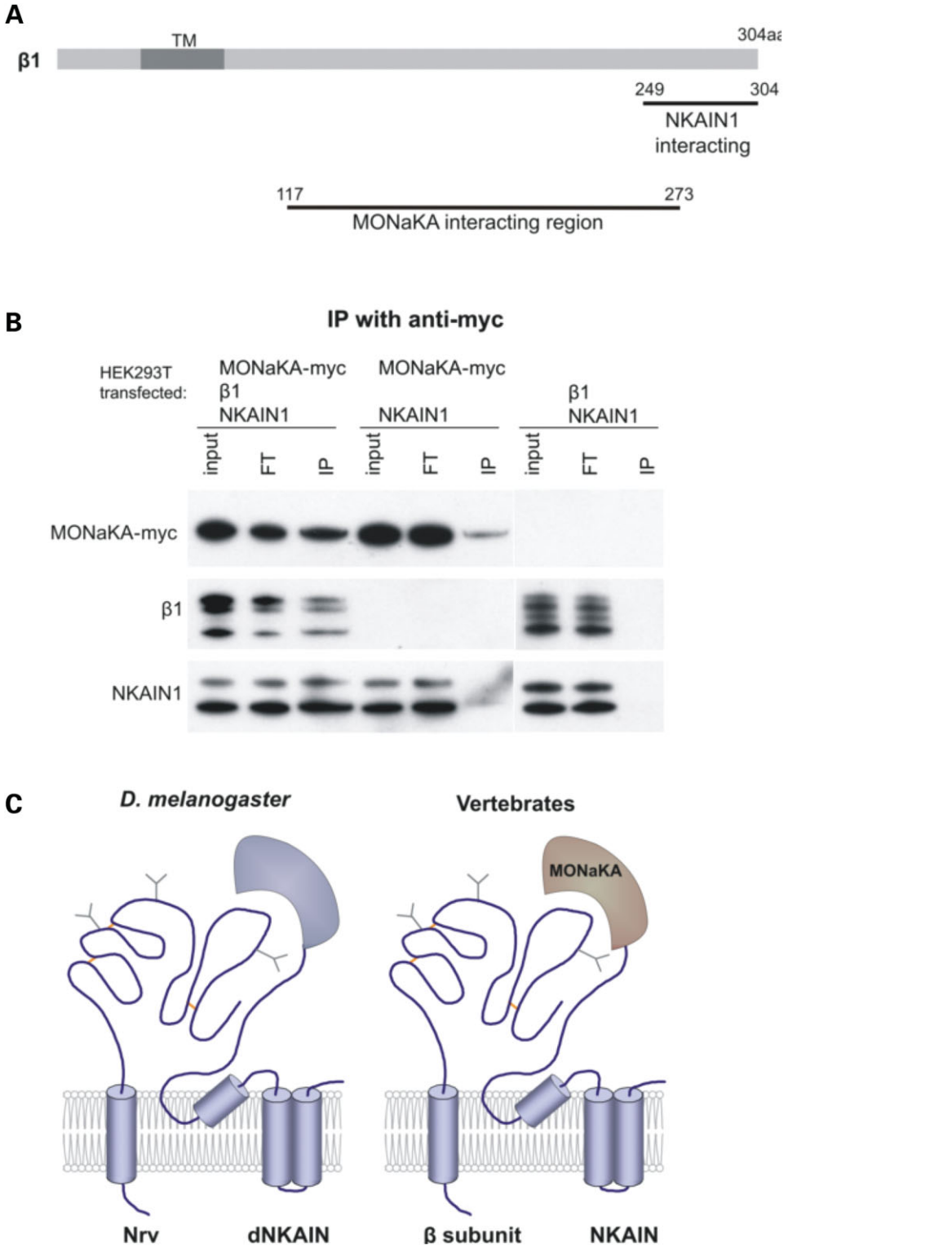

Figure 5. MONaKA, NKAIN1 and the Na,K-ATPase β1 subunit form a complex. (**A**) Regions of the β subunit containing the NKAIN and MONaKA interacting domains only partially overlap. Amino acid positions of the interacting domains are marked. The numbering corresponds to mouse β1 subunit. (**B**) MONaKA, NKAIN1 and the β1 subunit form a complex in transfected cells. HEK293T cells were transfected with all three constructs, MONaKA-myc + NKAIN1 or β1 subunit + NKAIN1. Anti-myc antibody was used for immunoprecipitation. Both β1 subunit and NKAIN1 precipitate with MONaKA-myc. Small amount of NKAIN1 is co-immunoprecipitated with MONaKA in the absence of β1 subunit. No NKAIN1 or β1 subunit is bound to the beads in the absence of MONaKA-myc. (**C**) Model of the dNKAIN-Nrv complex in *Drosophila* and the equivalent complex in mammals. MONaKA and mammalian NKAIN bind to distinct sites on the β1 subunit of the Na,K-ATPase. We propose that functions of MONaKA and mammalian NKAINs could be performed in *Drosophila* by a single protein that combines both domains—dNKAIN.

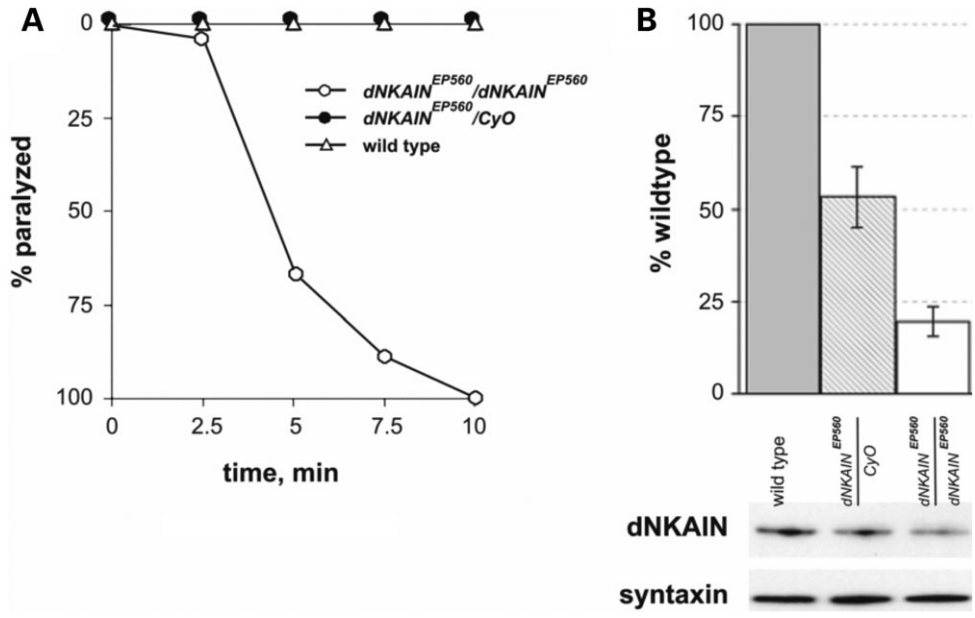

Figure 6. Flies expressing lower level of dNKAIN are temperature-sensitive. (**A**) *dNKAIN*^{EP560} homozygous flies become severely uncoordinated and paralyzed when exposed to 38°C, whereas heterozygous and wild-type flies behave normally. Sixty homozygous flies, 30 heterozygous and 30 wild-type flies were used for this experiment. (**B**) *dNKAIN*^{EP560} homozygous flies express lower level of dNKAIN when compared with wild-type control flies. Anti-dNKAIN and anti-syntaxin antibodies were used for the western blot on *Drosophila* head extracts. The intensity of the dNKAIN bands, scanned with Typhoon phosphoimager, was normalized against the intensity of the loading control syntaxin bands. A representative western blot and the average of three experiments are shown.

lethality. However, when a precise P-element excision line *dNKAIN*^{EP560rv2} was crossed to *Df(2R)ED4071*, heterozygous flies were viable and not temperature-sensitive. The results of these experiments demonstrate that the temperature-sensitive phenotype of *dNKAIN*^{EP560} flies is due to the disruption of *dNKAIN* gene by a P-element insertion.

As the P-element insertion does not disrupt the open reading frame of the *dNKAIN* gene, we used anti-dNKAIN antibody to determine if expression of dNKAIN was affected in *dNKAIN*^{EP560} flies. As shown in Figure 6B, the intensity of dNKAIN band in *Drosophila* head extracts from flies homozygous for P-element insertion is only 19% of wild-type dNKAIN band intensity. dNKAIN expression from heterozygous animals is decreased by 47%, when compared to wild-type. Antibody specific to *Drosophila* syntaxin was used to control for loading. The average of three independent experiments and a representative Western blot are shown in Figure 6B. These experiments demonstrate that insertion of a P-element in the first exon of *dNKAIN* gene decreases the expression of dNKAIN protein. Thus, decrease in dNKAIN expression leads to temperature-sensitive paralysis, a *Drosophila* phenotype often caused by mutations in ion channels or proteins affecting ion channel function (13–16).

Expression of dNKAIN, but not mouse NKAIN1, induces a Na^+ conductance in *Xenopus* oocytes

The temperature-sensitive phenotype of *Drosophila* mutants, high evolutionary conservation of NKAIN transmembrane domains and interaction between NKAINs and β subunits of the Na,K-ATPase prompted us to test the functional properties of NKAIN proteins by electrophysiology in *Xenopus* oocytes. We first tested if NKAIN1 or dNKAIN affected mammalian or *Drosophila* Na,K-ATPase function, respectively. Owing to the low expression level of the Na,K-ATPase subunits in the presence of NKAINs, detected by ouabain binding and electrophysiological recordings (data not shown), it was not possible to reliably measure the transport properties, e.g. the apparent K^+ or Na^+ affinity of the Na,K-ATPase. We then tested if the expression of dNKAIN or NKAIN1 would affect ion conductance of *Xenopus* oocyte membranes. Oocytes were injected with either NKAIN1 cRNA, dNKAIN cRNA or water. Small currents induced by raising the extracellular concentration of chloride from 26.8 to 106.8 mM, potassium from 3 to 15 mM or sodium from 20 to 80 mM were recorded over a potential range of +30 to −130 mV. In these experiments, the endogenous oocyte Na,K-ATPase was inhibited by the presence of 1 μM ouabain. NKAIN1-expressing oocytes and control-water-injected oocytes displayed identical small currents (Fig. 7A–C). Surprisingly, expression of dNKAIN resulted in an increase in Na^+-induced conductance (Fig. 7F), whereas Cl^- and K^+ conductances were unaffected (Fig. 7D and E). dNKAIN-induced conductances showed no or little voltage dependence. Over a large range of membrane potentials, dose–response curves of extracellular Na^+ revealed non-saturable currents in dNKAIN-expressing oocytes between 10 and 90 mM Na^+ (Fig. 8A and B) consistent with a channel-like activity. Lanthanum (1 mM), an ion channel blocker, completely abolished dNKAIN-mediated currents at

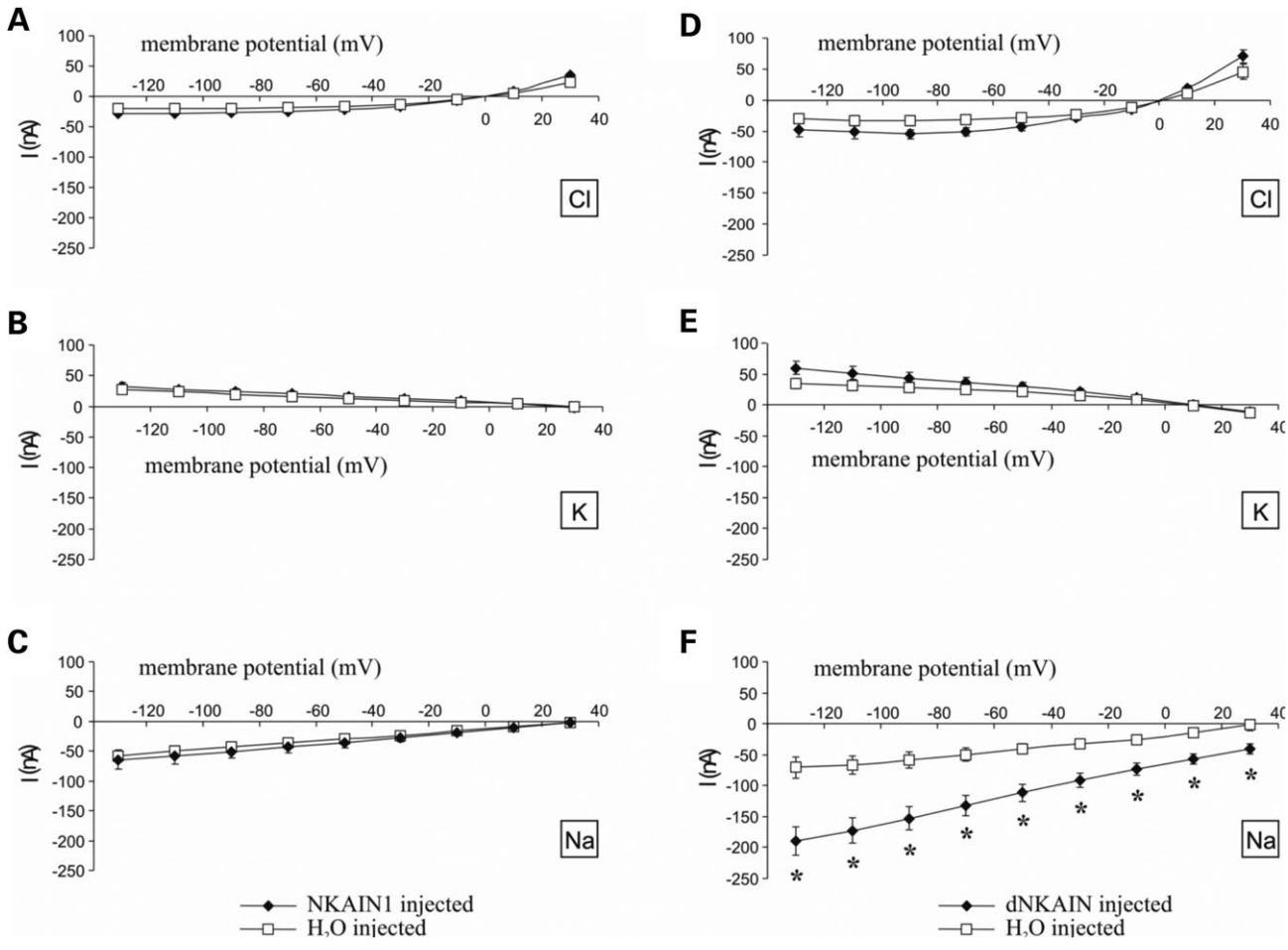

Figure 7. dNKAIN but not mouse NKAIN1 expression induces Na^+-specific conductance in *Xenopus* oocytes. One day after injection of NKAIN1 (5 ng) (**A**–**C**) or dNKAIN (5 ng) (**D**–**F**) cRNA, the current–voltage relationship was determined by patch clamp techniques by decreasing the concentration of chloride from 106.8 to 26.8 mM (A, D), by increasing the concentration of potassium from 3 to 15 mM (B, E), and by decreasing the concentration of sodium from 80 to 20 mM (C, F). Also shown are means ± SE of 20 oocytes from three different batches. (F) $P < 0.001$, dNKAIN versus H_2O at −130 to +30 mV.

negative potentials (Fig. 8C and D). dNKAIN-induced Na^+ conductance was insensitive to amiloride, a blocker of epithelial Na^+ channels (ENaC) (Fig. 8E and F). Taken together, these results indicate that dNKAIN, but not mouse NKAIN1, can induce a small but significant amiloride-insensitive Na^+ conductance when expressed in *Xenopus* oocytes.

DISCUSSION

We present cloning and characterization of a novel family of four mammalian proteins NKAIN1, 2, 3 and 4. *NKAIN2* was previously named *TCBA1* (T-cell lymphoma breakpoint-associated target 1), because it contains a chromosomal translocation site in two T-cell lymphoma/leukemia cell-lines, but no function was proposed for this protein (23). A single ortholog of the NKAIN proteins (dNKAIN) is present in the *Drosophila* genome. *NKAIN* genes were *in silico* cloned as part of a bioinformatics screen for novel brain-specific genes and were selected for further biochemical analysis based on novelty, membrane localization, neuron specificity and striking evolutionary conservation of NKAIN proteins. Remarkable amino acid conservation of the first two transmembrane domains suggested that NKAIN proteins might function within membrane bilayer.

Our results demonstrate that mouse NKAIN1 interacts with the β1 subunit of the Na,K-ATPase, whereas the *Drosophila* ortholog, dNKAIN, associates with Nrv2.2, a *Drosophila* homolog of the Na,K-ATPase β subunit. On the basis of the results of a yeast-two-hybrid screen, the most C-terminal 56 amino acids of the β1 subunit are sufficient for interaction with NKAIN1. Even though several regions of the β subunit have been implicated in the interaction with the α subunit (4,24–26), most studies suggest that the region following the transmembrane domain of the β subunit is the major region contributing to α–β interaction (27–30). Thus, NKAIN1 and the α subunit interact with the β subunit at distinct sites (Fig. 9). The region of the α subunit responsible for interaction with β subunits is more clearly defined: it consists of 26 amino acids in the extracellular loop between M7 and M8 transmembrane domains of the α subunit, with the highly conserved amino acids DSYG (893–896) playing a

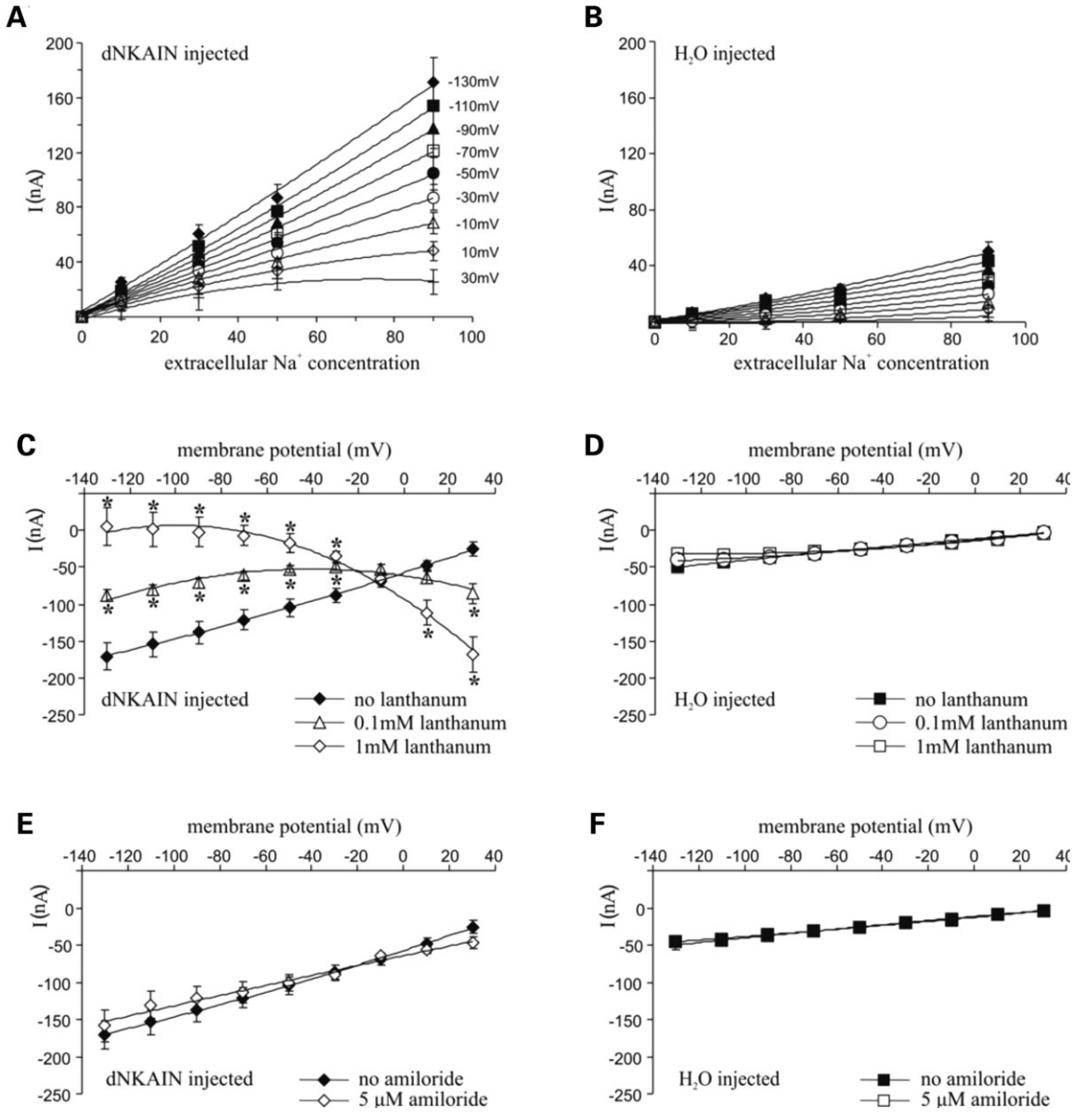

Figure 8. Effect of extracellular Na^+ and lanthanum on dNKAIN-mediated current. One day after injection of dNKAIN (5 ng) cRNA or H_2O, current was recorded at indicated membrane potentials using two-electrode voltage clamp techniques by raising the concentration of extracellular Na^+ from 0 to 90 mM. The endogenous oocyte Na,K-ATPase was inhibited by the presence of 1 μM ouabain. (**A**) Dose–response curves of extracellular Na^+-dependent current in dNKAIN-expressing oocytes. (**B**) Dose–response curves of extracellular Na^+-dependent current in H_2O-injected oocytes. Also shown are means ± SE of 10 oocytes from two batches. One day after injection of dNKAIN (5 ng) cRNA or H_2O, the current was measured by two-electrode voltage clamp techniques using extracellular Na^+ concentration of 90 mM with or without pharmacological agents. Means ± SE of 15 oocytes from two different batches are shown. (**C**) Currents from dNKAIN-injected oocytes with or without 0.1 or 1 mM lanthanum. $^*P < 0.04$, lanthanum-treated versus untreated. (**D**) Currents from H_2O-injected oocytes with or without 0.1 or 1 mM lanthanum. (**E**) Currents from dNKAIN-injected oocytes with or without 5 μM amiloride. (**F**) Currents from H_2O-injected oocytes with or without 5 μM amiloride.

critical role in subunit assembly (27,31,32). Interestingly, the same DSYG peptide is present in the C-terminal β-interacting domain of NKAIN1 and 2. It is unlikely that the DSYG motif itself is responsible for NKAIN-β interaction, as NKAIN4, which lacks it, still interacts with β1 subunit. A model of the NKAIN–Na,K-ATPase complex is shown in Figure 9.

The N-terminal part of the *Drosophila* ortholog, dNKAIN, is highly homologous to mammalian NKAINs, whereas the C-terminal part contains regions of amino acid similarity to another β subunit binding protein, MONaKA (19). On the basis of our yeast-two-hybrid experiments, NKAIN1 and MONaKA are likely to interact with the β1 subunit at distinct sites, because the 156-amino-acid-long MONaKA-interacting domain of the β1 subunit only partially overlaps with the 56 amino acid NKAIN1-interacting region. We also demonstrate that both mouse NKAIN1 and MONaKA interact with the extracellular domain of the β1 subunit of the Na,K-ATPase and form a complex with the β1 subunit in transfected cells.

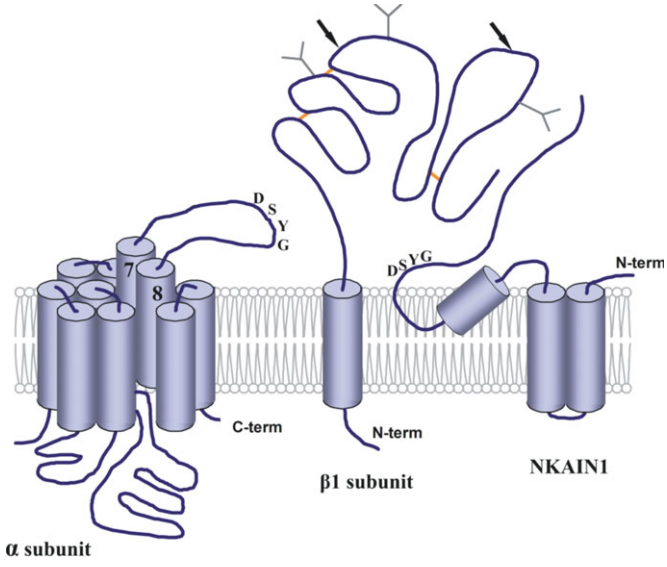

Figure 9. Model of the Na,K-ATPase and NKAIN complex. C-terminal region of NKAINs interacts with the C-terminal domain of the Na,K-ATPase β subunit. The longest and the shortest NKAIN1 interacting C-terminal fragments of the β1 subunit identified in the yeast-two-hybrid screen are marked by arrows. M7–M8 loop of the α subunit, specifically DSYG peptide, interacts with the extracellular domain of the β subunit. The same amino acid sequence DSYG is present in the C-terminal domains of NKAIN1 and NKAIN2.

On the basis of these results, we propose that dNKAIN function is performed by two separate proteins in mammals—mammalian NKAIN1 and MONaKA (Fig. 5C). There are many examples of a function carried out by two proteins containing separate functional domains in some species, whereas an equivalent function in another organism belongs to a protein that has both these domains fused. For example, two structural domains of an eukaryotic enzyme imidazole glycerophosphate synthase are encoded by two separate genes in archea (33).

Expression of dNKAIN in *Xenopus* oocytes induced a voltage-independent Na^+-specific current that was blocked by lanthanum and unaffected by amiloride. This conductance was independent of the Na,K-ATPase function, as recordings were done in the presence of ouabain at concentrations that block endogenous oocyte Na,K-ATPase. Remarkably, mouse NKAIN1 did not induce any conductance in oocytes. As dNKAIN contains a C-terminal domain that is absent from mouse NKAIN1, it is possible that both NKAIN1-homologus N-terminal domain and MONaKA-related C-terminal domain of dNKAIN are needed for generation of the observed Na^+ conductance. Testing of whether the expression of NKAIN1 and MONaKA protein fusion in oocytes would induce a current similar to dNKAIN-induced current would confirm this hypothesis.

High levels of several heterologously expressed structurally unrelated membrane proteins can induce chloride currents and hyperpolarization-activated cation-selective currents in *Xenopus* oocytes (34). Small integral proteins IsK, FXYD1, SYN-C and MB of influenza B virus have been shown to induce hyperpolarization-activated chloride currents, presumably by activating endogenous chloride channels (35,36). In addition, heterologous expression of FXYD2 in *Xenopus* oocytes was shown to induce endogenous Ca^{2+} and voltage-gated large-diameter non-selective pores (37). It is unlikely that dNKAIN-induced conductance represents these currents, because expression of dNKAIN in *Xenopus* oocytes induces voltage-independent Na^+-selective conductance. Furthermore, the amount of *dNKAIN* cRNA injected in our experiments was small compared with the amount necessary for the induction of hyperpolarization-induced currents in *Xenopus* oocytes. However, we cannot rule out a possibility that dNKAIN expression influences uncharacterized endogenous Na^+ channels leading to a change in Na^+ conductance upon dNKAIN injection. Further experiments, such as the reconstitution of purified dNKAIN in lipid bilayers, are needed to determine if NKAINs can independently induce Na^+ currents.

It has been previously suggested that Na^+ ion influx activates Na,K-ATPase (38). Recently, a direct interaction between Na_x, a Na^+ channel that opens in response to an increase in extracellular Na^+ concentration, and the α subunit of the Na,K-ATPase was demonstrated in glial cells (39). It was proposed that Na_x activates Na,K-ATPase by direct interaction as well as by Na^+ influx through Na_x channel, leading to stimulation of anaerobic metabolism (39). As dNKAIN induces Na^+ conductance in *Xenopus* oocytes and interacts with the β subunit of the Na,K-ATPase, a similar functional mechanism is possible for NKAIN proteins. Alternatively, NKAINs could function as subunits of a channel or a transporter coupled to the electrochemical gradient created by Na,K-ATPase and brought to Na,K-ATPase proximity by NKAIN-β interaction. This process would be similar to coupling of K–Cl transporter KCC2 with the Na,K-ATPase α2 subunit (40) or Na–Ca exchanger with α1 and α2 subunits (41).

In order to determine the function of NKAIN proteins *in vivo*, we analyzed *Drosophila* mutants with decreased expression of dNKAIN protein due to a P-element insertion in the *dNKAIN* gene. These mutants had a pronounced temperature-sensitive phenotype, with marked uncoordination and paralysis upon exposure to 38°C. Two lines of evidence confirm that the temperature-sensitive phenotype present in these flies is due to P-element insertion in *dNKAIN* gene. First, when the P-element is excised, flies are no longer temperature-sensitive. Second, flies heterozygous for P-element insertion and a deficiency chromosome *Df(2R)ED4071* that deletes *dNKAIN* are also temperature-sensitive. It is unlikely that P-element insertion affects a downstream putative sodium channel gene *DSC1*, as mutations and deletions in *DSC1* gene do not cause temperature-sensitive paralysis (42,43). A number of *Drosophila* mutants with temperature-sensitive phenotype have changes in cell excitability. In fact, two seemingly opposite processes lead to similar temperature-sensitive phenotypes. First, increased neuronal excitability upon high temperature exposure causes seizures followed by paralysis. For example, mutations in the Na,K-ATPase α subunit ($ATPalpha^{DTS}$) and two different K^+ channels (*sei* and *slo*) cause membrane hyperexcitability and temperature-sensitive paralysis (10,15,44,45). Second, decreases in sodium conductance leads to neuronal hypoexcitability, affecting propagation of action potentials and thus causing paralysis of flies (16).

Indeed, mutations in *Drosophila* Na^+ channel (*para*) as well as mutations that decrease Na^+ channel activity by affecting channel expression or localization (*nap*^{ts}, *tipE*, *khc* or *axo*) cause temperature-sensitive phenotypes (13,14,46–49). It is possible that both these processes at least partially contribute to temperature-sensitivity in *dNKAIN* mutants, because dNKAIN protein interacts with a component of the Na,K-ATPase and also induces Na^+ conductance in *Xenopus* oocytes. Electrophysiological recordings from *dNKAIN* mutants might help to determine if the temperature-sensitive phenotype of these flies is caused by neuronal hyperexcitability.

The correlation between neuronal excitability and seizure phenotype in humans is also complex. For example, persistent sodium currents due to abnormal channel inactivation caused by mutations in Na^+ channel genes *SNC1A*, *SNC1B* or *SNC2A* lead to neuronal hyperexcitability and epilepsy with febrile and non-febrile seizures (50). However, loss-of-function mutations in Na^+ channels and loss of sodium currents can also cause epilepsy, most likely due to decreased function of inhibitory neurons (51–53). Seizures in patients with mutations in Na^+ channels are often precipitated by fever, suggesting an underlying mechanism similar to the one causing temperature sensitivity in *Drosophila* mutants. Similarly, symptoms of Rapid-onset Dystonia Parkinsonism, caused by mutations in the α3 subunit of the Na,K-ATPase, often start after fever or exposure to heat as well as other physiologic or psychologic stressors (54).

Two unrelated patients with NKAIN2 truncations have been described as having severe neurological impairment including febrile and afebrile seizures, difficulties in motor skills and expressive speech, muscle weakness, psychomotor retardation as well as other clinical symptoms (55,56). In these patients, NKAIN2 protein is truncated after the second transmembrane domain by a chromosomal translocation in *NKAIN2* gene. No other genes were disrupted by the chromosomal translocations, suggesting that the clinical presentation of these patients is due to *NKAIN2* truncation (55,56). On the basis of our findings, the deleterious effect of truncated NKAIN2 on neuronal function could be explained by either abnormal Na^+ conductance or changes in the Na,K-ATPase function.

In summary, we describe a novel family of membrane proteins (NKAINs) that localize to neurons in mammalian brain. Both mammalian and *Drosophila* NKAINs interact with the β subunits of Na,K-ATPase. *Drosophila* NKAIN induces small but significant Na^+ currents when expressed in *Xenopus* oocytes. dNKAIN *Drosophila* mutants exhibit temperature-sensitive paralysis. Temperature-sensitive phenotype and severe neurological impairment of patients with NKAIN2 truncations could, in part, be explained by abnormal Na^+ conductance or disrupted Na,K-ATPase function.

MATERIALS AND METHODS

Cloning and multiple alignment

Initial sequence information for mouse *NKAIN* genes was obtained from extensive EST sequence data available in GenBank (205 ESTs and full-length mRNAs for *NKAIN1*, 117 for *NKAIN2*, 104 for *NKAIN3* and 56 for *NKAIN4*). Primers were then designed to confirm the sequence of *NKAIN* family members by RT–PCR and sequencing. If a sequence variation in an EST was observed, primers were designed to determine if this variant represents a true splice form, or a sequencing error. Primers in the corresponding regions were then designed for other family members to determine if other NKAIN genes also have similarly spliced mRNA variants. Any additional bands observed on RT–PCR gel were excised and sequenced to determine if they represent additional splice forms. Primers used for RT–PCR and sequencing, accession numbers, chromosomal locations as well as exon–intron structure of mouse *NKAIN* genes are shown in Figure 1.

Drosophila ortholog of mammalian *NKAIN* genes (*dNKAIN*) corresponded to a predicted *Drosophila* gene *CG9047*. There is one full-length mRNA partially identical to *CG9047* present in GenBank (AY051438). Coding region sequence of *dNKAIN* mRNA was confirmed by RT–PCR and sequencing using the following primers: 5′-GACGGG GAGAGTGGATTTATT, 5′-TATACCCACAACATCCGCA, 5′-CGCTTGCATGGGAAGCTATGG. Single variant of the protein-coding region of *dNKAIN* was detected. Except for three amino acid difference (amino acids 94–96 of CG9047 are deleted in dNKAIN), dNKAIN is identical to the predicted protein CG9047.

For multiple alignment of NKAIN proteins, *Xenopus tropicalis* and *Galus galus* NKAIN protein sequences were determined from ESTs and predictions from genomic DNA using GeneScan program (57). *C. elegans* NKAIN ortholog corresponds to a hypothetical protein T13H5.6 (NP_495801). NKAIN protein family corresponds to Protein Family database Pfam entry DUF798, a family of proteins of unknown function (58).

RT–PCR and NKAIN expression

RNA was isolated from nine different mouse tissues as well as six brain regions using Trizol® reagent (Life Technologies, Inc., Gaithersburg, MD, USA). Reverse transcription was performed using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and the following primers were used for PCR: NKAIN1 5′-CCTGGTGAC TCCTGTTCTGAA and 5′-CCAACCCTGAAGTCCTGCTA; NKAIN2 5′-GTGACACCTGCCCCAGACT and 5′-AGTCCA TGGGCTGACAGACA; NKAIN3 5′-GGAGAGTGCTACCA CCATCAG and 5′-ACACAGGGCTGAGACCAAAG; NKAIN4 5′-CGAGGAGGAGGCCACAGC and 5′-ATGTTAGGCAGG CAGGGAAG. In order to ensure family member specificity, forward primers were designed in the low similarity region in exon 4, and the reverse primers were designed in 3′ untranslated region. Beta-actin primers were used to control for RNA quality.

In situ hybridization data for *NKAIN1, 3* and *4* were obtained from BGEM (http://www.stjudebgem.org), using the following accession numbers: *NKAIN1*—2610200G18Rik, *NKAIN3*—E130310K16Rik, *NKAIN4*—C030019F02Rik (17). Expression data for *NKAIN2* and *3* BAC-transgenic mice were obtained from GENSAT (http://www.gensat.org), using the same accession numbers (18).

cDNA constructs

Full-length cDNAs were amplified by RT–PCR from mouse or *Drosophila* RNA using the SuperScript First-Strand Synthesis System (Invitrogen) and Platinum *Pfx* DNA polymerase (Invitrogen) according to the manufacturer's instructions. Mammalian expression vectors pFLAG-CMV5.1 (Sigma, St. Louis, MO, USA) and pcDNA3.1/Myc-His+A (Invitrogen) were used for flag- and myc- tagging, respectively, with tags placed at the C-terminus. pGBKT7 and pACT2 (Clontech, Palo Alto, CA, USA) were used for yeast-two-hybrid screen. pBSK (Stratagene, La Jolla, CA, USA) was used for *in situ* probe generation. pGEMHE vector (kindly provided by Dr D. Gadsby, Rockefeller University, New York, NY, USA) was used for protein expression in *Xenopus* oocytes. Primers used for cloning are shown in Supplementary Material. All constructs were confirmed by sequencing.

Antibody generation

Anti-mouse NKAIN1 polyclonal peptide antibodies were prepared by immunizing rabbits with peptides containing amino acids 116-LVTPVLNSRLALEDHHVISVT-136 (anti-NKAIN1) and 186-SYGYQAPQKTSHLQLQPLY-204 (anti-NKAIN1/2). The peptides were selected using the ANTIGENIC program from EMBOSS (59). Peptide synthesis and rabbit immunization were performed by Invitrogen. Anti-NKAIN1 antibody was affinity-purified using NKAIN1 peptide coupled to cyanogen bromide-activated Sepharose 4B (Sigma) according to the manufacturer's instructions. Polyclonal anti-dNKAIN antibody was made using peptide containing amino acids 183-GDAKSPQHTVVHPMY-197 of dNKAIN protein. Antibody specificity was confirmed by peptide competition assay and western blot on mouse brain extracts and transfected cell extracts.

Immunoprecipitation and western blotting

HEK293T cells were transfected using the standard calcium phosphate method. Extracts of HEK293T-transfected cells were obtained by homogenizing the cells grown in a 60 mm dish in 1 ml of homogenization buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, protease inhibitors (Sigma), pH 7.4). Homogenates were centrifuged for 5 min at 14 000 g at 4°C, and pellets were resuspended in 200 μl of homogenization buffer. To solubilize the membrane proteins, 20 μl of 0.3 M DHPC (Avanti, Alabaster, AL, USA), 60 μl 5% Na deoxycholate and homogenization buffer were added to the final volume of 600 μl (final concentration of 10 mM DHPC, 0.5% Na deoxycholate). Extracts were then rotated at 4°C for 30 min and centrifuged for 10 min at 8000 *g* at 4°C. Supernatants were used for western blotting or immunoprecipitation. Two milligrams of Dynabeads M270 (Invitrogen) were coupled with 10 μl of M2 anti-flag or anti-myc antibodies (Sigma) according to the manufacturer's instructions. The coupled beads were washed four times with phosphate-buffered saline (PBS), pH 7.4, and four times with the homogenization buffer. Two hundred microliters of cell extract were added per 1 mg of beads and rotated at 4°C for 1 h. The beads were then washed five times with washing buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, protease inhibitors (Sigma), 0.5% Na deoxycholate pH 7.4). To elute immunoprecipitated proteins, the beads were boiled for 5 min in the sample loading buffer (Invitrogen). NuPAGE Bis–Tris 4–12% gels and the MOPS or MES running buffers (Invitrogen) were used for western blot. Primary antibodies were used at the following dilutions: HRP-M2 (Sigma) 1:1000, HRP-myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:1000, polyclonal anti-β1 (cat# 06-170, Upstate Biotechnology, Lake Placid, NY, USA) 1:5000, anti-NKAIN1 1:2000, anti-dNKAIN 1:5000, monoclonal anti-Nrv (5F7, Developmental Studies Hybridoma Bank) 1:1000. HRP-coupled secondary anti-rabbit and anti-mouse antibodies (Pierce, Rockford, IL, USA) were used at 1:25 000 dilution. The signal was then revealed using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Boston, MA, USA) or Supersignal Femto Maximum Sensitivity Substrate (Pierce), and photographed using BioMax MR film (KODAK, Rochester, NY). Anti-syntaxin (8C3) and anti-Nrv (5F7) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA, USA.

Immunohistochemistry

Transfected HEK293T cells were fixed in 4% paraformaldehyde (PFA) in PBS at room temperature (RT) for 15 min, washed three times with PBS and incubated for 30 min at RT in blocking solution containing 10% normal goat serum (NGS) in PBS. Cells were then incubated for 1 h at RT with primary antibodies diluted in 10% NGS in PBS at the following concentrations: anti-NKAIN1 1:1000, anti-NKAIN1/2 1:1000, pre-immune serum for anti-NKAIN1 1:1000, pre-immune serum for anti-NKAIN1/2 1:1000. After washing three times with PBS, cells were incubated with goat anti-rabbit Alexa Fluor 568-conjugated secondary antibody (Invitrogen) at 1:1000 dilution in 1% NGS in PBS for 1 h at RT. Cells were then washed three times in PBS and mounted using Gel/Mount (Biomeda Corp., Foster City, CA, USA). Untransfected cells were used as a control.

Mouse brains were perfused with 4% PFA and postfixed in 4% PFA overnight at 4°C. Seventy micrometers of vibratome sections were then incubated for 30 min at RT in blocking solution containing 5% NGS and 0.1% Triton X-100 in PBS. Sections were then incubated with the primary antibodies in blocking solution for 1 h RT at the following dilutions: anti-NKAIN1/2 1:1000, anti-NeuN 1:100, pre-immune serum for anti-NKAIN1/2 1:1000 (Chemicon, Temecula, CA, USA). After washing three times with PBS, sections were incubated with goat anti-rabbit Alexa Fluor 568 or goat anti-mouse Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) at 1:1000 dilution in 1% NGS in PBS for 1 h at RT. Sections were then washed three times in PBS and mounted using Gel/Mount (Biomeda Corp., Foster City, CA, USA). Confocal imaging of the stained cells and mouse brain sections was done on LSM 510 Axioplan (Zeiss).

Yeast-two-hybrid screen

MATCHMAKER Gal4 Two-Hybrid System 3 (Clontech) was used to screen the mouse brain cDNA library ML4008AH (Clontech) for interactions with the C-terminal domain of NKAIN1. The C-terminal part of NKAIN1-encoding amino acids 177–207 was cloned into pGBKT7 vector and co-transformed together with the cDNA library into AH109 yeast strain. The highest stringency selection SD/-Ade/-His/-Leu/-Trp/X-α-Gal was used. After screening approximately 170 000 clones, 72 positive blue colonies were picked and restreaked on SD/-Leu/-Trp/X-α-Gal plates three times to eliminate the clones transformed with multiple library plasmids. Plasmids from the positive clones were isolated and sequenced. True positive clones were then re-transformed along with the bait to confirm the interaction. NKAIN2 and 4 were also cloned into the pGBKT7 vector and tested for the interaction with the β1 subunit of the Na,K-ATPase. Primer sequences used in cloning are listed in the Supplementary Material.

Fly stocks

All fly stocks were maintained on conventional cornmeal-agar-molasses medium under a 12 h light:12 h dark cycle at 18 or 25°C. *EP(2)0560* stock (21) was obtained from Exelixis, South San Francisco, CA, USA. Position of the P-element insertion was confirmed by PCR and sequencing using the following primers: 5′-TCCTATGGCCTGC GAAAATA and 5′-CACCCAAGGCTCTGCTCCCACAAT. As *EP(2)0560* stock contained an unlinked recessive lethal mutation on the second chromosome, *EP(2)0560/CyO* males were mated to *yw* females. *EP(2)0560*/+ F1 females, in which recombination between the second chromosomes could occur, were then mated to *Bl/CyO* males. Individual *EP(2)0560/CyO* males were then crossed to *Bl/CyO* females to establish new *EP(2)0560/CyO* stocks, which were then screened for the presence of the homozygous *EP(2)0560/EP(2)0560* animals. A new homozygous viable line was established, hereafter referred to as $dNKAIN^{EP560}$ and was used in subsequent tests. Presence of the original P-element at the *dNKAIN* locus in the $dNKAIN^{EP560}$ line was confirmed by PCR and sequencing as described above. *Df(2R)ED4071*, which belongs to DrosDel collection (22), was obtained from the Bloomington Stock Center.

P-element excision

Mobilization of the P-element, which contains a mini-white gene, was achieved by mating $dNKAIN^{EP560}$*/CyO* females to *Bl/CyOΔ2-3[w+]; ry e ca/TM6B* males, and then mating the individual dysgenic males $dNKAIN^{EP560}$*/CyOΔ2-3[w+]* to *Bl/CyO*; *TM2/TM6B* females. The individual $dNKAIN^{EP560}$*/CyO* males, in which the P-element has been excised through the action of Δ2-3 transposase, were selected based on their w-phenotype and crossed to *Bl/CyO*;*TM2/TM6B* females. 21 P-element excision lines were created. Most of the imprecise P-element excisions resulted in deletions larger than 10 kb as determined by PCR using the following forward primers: 5′-TCCTATGGCCTGCGAAAATA, 5′-CAATAATTTCCCTGCACATTCA, 5′-TACCAGAAGAT GGTGCCGTAG and reverse primers: 5′-ACGCTGCGT TTAGATTCCAT, 5′-ACCCTGCACGCCCACTATCGAG, 5′-ACGGAACCAGCAACAAAAAC. For one of these *dNKAIN*-null lines, $dNKAIN^{EP560rv9}$, the excision site was PCR-amplified using the following primers: upstream of excision—5′ TCCTATGGCCTGCGAAAATA and downstream of excision 5′-TACGATTTCCGATCCCCA using Expand High Fidelity PCR System (Roche, Indianapolis, IN, USA). The resulting 17 kb PCR fragment was then subcloned into pCR2.1-TOPO plasmid using TOPO TA Cloning (Invitrogen) according to the manufacturer's instructions. Deletion of *dNKAIN* gene in this line was then confirmed by sequencing. Precise excision of the P-element in $dNKAIN^{EP560rv2}$ was confirmed by PCR using the following primers: 5′-TCCTAT GGCCTGCGAAAATA and 5′-ACGCTGCGTTTAGATTC CAT, and sequencing.

Adult fly brain *in situ*

Antisense digoxigenin riboprobes were made by cloning the 3′-UTR of *dNKAIN* gene into the pBSK vector (Stratagene) and DIG labeling using DIG DNA Labeling kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Primers used for the cloning are listed in the Supplementary Material. Adult fly heads were frozen in OCT compound (Miles, Elkhart, IN, USA) and cryostat sectioned at 15 μm. Alkaline-phosphatase-visualized *in situ* hybridization was performed as previously described (60). Sense probe was used for control experiments.

Drosophila behavioral testing

Six 4–6-day-old flies were placed in glass vials and immersed in a 38°C water bath. The number of paralyzed animals was counted every 2.5 min for 10 min. A total of 60 $dNKAIN^{560}$ homozygous flies were used, and 30 heterozygous as well as wild-type controls were examined.

dNKAIN expression level

Fifty fly heads of each genotype were collected, washed in cold PBS with 0.1% Triton X-100 and homogenized in 200 μl of homogenization buffer [150 mM NaCl, 10 mM Tris, 5 mM EDTA, 0.5% Triton X-100, protease inhibitors cocktail (P8340, Sigma)]. The homogenate was centrifuged for 15 min at 16 000*g* at 4°C. The supernatant was collected and diluted 3× before adding the loading buffer (Invitrogen) for western blot as described above. Anti-dNKAIN antibody was used at 1:5000 dilution, and the anti-syntaxin (8C3, Hybridoma Bank) was used for control at 1:1000 dilution. Western blot membranes were scanned using Typhoon 9200 Imager (Amersham Biosciences, Sunnyvale, CA, USA) and the resulting images were analyzed using ImageQuant software. dNKAIN band intensity values were normalized to control syntaxin band intensity for each lane. In three separate experiments, the intensity of the dNKAIN protein bands in the homozygous and heterozygous flies were compared to wild-type control.

Analysis of NKAIN1 and dNKAIN function in *Xenopus* oocytes

cDNAs of NKAIN1, dNKAIN, Nrv2.2 and ATPalpha were subcloned into a pGEMHE vector (kindly provided by Dr D. Gadsby, Rockefeller University, New York, NY, USA). cDNAs of human Na,K-ATPase $\alpha1$, $\alpha2$, $\alpha3$, $\beta1$ subunits (61) were subcloned into a pSD5 vector. cRNAs were prepared by *in vitro* translation (62). Stage V–VI *Xenopus* oocytes were obtained as described (63). Electrophysiological measurements of NKAIN1 and dNKAIN currents were performed 1 day after oocyte injections with 5 ng of NKAIN1 or dNKAIN cRNAs using the two-electrode voltage clamp technique. NKAIN1 and dNKAIN currents were determined after deducing the current measured in H_2O-injected oocytes. Briefly, oocytes were placed in a recording chamber that was initially perfused with a solution containing NaCl 80 mM, NMDG-Cl 20 mM, KCl 3 mM, NMDG-HEPES 10 mM, $CaCl_2$ 0.9 mM, $MgCl_2$ 1 mM, pH 7.4 and 1 μM ouabain to inhibit endogenous oocyte Na,K-ATPase. The perfusate was sequentially changed to a series of solutions in which the Na^+ concentration was decreased from 80 to 20 mM, the K^+ concentration was increased from 3 to 15 mM and the Cl^- concentration was decreased from 106.8 to 26.8 mM by adjusting the concentration of NaCl, NMDG-Cl and KCl. Currents were activated by 200 ms steps from +30 to −130 mV.

Na^+ activation was measured in a basal solution composed of NMDG-Cl 100 mM, KCl 3 mM, NMDG-HEPES 10 mM, $CaCl_2$ 0.9 mM, $MgCl_2$ 1 mM, pH 7.4 and 1 μM ouabain which was sequentially changed to a series of solutions containing increasing Na^+ concentrations from 0 to 90 mM and adjusted in the concentration of NMDG-Cl.

To measure the effect of lanthanum, oocytes were perfused with the basal solution used for Na^+ activation experiments, containing 0 mM Na^+ and then sequentially perfused with a solution containing 90 mM Na^+ without or with 0.1 or 1 mM lanthanum. Reversibility of the effect was tested after perfusion of the oocytes with a lanthanum-free solution. To measure the effect of amiloride, oocytes were perfused with the basal solution used for Na^+ activation experiments, containing 0 mM Na^+ and then sequentially perfused with a solution containing 90 mM Na^+ without or with 5 μM amiloride.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None of the authors have a conflict of interest.

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