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NKAINs: A Novel Family of Membrane Proteins that Interact with the Na, K-ATPase and Affect Membrane Excitability

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NKAINs: a novel family of membrane proteins that interact with the Na,K-ATPase and affect membrane excitability

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Dedicated to my parents.

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Abstract

A novel family of Na,K-ATPase interacting (NKAIN) proteins was cloned and characterized. These proteins are highly conserved across species and do not resemble any known proteins in the genome. Aside from the conserved transmembrane domains, NKAINs contain no known functional domains. Striking amino acid conservation in the first two transmembrane domains suggests that the function of these proteins is concentrated in the membrane bilayer. NKAIN1, 2, 3 and a splice form of NKAIN4 are brain and testis specific; another splice form of NKAIN4 is expressed ubiquitously. In the central nervous system, NKAINs localize to neurons. The C-terminal tail of NKAIN proteins interacts with the β subunit of the Na,K-ATPase. The Na,K-ATPase β1 subunit, NKAIN1 and another β subunit interacting protein MONaKA form a complex in transfected cells. Since MONaKA shares sequence similarity with the C-terminal part of the *Drosophila* NKAIN homolog (dNKAIN), it is proposed that dNKAIN function is carried out by two mammalian proteins – NKAIN and MONaKA. Flies with decreased dNKAIN expression show temperature-sensitive seizures, motor deficit and paralysis a phenotype of neuronal hyperexcitability. In contrast, dNKAIN overexpression leads to a phenotype identical to the phenotype of flies with inhibited cellular excitability. Thus, dNKAIN function is likely to decrease membrane excitability, possibly by affecting the Na,K-ATPase function. Neuronal expression of NKAIN proteins and a possible role in membrane excitability strongly suggest that this novel protein family could be critical for neuronal function.

Chapter 1. Introduction

Since complete genome sequences from several organisms became available, modern biology has entered a post-genomic era. It is estimated that human or mouse genomes contain approximately 20,000 to 25,000 genes (IHGSC, 2004). It is believed that more than half of these genes are expressed in the central nervous system (CNS) (Sandberg et al., 2000). Comparing to the total number of proteins that could potentially play a role in brain function, only a small percentage of genes with known CNS-specific function have been identified so far (Insel and Collins, 2003). 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 previously uncharacterized proteins (NKAIN1, 2, 3 and 4) was selected for further biochemical analysis.

NKAIN protein family was predicted to be important for neuronal function, since NKAINs show remarkable evolutionary conservation and localize to neuronal plasma membranes. The highest amino acid identity between NKAIN family members from different species is found in their transmembrane domains suggesting that NKAINs function is confined to neuronal membranes, such as forming channels or pore structures, or affecting function of other neuronal membrane proteins. A *Drosophila* mutant with P-element disruption of single Drosophila homolog dNKAIN exhibits temperature sensitive paralysis, a phenotype often present in ion channel or synaptic transmission fly mutants (Ganetzky, 2000). Since NKAIN proteins have no known domains and show no similarity to any characterized proteins, their function could not be predicted from their

amino acid sequences. A yeast-two-hybrid screen of a mouse brain library, performed in order to identify NKAIN interacting proteins, resulted in multiple clones of β1 subunit of the Na,K-ATPase. The interaction between NKAIN proteins and the β subunit of the Na,K-ATPase was further confirmed for both mouse and *Drosophila* proteins by co-immunoprecipitation in transfected cells. Another finding that supports this interaction is that *Drosophila* NKAIN homolog (dNKAIN) contains an additional domain homologous to a recently discovered β subunit binding protein MONaKA. Since both mouse NKAIN and MONaKA form a complex with the β subunit of the Na,K-ATPase in transfected cells, we propose that function of two mammalian proteins, MONaKA and NKAIN is carried out by a single protein dNKAIN in *Drosophila.*

Based on the phenotypes of fly mutants expressing lower levels of dNKAIN or overexpressing dNKAIN, it seems that NKAINs function is to decrease cell excitability. Mechanisms that prevent neurons from depolarizing inappropriately are particularly important for the central nervous system function. Increase in neuronal excitability can lead to such diseases as epilepsy and migraine (Bussone, 2004; Steinlein, 2004). For example, a mutation responsible for febrile seizures and generalized epilepsy causes decrease in rate of inactivation of a voltage-gated sodium channel which leads to hyperexcitability (Wallace et al., 1998). Mutations in potassium channels that are responsible for repolarization of neuronal membranes also cause neuronal hyperexcitability. A number of such mutations have been identified in patients with epilepsy (Biervert et al., 1998; Charlier et al., 1998; Du et al., 2005; Singh et al., 1998). Thus, mutations in NKAIN proteins could also cause seizure disorders by lowering cell excitability threshold. Indeed, two recent clinical reports describe patients with

chromosomal translocations in *NKAIN2* gene affected with profound neurological dysfunction including seizures (Bocciardi et al., 2005; Yue et al., 2005). Evidence of NKAINs role in cell excitability taken together with their remarkable evolutionary conservation and neuronal membrane expression suggest that these novel proteins are likely to play a role crucial for CNS function and development. They might also potentially be used as drug targets in a number of neuronal hyperexcitability diseases such as epilepsy and migraine.

Chapter 2. Search for novel proteins expressed in the central nervous system

INTRODUCTION

Since development of techniques that allowed large scale sequencing, modern biology has changed dramatically. There are currently two ways of discovering new proteins. Traditionally, the new proteins have been discovered through interactions with already known proteins. Naturally, a newly discovered protein usually belongs to a studied pathway, allowing deeper understanding of previously identified biological phenomena. Currently there are about 14,000 mammalian proteins that have been discovered using this approach, according to the RefSeq database of functionally annotated genes at NCBI. However, the number of genes with well-studied function is much smaller, since many of these genes have been given a name, but detailed functional analysis has not been carried out.

In addition to this "classical" method of protein discovery, there are numerous large-scale sequencing projects that identify thousands of novel proteins. For example, 60,770 full-length mouse cDNAs were recently sequenced and annotated by RIKEN Mouse Gene Encyclopedia Project (Okazaki et al., 2002). The total number of protein-coding human genes is estimated to be 20,000-25,000 according to the International Human Genome Sequencing Consortium (IHGSC, 2004). Thus, at least half of genes in the genome remain uncharacterized. Undoubtedly, a number of these genes will be crucial for understanding of human biology and disease.

There are approximately 15,000 to 16,000 genes expressed in brain (Sandberg et al., 2000). Currently, the majority of these genes remain uncharacterized, since more than

99% of neuroscience literature has focused on less than 1% of the genome (Insel and Collins, 2003). Many of these uncharacterized genes are likely to be important for brain function.

The mammalian central nervous system is comprised of a remarkable array of neuronal and glial cell types. There is a variety of neurons with different morphologies and electrophysiological properties. In addition to these differences between neuronal cell types, neuronal connections add to the complexity of the central nervous system. Very little is known about how a certain group of neurons acquires its function and decides on connecting partners. Molecular factors that establish these properties and distinguish one group of cells from another remain largely unknown, since until recently it has been difficult to discern these subpopulations.

The BAC transgenic system allows labeling a subpopulation of cells expressing a certain protein. Transgenic mice for a particular gene are generated using vectors containing a bacterial artificial chromosome (BAC) that includes a large region of genomic DNA encoding most of the regulatory elements of a given gene (Yang et al., 1997). The resulting BAC transgenic mice express enhanced green fluorescent protein (EGFP) in a pattern identical to the expression pattern of that endogenous gene. The Gene Expression Nervous System Atlas (GENSAT) was designed to create an expression map of multiple central nervous system genes using this technology (Gong et al., 2003). The atlas as well as transgenic mice and BAC vectors created in this project provide resources for investigating brain function. First, the expression pattern for a particular gene obtained from BAC-transgenic mice expressing EGFP can help elucidating the function of that gene. Second, labeling a specific subpopulation of brain cells with EGFP

allows distinguishing the cells of interest from other cells in brain. These mice can then be used to study function of the labeled structure by different techniques, such as electrophysiological recording from the labeled neurons. EGFP labeling can also be used to isolate these cells in order to compare expression profiles between EGFP expressing neurons and other cells in the central nervous system. Cell-type specific BACs can also be used to drive expression of other proteins in a cell-type specific manner. For example, tethered toxins can be expressed to inactivate specific ion channels in a population of cells (Ibanez-Tallon et al., 2004).

A very limited number of genes expressed in specific brain regions or neuron types are known. Since many of the brain-expressed genes are still uncharacterized, it is likely that numerous genes with interesting expression patterns remain to be discovered.

There is a number of ways to find brain-expressed genes using publicly available electronic databases. Full-length mRNA databases that contain sequences from brain libraries are available; the Database of Human Unidentified Gene-Encoded Large Proteins (HUGE) from Kazusa Human cDNA Project is one example of such database (Kikuno et al., 2004). Brain expression of a novel mRNA sequence from electronic databases can also be inferred from analyzing expressed sequence tags (ESTs) that correspond to that mRNA if the majority of ESTs derive from brain libraries, then the mRNA is likely to be brain-expressed. Next, genes with brain-specific regulatory elements or domains are likely to be expressed in brain. Finally, gene array expression databases, such as ArrayExpress at European Bioniformatics Institute, can be used to find genes expressed in brain.

RESULTS

In silico cloning of novel brain-expressed proteins and protein families

In order to find novel proteins and protein families involved in brain development and function, bioinformatic search for genes that are predominantly brain-expressed was performed. Full-length brain mRNAs from HUGE database (Kikuno et al., 2004) were individually examined for brain-specific expression based on brain EST matches and RT-PCR expression data available in that database. Only genes that had less than 60% amino acid similarity to known proteins were selected in order to focus on "novel" proteins. 140 novel brain-specific genes were identified and ranked according to their level of brain expression.

Some of the selected genes were subjected to further sequence analysis in order to find related proteins and identify novel protein families. Surprisingly, almost half of the analyzed genes had at least one additional protein family member, often also uncharacterized. A total number of 22 protein families with 2 to 5 family members sharing above 50% amino acid sequence similarity were identified. 173 genes including the original 140 gene set and the newly discovered protein family members were submitted to GENSAT project.

Most of *in silico* cloned genes have localized expression pattern in the CNS

Of the 173 novel *in silico* cloned genes, 34 genes were included in the first release of GENSAT database (Gong et al., 2003) and 46 genes are currently in some stages of BAC-transgenic mice development (Figure 2-1A). Images of brain expression patterns

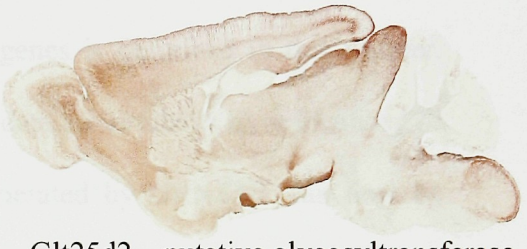
Figure 2-1. Novel *in silico* cloned brain-expressed genes.
A. 46 genes of 173 novel *in silico* cloned genes are currently in the GENSAT database. Expression pattern data is available for 26 genes (colored). 19 genes (red) had region or cell-specific pattern of expression. Some genes were given names based on domains present in their sequence, however little functional information is currently available for most of these genes. B. GENSAT brain expression images for several *in silico* cloned novel genes with interesting patterns of expression.

A

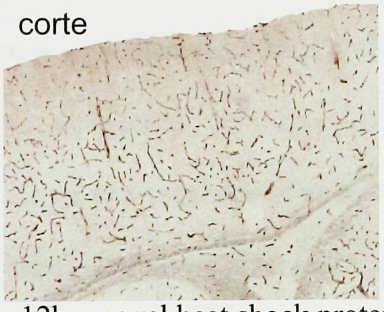
Sez6	FLRT2 precursor	Sez6l2
Scrn1	P301	Lrfn2
Irfn5	Ssx2ip	Satb2
satb1	9630025P05Rik	P300
P254	9330175B01Rik	P309
P271	5330427M13Rik	AI427515
P272	4930538C18Rik	Igsf10
Hspa12a		LOC240233
Hsp12b		Prosapip1
Lrig2		Peg10
Slitrk2		Trim2
Sema6d		1700051I12Rik
Slc7a14		1200009O22Rik
Glt25d2		B230339M05Rik
6430573F11Rik		2310022K01Rik
2900046G09Rik		4831444O18Rik
3732412D22Rik		2900010D03Rik
6330571D19Rik		B930008A12Rik
E130310K16Rik		9430073A21Rik

B

Secernin1 – a novel regulator of exocytosis

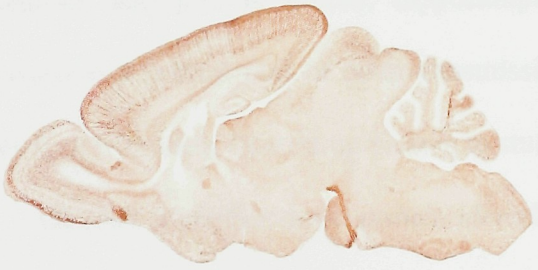


Glt25d2 – putative glycosyltransferase

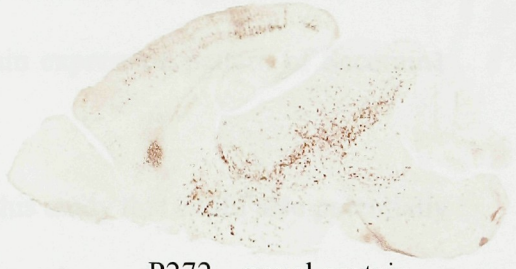


Hspa12b – novel heat shock protein

2900046G09Rik – novel protein



P271 – novel protein



P272 – novel protein

are currently available for 26 of these novel proteins. Remarkably, 19 of the 26 genes (73%) had cell or region-specific expression. Several examples of these expression patterns are included in figure 2-1B. Novel *in silico* cloned genes described in this chapter were highly enriched for genes with localized pattern of expression, since usually only 20-25% of randomly selected brain-expressed genes have this type of expression (Pr. Heintz, personal communication).

DISCUSSION

In silico cloning of 173 novel brain-specific genes is described in this chapter. More than 70% of genes for which expression pattern data are available have localized or region-specific expression. BAC-transgenic mice generated by GENSAT can now be used to determine functions of these novel proteins.

For several of these genes, functional information obtained from studies in other systems is now available. Combined with brain expression pattern, these genes now appear to have a potentially critical role in brain function. For example, Secernin1 was recently identified as a regulator of exocytosis in peritoneal mast cells. It was shown that this protein increases mast cell secretion and enhances sensitivity to calcium stimulation (Way et al., 2002). From GENSAT data, it appears that Secernin1 is expressed in a subset of neurons and glia in cortex, hippocampus and brainstem (Figure 2-1B). Since exocytosis is a critical part of neurotransmission, brain expression pattern of Secernin1 makes it an extremely interesting protein to study.

Glt25d2 is another novel protein identified in this study that could also potentially play a critical role in brain function. This protein contains a domain characteristic of

glycosyltransferases. According to GENSAT expression data, Glt25d2 is expressed in layer 5 pyramidal neurons in cortex, as well as several other brain region (Figure 2-1B). Many proteins critical for brain development and function, such as neural cell adhesion molecules, are glycosylated. Specific expression of a glycosyltransferase, a protein that is necessary for glycosylation, could be important for development and connectivity of Glt25d2 expressing neurons.

Finally, Hspa12b, a putative heat shock protein, is expressed specifically in brain blood vessels (Figure 2-1B). This expression pattern suggests that Hspa12b might be important for blood-brain barrier or response to brain ischemia.

BAC constructs generated for novel genes with interesting expression patterns can now be used to study brain regions where these genes are expressed. Several examples of interesting expression patterns, such as 2900046G09Rik, P271 and P272, are shown in Figure 2-1B. In fact, BAC construct for novel protein P271 is used to drive gene expression in the cortical layers 2 and 5 in several current projects in the lab of Pr. Heintz.

Most of the novel genes described in this chapter had localized or region-specific expression. Thus, the criteria used in the gene selection enrich for this type of expression. In the future, such simple pre-screening process as RT-PCR or analyzing brain vs. non-brain EST matches can help to focus the resources on potentially more interesting genes.

Chapter 3. NKAINs – a novel brain-expressed transmembrane protein family

INTRODUCTION

One novel protein family consisting of four mammalian proteins (NKAIN1, 2, 3 and 4) was selected from the genes described in the previous chapter for further biochemical analysis. Central nervous system expression, transmembrane localization and a remarkable evolutionary conservation suggest that NKAINs might play a critical role in brain function.

RESULTS

NKAINs – a family of four evolutionary conserved novel proteins

Initially, sequences of four mouse NKAIN proteins were determined from ESTs and genomic sequence predictions combined with RT-PCR confirmation. Later, however, several full-length cDNA sequences corresponding to NKAIN proteins were deposited in GenBank as part of the large-scale effort to determine mouse transcriptome (Okazaki et al., 2002). Proteins encoded by *NKAIN* genes are 181 to 233 amino acids long, depending on the splice variant.

When NKAIN1, 2, 3 and 4 proteins are compared to each other within one species, the amino acid similarity ranges from 71 to 81% for the full-length sequence. The highest similarity is found in the first 120 amino acid region, which contains a cleavable signal peptide and two putative transmembrane domains. This highly conserved part is followed by a short stretch of 15 amino acids without any homology, which was

used to design family member-specific primers and a peptide antibody specific to NKAIN1. Finally, there is a relatively high similarity region containing the third putative transmembrane domain followed by an alternatively spliced C-terminal tail (Figure 3-1).

There are four family members in vertebrates, one in *Drosophila* and one in *C. elegans*. Multiple alignment of NKAIN proteins from mouse (*M. musculus*), chicken (*G. galus*), frog (*X. tropicalis*), fruit fly (*D. melanogaster*) and *C. elegans* is shown in Figure 3-1. As seen from the alignment, this family is highly conserved across species. There is 81-96% amino acid similarity between mouse, chicken and frog homologs. *Drosophila* homolog (CG9047) dNKAIN shares 59-60% similarity with mouse NKAINs in the first 200 amino acids of the protein and has an extra 458aa C-terminal tail that is absent from vertebrate NKAINs. Amino acid similarity between fly and mammalian homologs is even higher in the first two putative transmembrane domains (70-74% to mouse NKAINs) and almost identical in the N-terminal 25 amino acid region (positions 25-46) (Figure 3-1). This remarkable evolutionary conservation of protein sequence suggests that NKAINs are important for cell function.

Another fact that makes this protein family interesting to study is that it shows no similarity to any known proteins in GenBank. BLAST as well as PHI- and PSI-BLAST searches did not generate any hits besides uncharacterized and predicted proteins corresponding to NKAIN family members obtained from large-scale cDNA clone and genome sequencing projects. Recently, *NKAIN2* was named *TCBA1* (T-cell lymphoma breakpoint associated target 1), since this gene contains the site of a chromosomal translocation in two T-cell lymphoma/leukemia cell-lines and in two clinical cases

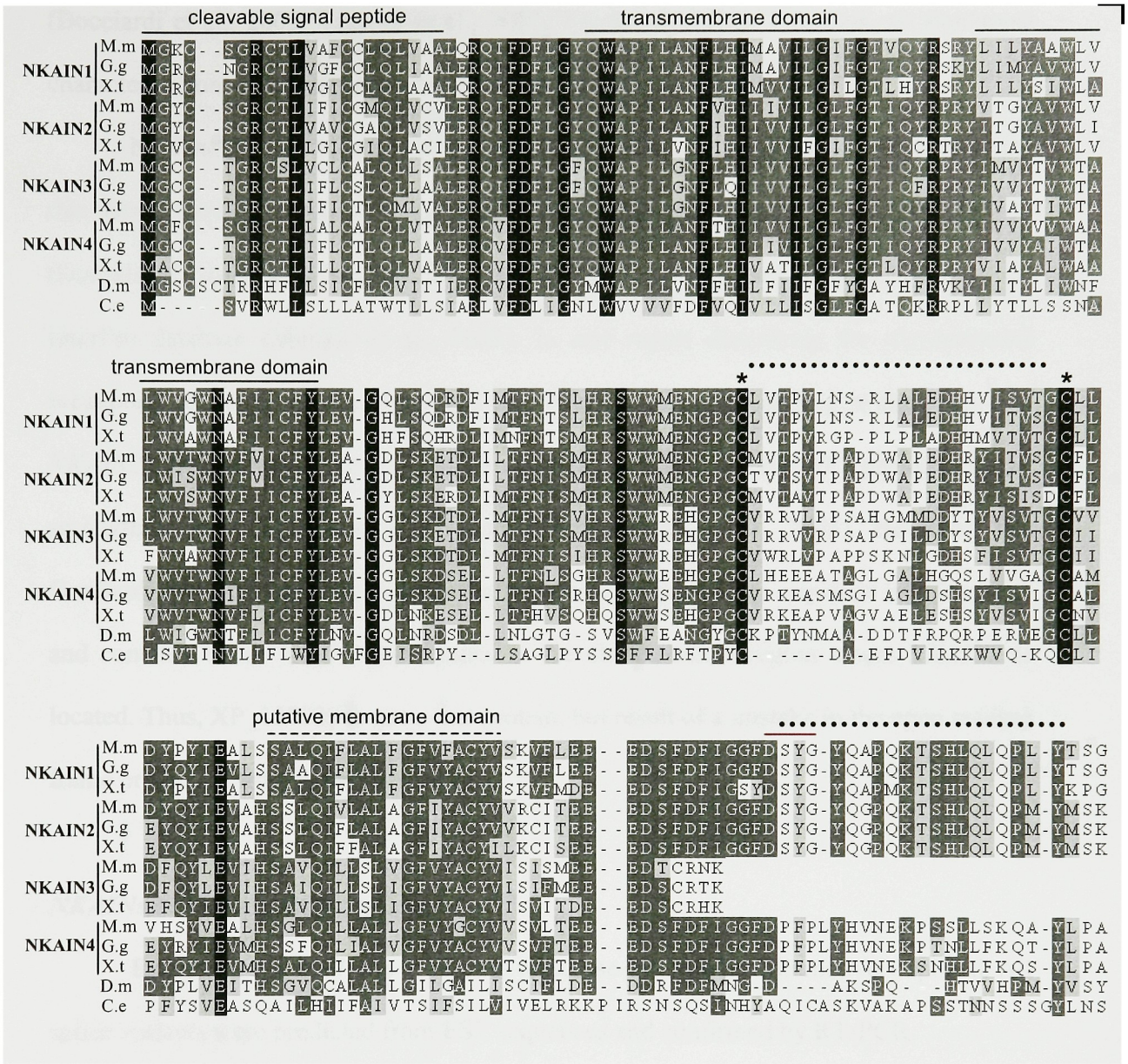


Figure 3-1. Multiple alignment of the NKAIN protein family.
NKAIN proteins from *M. musculus* (M.m), *G. galus* (G.g), *X. tropicalis* (X.t), *D. melanogaster* (D.m) and *C. elegans* (C.e) are included in this multiple alignment. For the *D. melanogaster* and *C. elegans* NKAINs, the first 200aa and 199aa respectively are included in the alignment. Putative transmembrane domains are labeled with solid lines, 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 cysteines 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 red line.

(Bocciardi et al., 2005; Tagawa et al., 2002; Yue et al., 2005). However, no functional characterization was reported for NKAIN2 or other family members.

No function can be predicted based on NKAIN amino acid sequences alone, since this family does not contain any characterized domains. In fact, a novel domain DUF798 (Domain of Unknown Function 798) is defined by proteins from NKAIN family in the InterPro database (Mulder et al., 2005). In one report describing the chromosomal breakpoint in *NKAIN2*, it is suggested that 70 residues corresponding to the exon 4 of *NKAIN2* are identical to a mouse protein similar to B-RAF proto-oncogene serine/threonine-protein kinase (XP_137033) (Bocciardi et al., 2005). However, this GenBank entry corresponds to a protein predicted from genomic sequence by GeneScan and contains exons from several genes in the chromosomal region where *NKAIN2* is located. Thus, XP_137033 is not a real protein, but result of a mistake in the open reading frame prediction from genomic sequence.

_NKAIN_s are alternatively spliced

Except for *NKAIN1*, the 3' regions of mouse *NKAINs* are alternatively spliced. All splice variants were predicted from EST sequences and confirmed by RT-PCR.

7 exon splice forms with identical exon-intron boundaries exist for *NKAIN1, NKAIN2* and *NKAIN4*. Exon 6 is skipped in *NKAIN3a* and *NKAIN4b* and an extra exon 6 is included in *NKAIN2b* (Figure 3-2A, blue). In addition to these splice forms, several additional variants can also be detected, though they are expressed at much lower levels. An alternatively spliced form terminating with exon 4 is detected for *NKAIN2*, *NKAIN3* and *NKAIN4*, but not *NKAIN1*. Finally, an alternative exon 5 is included in *NKAIN3b*

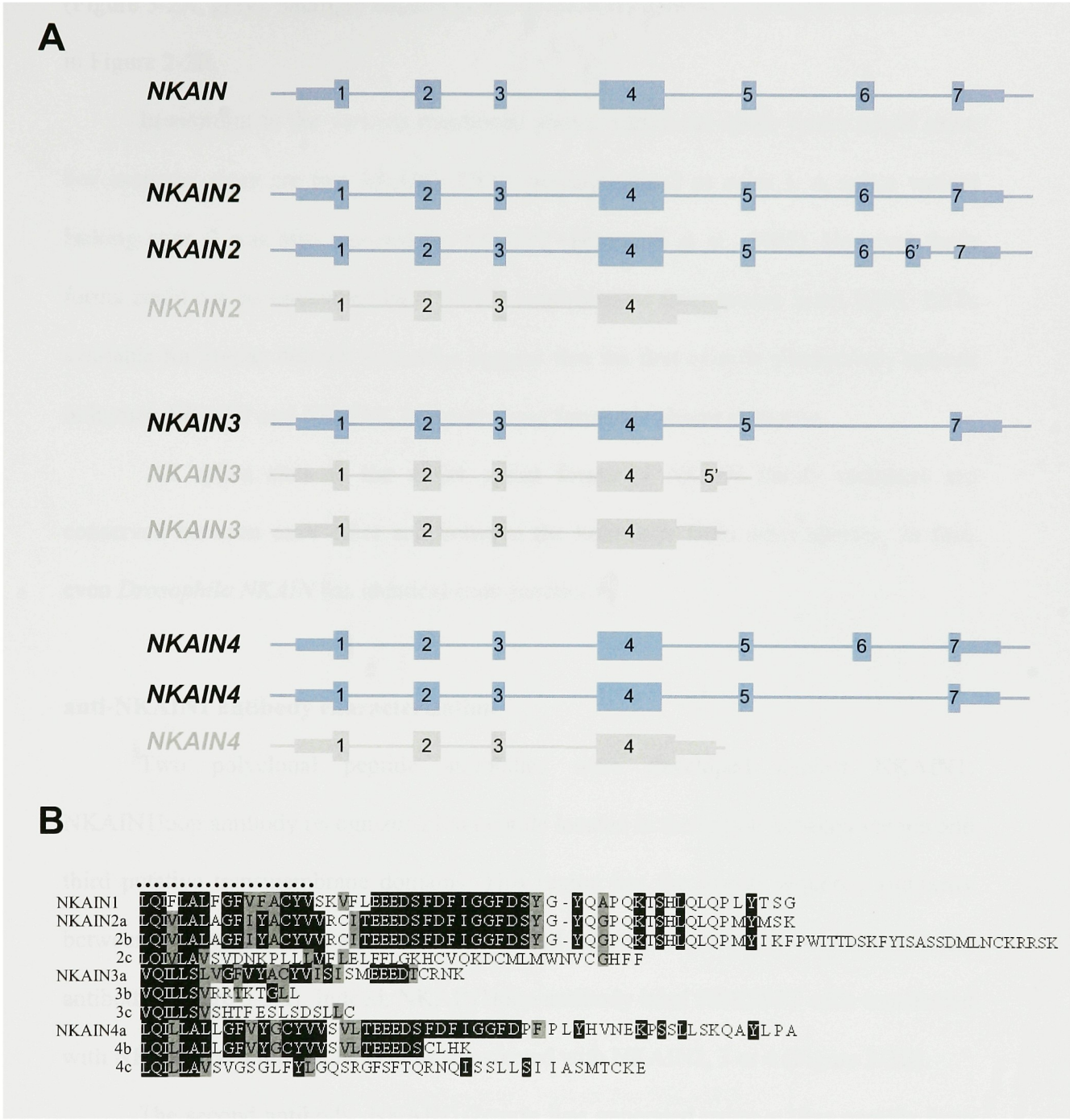


Figure 3-2. C-terminal tail of NKAINs is alternatively spliced.
A. Exon-intron structure of *NKAIN* genes. The splice variants were predicted from EST sequences and confirmed by RT-PCR. The splice variants that are expressed at a much lower level compared to the major highly expressed splice forms are written in gray. B. Multiple alignment of the alternatively spliced C-terminal regions of NKAIN proteins. Putative third transmembrane domain (dotted line) is absent in several isoforms.

(Figure 3-2A, gray). Multiple alignment of alternatively spliced C-terminal tails is shown in Figure 2-2B.

In addition to the variants mentioned above, additional splice forms might exist. For example, there are two *NKAIN4* ESTs missing exon 2 or exon 3. A splice variant lacking exon 2 was also reported for *NKAIN2* (Bocciardi et al., 2005). However these forms could not be confirmed by RT-PCR amplification from mouse brain RNA. ESTs available for human *NKAIN* homologs suggest that the first exon is alternatively spliced in human *NKAIN1* and *NKAIN4*, however these forms are absent in mouse.

All splice sites in the major splice forms of *NKAIN* family members are conserved between each other and between the homologs from other species. In fact, even *Drosophila NKAIN* has identical exon junctions.

anti-NKAIN1 antibody characterization

Two polyclonal peptide antibodies were developed against NKAIN1. NKAIN1loop antibody recognizes 21aa peptide located in the region between second and third putative transmembrane domains. This region has the lowest sequence similarity between family members, and thus was selected for generation of the NKAIN1 specific antibody (Figure 3-3A). Indeed, NKAIN1loop antibody label HEK293T cells transfected with NKAIN1 and did not label cells transfected with NKAIN2, 3 or 4 (Figure 3-3B).

The second antibody, NKAIN1/2cterm was generated using a 19aa peptide from the C-terminal tail of NKAIN1. This region, however, is highly conserved between NKAIN1 and NKAIN2. Only 2 out of 19 amino acids are different between these two

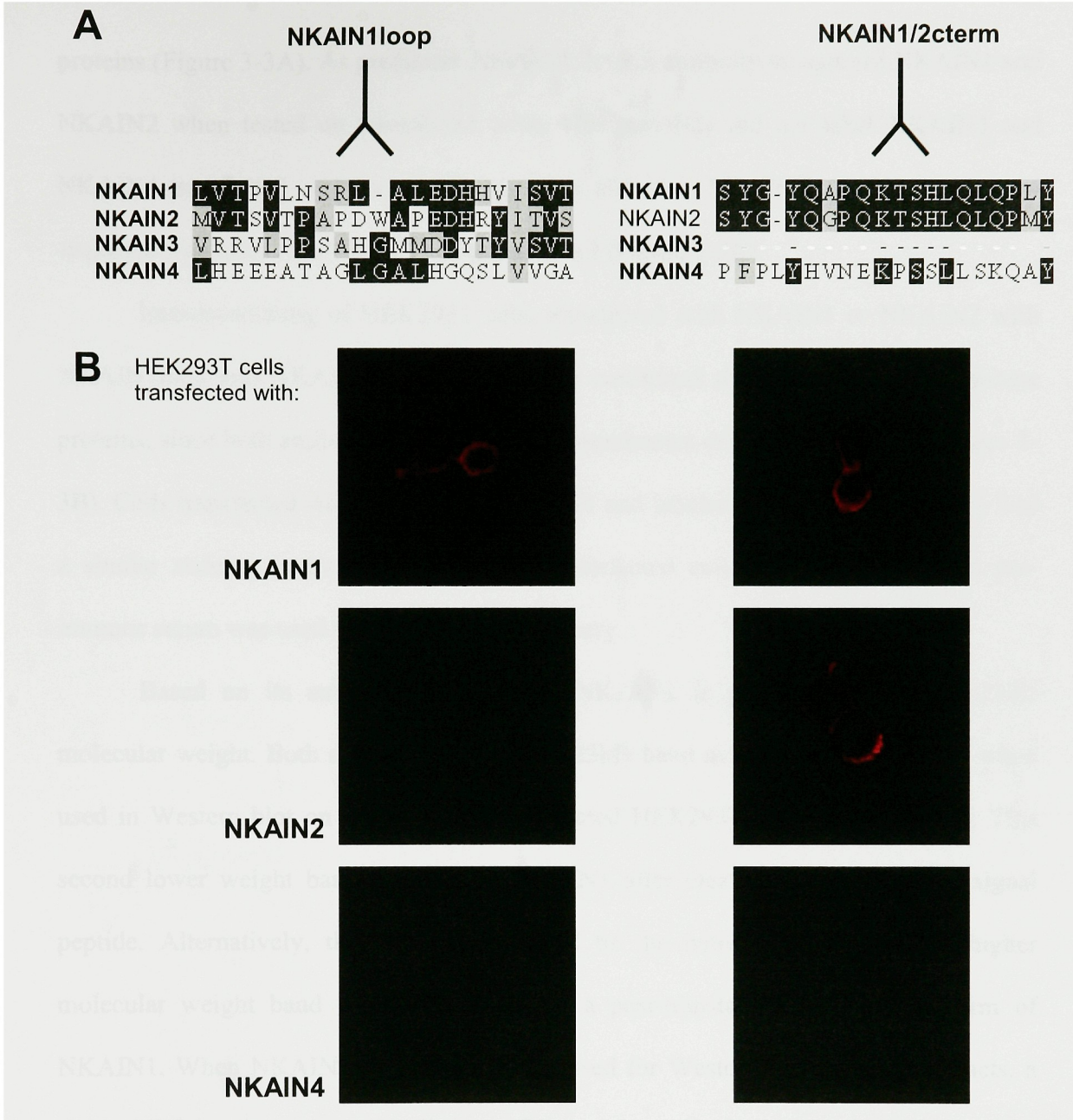


Figure 3-3. Two polyclonal antibodies were raised for NKAIN1.
A. Protein sequence alignment of NKAIN1 peptides used for the antibody generation and the corresponding regions from other family members. 21aa peptide (116-136) used to generate NKAIN1loop antibody is unique to NKAIN1. 19aa peptide (186-204) used to generate NKAIN1/2cterm antibody is highly similar to NKAIN2. B. NKAIN1loop is NKAIN1 specific, while NKAIN1/2cterm recognizes both NKAIN1 and NKAIN2. HEK293T cells were transfected with NKAIN proteins and labeled with either NKAIN1loop or NKAIN1/2cterm antibodies. Untransfected cells were not labeled. No staining was observed when pre-immune serum was used for immunocytochemistry.

proteins (Figure 3-3A). As predicted, NKAIN1/2cterm antibody recognized NKAIN1 and NKAIN2 when tested on transfected cells. This antibody did not label NKAIN3 and NKAIN4 transfected cells, since this region is absent in NKAIN3 and has a very low sequence similarity with NKAIN4 C-terminal tail (Figure 3-3B).

Immunostaining of HEK293T cells transfected with NKAIN1 or NKAIN2 with NKAIN1loop and NKAIN1/2cterm antibodies confirmed that NKAINs are membrane proteins, since both antibodies labeled plasma membranes of transfected cells (Figures 3-3B). Cells transfected with flag-tagged NKAIN4 and labeled with anti-flag antibody had a similar staining pattern. No labeling of transfected cells was observed when pre-immune serum was used for immunocytochemistry.

Based on its amino acid sequence, NKAIN1 is predicted to have a 23kD molecular weight. Both antibodies detected a 23kD band as well as a 21kD band when used in Western blot on extracts from transfected HEK293T cells (Figure 3-4A). This second lower weight band is probably NKAIN1 after cleavage of the putative signal peptide. Alternatively, the lower band could be the native protein, and the higher molecular weight band could correspond to a post-translationally modified form of NKAIN1. When NKAIN1loop antibody was used for Western blot on brain extracts, a single 55kD band was detected. The specificity of the antibody was confirmed by peptide competition assay (Figure 3-4B). Both 23kD band in transfected cells and 55kD brain extract band disappeared when NKAIN1loop antibody was pre-incubated with the peptide used to raise this antibody. Pre-incubation with an unrelated control peptide did not interfere with NKAIN1loop labeling. NKAIN1,2cterm antibody detected bands of the same size as NKAIN1loop, however it worked much better on transfected cells than on

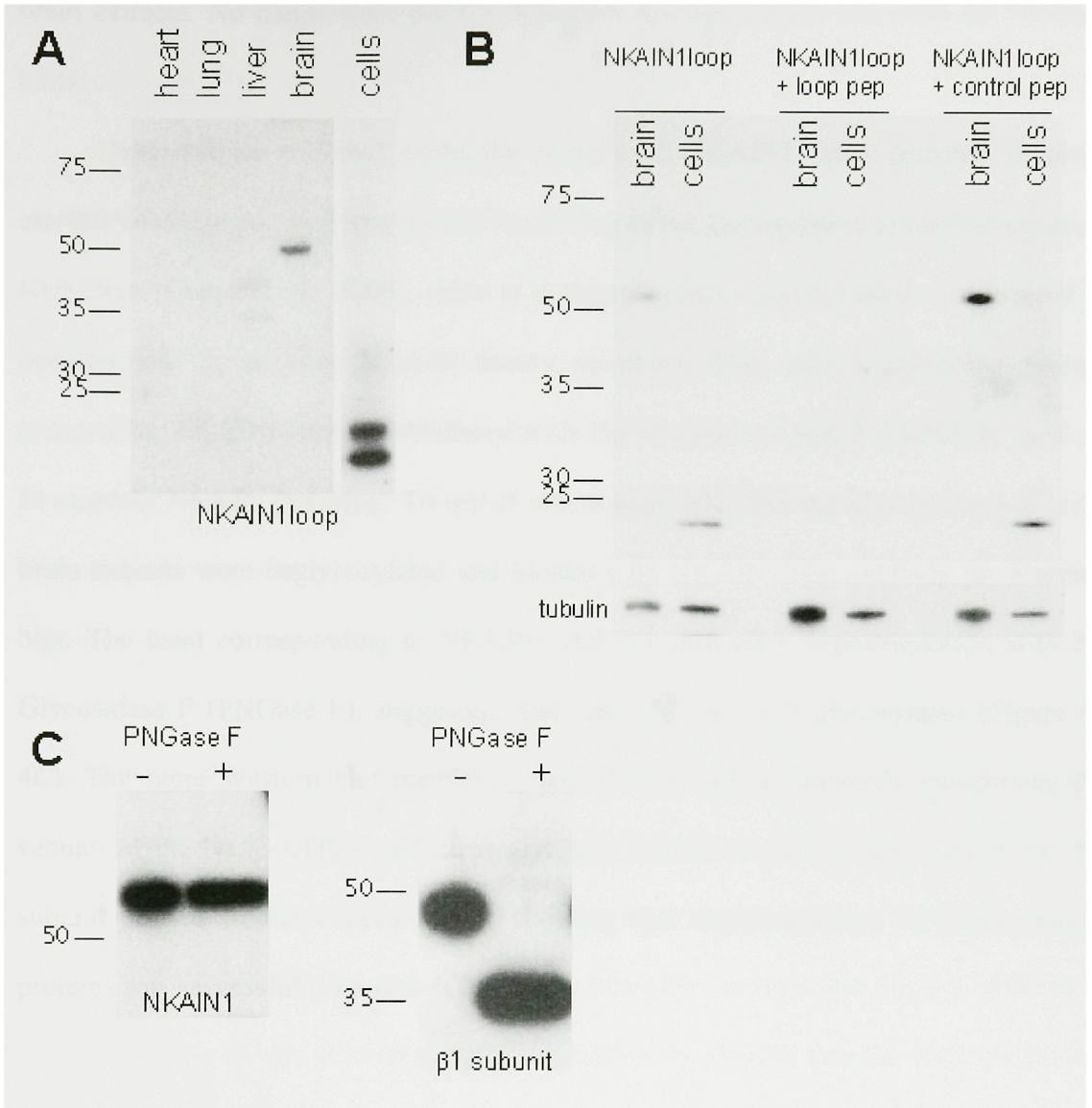


Figure 3-4. Anti-NKAIN1 antibody detects specific bands in brain and cell extracts. A. NKAIN1 antibody detects a 55kD band in brain extracts, but not in extracts from other tissues. Predicted molecular weight of NKAIN1 is 23kD. Doublet detected in HEK293T cells transfected with NKAIN1 is probably due to cleaving of the signal peptide or due to other post-translational modification. B. The bands detected by NKAIN1loop antibody disappear when the antibody is pre-incubated with NKAIN1 peptide, but not after pre-incubation with an unrelated control peptide. Anti-tubulin antibody was used for loading control. C. The higher molecular weight of NKAIN1 band in brain extracts cannot be explained by N-glycosylation. Brain extracts were treated with N-Glycosidase F (PNGase F). The band for the β1 subunit of the Na,K-ATPase, which is known to be glycosylated, is shifted in the deglycosylated brain extract, however the molecular weight of NKAIN1 band remains the same.

brain extracts. No bands were present when pre-immune serum was used for Western blots.

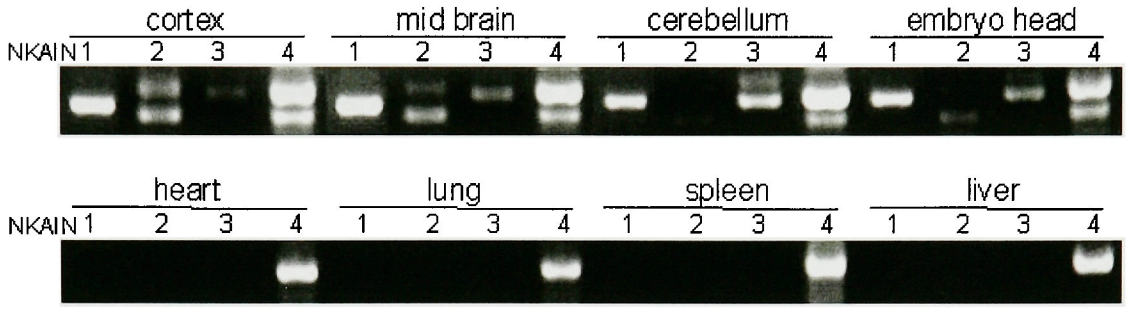
Higher than expected molecular weight of NKAIN1 band detected in brain extracts could be due to glycosylation. According to the glycosylation prediction program NetNGlyc (Gupta et al., 2004), there is a single putative glycosylation site located at position 100 in all four NKAIN family members. This site is conserved among mammalian NKAINs, but is substituted with His in frog and fish NKAIN4, as well as *Drosophila* NKAIN homolog. To test if mammalian NKAINs are glycosylated *in vivo*, brain extracts were deglycosylated and blotted with NKAIN1loop antibody on Western blot. The band corresponding to NKAIN1 did not shift after deglycosylation with N-Glycosidase F (PNGase F), suggesting that NKAIN1 is not N-glycosylated (Figure 3-4C). The same Western blot membrane was blotted with an antibody recognizing β1 subunit of the Na,K-ATPase, which is glycosylated. The band corresponding to the β1 subunit shifted to the expected size, showing that deglycosylation of brain extract proteins was successful (Figure 3-4C).

NKAIN family members are expressed predominantly in brain

Since most of ESTs corresponding to *NKAIN1, 2* and *3* were derived from brain libraries, it was predicted that expression of these family members is restricted to central nervous system, while *NKAIN4* is likely to be expressed ubiquitously. To confirm this prediction, RT-PCR was performed on RNA from different tissues, using specific primers located in the fourth exon and 3' UTR of *NKAIN* genes. In addition to determining expression patterns of these genes, this primer design also allowed to

analyze alternative splicing and expression of splice variants, since most splice forms differ between exon 4 and 3' UTR. The results of these experiments are shown in Figure 2-5. As predicted from EST data, all four family members are highly expressed in brain. Except for testis, *NKAIN1, 2* and *3* are not detectable outside of the brain. *NKAIN4* is expressed ubiquitously; however, the short alternatively spliced form (*NKAIN4b*) containing 6 exons is brain and testis-specific. Brain-specific expression of NKAIN1 was also demonstrated by Western blot using NKAIN1 specific antibody (Figure 3-4A).

NKAIN2 (TCBA1) was previously reported to be highly expressed in adult and fetal brain (Bocciardi et al., 2005; Tagawa et al., 2002; Yue et al., 2005). However, there are conflicting results regarding expression in other tissues. In one study, human NKAIN2 was barely detectable by RT-PCR in several tissues and highly expressed in skeletal muscle; while murine isoform was amplified from adrenal gland, testis and breast (Bocciardi et al., 2005). In another report, in addition to the high brain expression, NKAIN2 was also detected by RT-PCR in testis and liver, but not in other tissues (Yue et al., 2005). Finally, NKAIN2 was shown to be expressed in brain and thymus, and not in lung, parathyroid, liver, fetal liver, spleen, lymph node, skeletal muscle, stomach, ileum, colon and kidney (Tagawa et al., 2002). Thus, it is possible that some NKAINs might be expressed in other tissues at a barely detectable level. However, given the extremely high expression in the nervous system, it is likely that the major function of NKAIN proteins is in brain.



	whole brain	embryo head	cerebellum	cortex	mid-brain	brain-stem	olf. bulb	retina	kidney	liver	spleen	heart	lung	testis	ovaries
NKAIN1	−	−	−	−	−	−								−	
NKAIN2	=	=	−	=	=	=								=	
NKAIN3															
NKAIN4	=	=	=	=	=	=	−	−	−	−	−	−	−	=	−

Figure 3-5. Expression profile of *NKAIN* genes.
NKAIN1, 2 and *3* are brain and testis specific. *NKAIN4a* is expressed ubiquitously, while *NKAIN4b* is brain and testis specific. RT-PCR using primers located in the fourth exon and 3'UTR was performed on RNA from different tissues.

Brain expression patterns of NKAIN family members

Brain expression patterns of mouse NKAINs were determined by immunohistochemistry, analyzing BAC-transgenic mice from GENSAT (Gong et al., 2003), and *in situ* expression data available from Brain Gene Expression Map (BGEM) and Allen Brain Atlas databases (Gewin, 2005).

Using NKAIN1 specific antibody, it was determined that NKAIN1 is widely expressed in cortex, thalamus, hippocampus and cerebellum (Figure 3-6). NKAIN2 is expressed in neurons in cortex, olfactory bulb, hypothalamus, midbrain, brainstem and spinal cord (Figure 3-7). Contrary to NKAIN1, NKAIN2 is not expressed in hippocampus, and only weakly in cerebellum. NKAIN2 expression was determined from BAC-transgenic mice. It is possible that not all of the regulatory elements are included in the BAC used for the generation of these mice, since *NKAIN2* gene is extremely large. However, NKAIN2 expression data is reproducible in multiple mouse lines, suggesting that it is indeed the true expression pattern. Weak expression in BAC-transgenic mice cerebellum also correlates with the low intensity of RT-PCR band for that brain region. According to *in situ* data in a recent report, *NKAIN2* is expressed in hippocampus (Bocciardi et al., 2005). However, the probe that was used in these experiments included the open reading frame of *NKAIN2*, which has regions highly similar to *NKAIN1*. Thus, it is possible that reported labeling of hippocampus reflects both *NKAIN1* and *NKAIN2* expression.

NKAIN3 expression data, obtained from both *in situ* databases and GENSAT, is similar between these different sources. Similarly to other family members, NKAIN3 is expressed in multiple neurons scattered throughout cortex, hippocampus, thalamus, brain

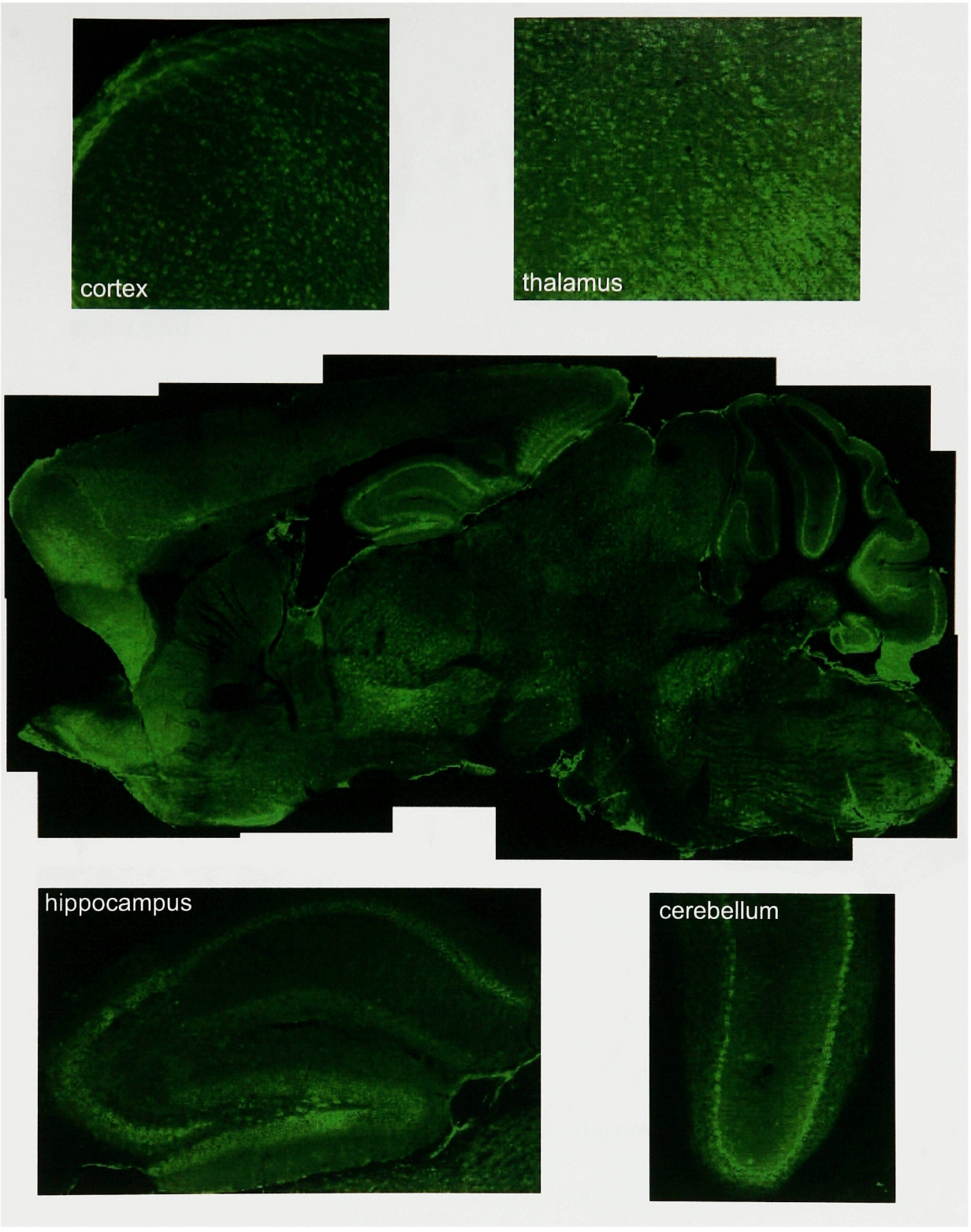


Figure 3-6. Mouse brain expression pattern of NKAIN1.
NKAIN1 specific antibody was used for immunohistochemistry on mouse brain sections. NKAIN1 is expressed in hippocampus, cerebellum, cortex and thalamus.

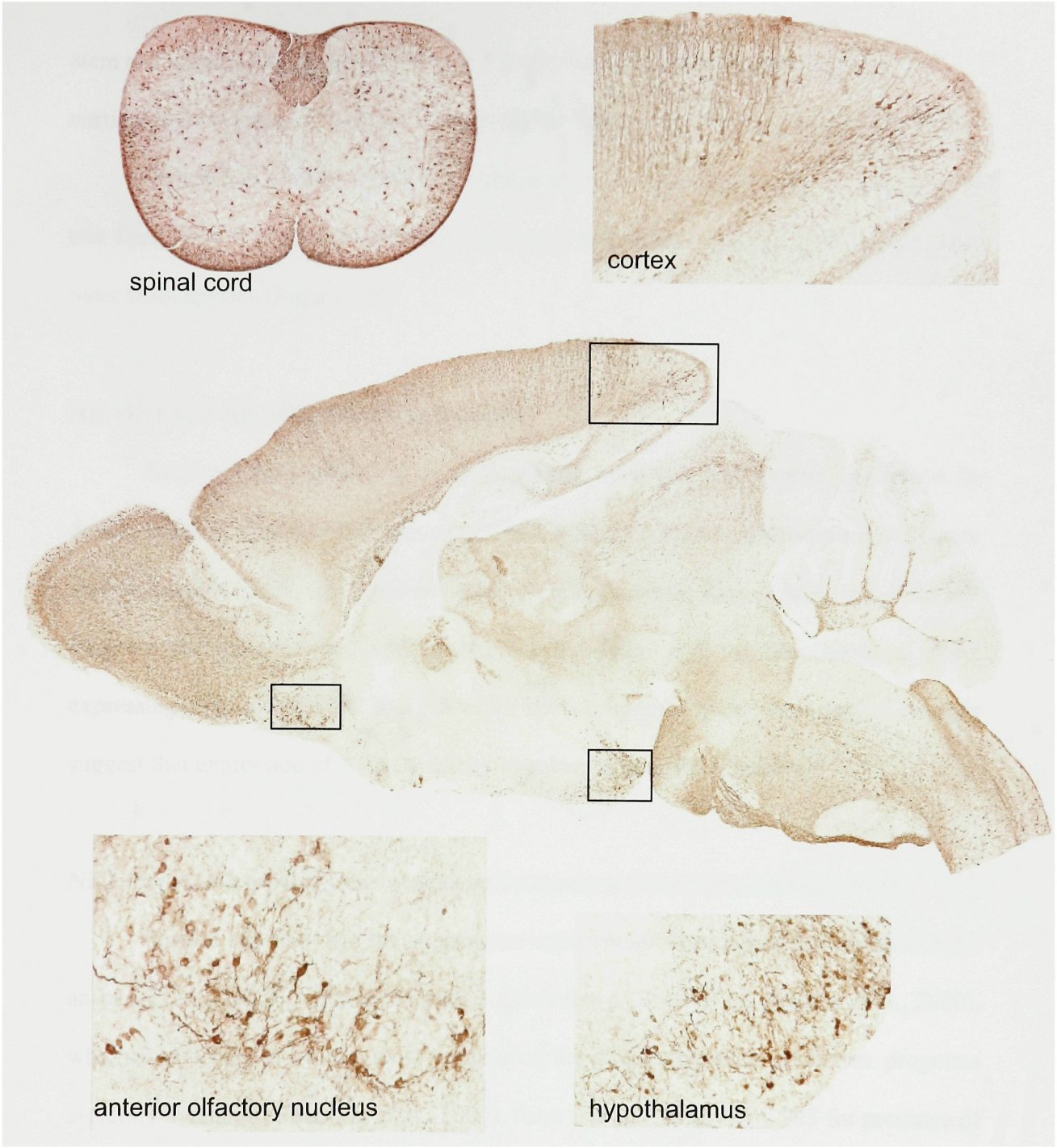


Figure 3-7. Mouse brain expression pattern of NKAIN2.

Brain expression data for NKAIN2 is available from GENSAT database. Several BAC mouse lines had identical expression patterns. NKAIN2 is expressed in multiple neurons in cortex, hypothalamus, brainstem and spinal cord.

stem and spinal cord. However, NKAIN3 expression in certain neurons, such as 5th cortical layer neurons or Purkinje cells, is higher than in other brain regions (Figure 3-8).

Expression pattern of NKAIN4 obtained from Allen Brain Atlas is similar to the one from BGEM. NKAIN4 is highly expressed in Purkinje cells, as well as neurons in most brain regions (Figure 3-9).

NKAIN1 and NKAIN2 are neuron-specific

Neuronal localization of NKAIN family members was further confirmed by double labeling of mouse brain sections with NKAIN1/2 specific antibody and a neuron-specific marker NeuN. As shown in Figure 3-10, cells expressing NKAIN1 and NKAIN2 (red) are also stained with NeuN (green). Taken together with the morphology of EGFP expressing cells in NKAIN2 and NKAIN3 BAC-transgenic mice, these results strongly suggest that expression of NKAIN family members is specific to neurons.

NKAIN protein topology and transmembrane domain sequence conservation

Several topology and transmembrane region prediction programs were used to get an insight into NKAIN protein structure. According to SignlaP 3.0 (Menne et al., 2000), which was found to be the most reliable of the signal sequence prediction programs currently available (Bendtsen et al., 2004), there is a probability of 0.993 for presence of a signal peptide in NKAIN proteins. Predicted cleavage site of the signal peptide is between amino acids 21 and 22 (Figure 3-1).

In addition to a signal peptide, all four NKAIN family members were predicted to have three putative transmembrane domains using multiple transmembrane region

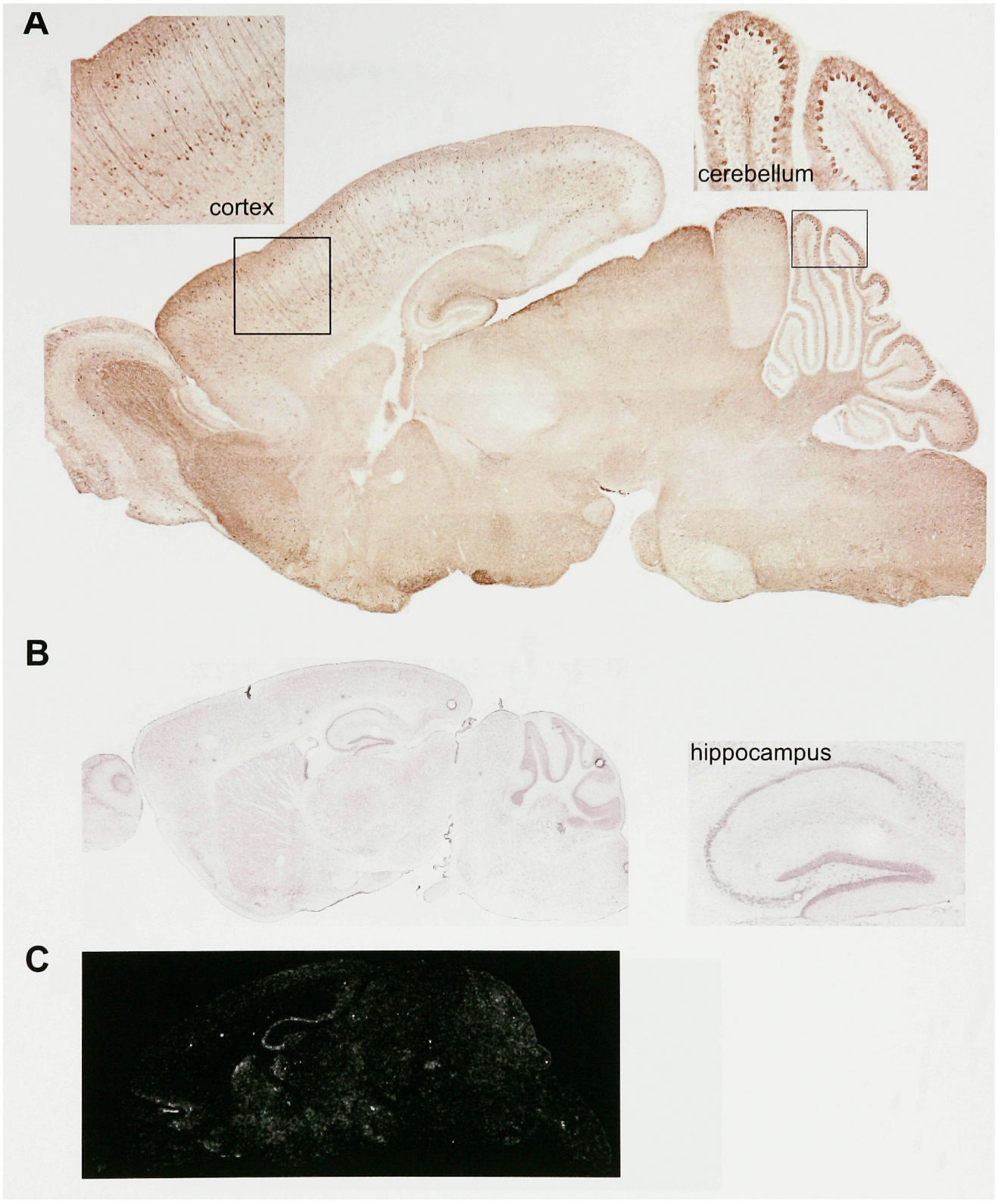


Figure 3-8. Mouse brain expression pattern of NKAIN3.
A. NKAIN3 expression data obtained from GENSAT BAC-transgenic mice. B. *NKAIN3* expression data from Allen Brain Atlas databases. C. *NKAIN3* expression data from Brain Gene Expression Map (BGEM). *NKAIN3* is expressed in Purkinje cells, pyramidal neurons in cortex and hippocampus.

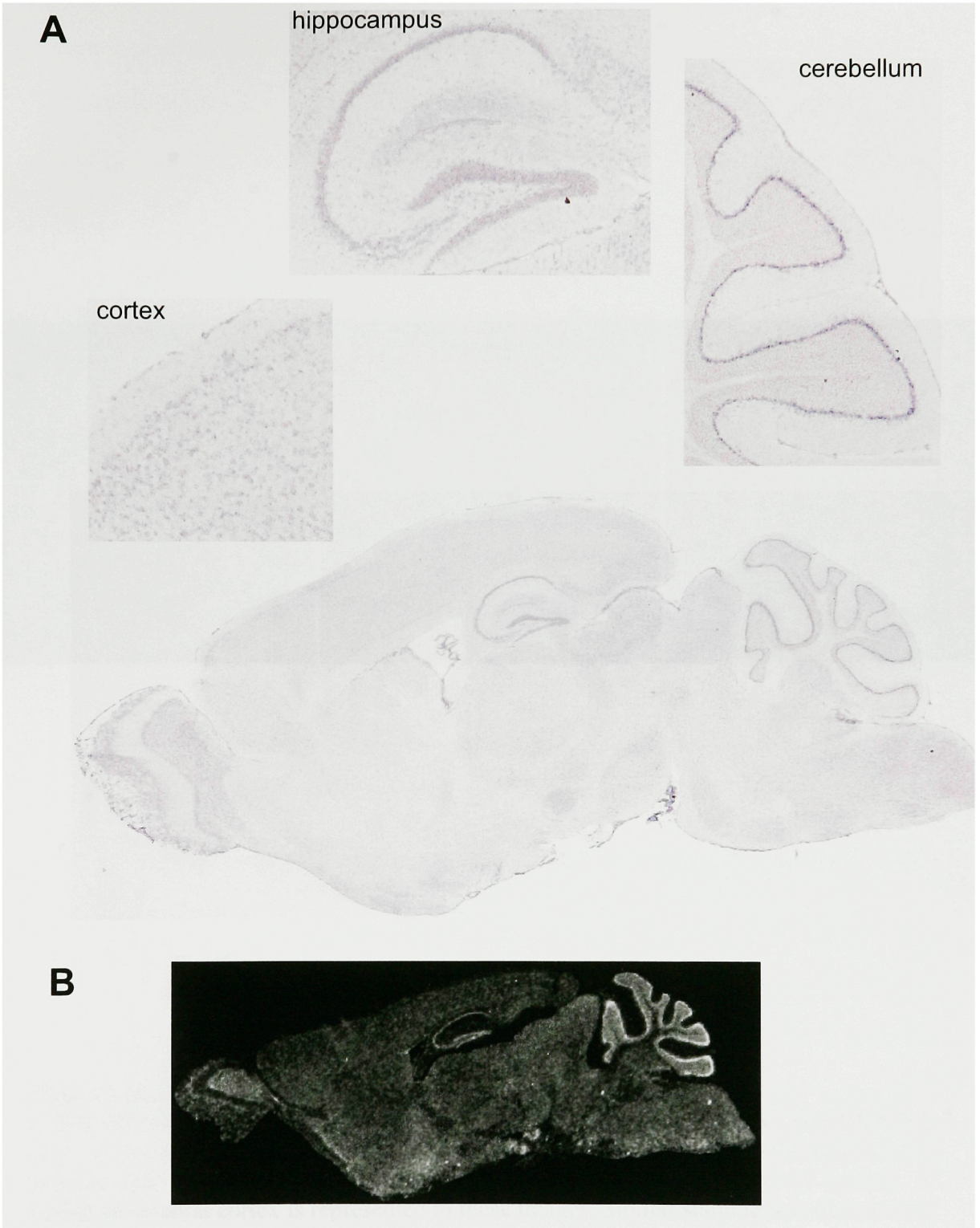


Figure 3-9. Mouse brain expression pattern of NKAIN4.
A. NKAIN4 expression data from Allen Brain Atlas databases. B. NKAIN4 expression data from Brain Gene Expression Map (BGEM). NKAIN4 is highly expressed in Purkinje cells, hippocampus and multiple cortical neurons.

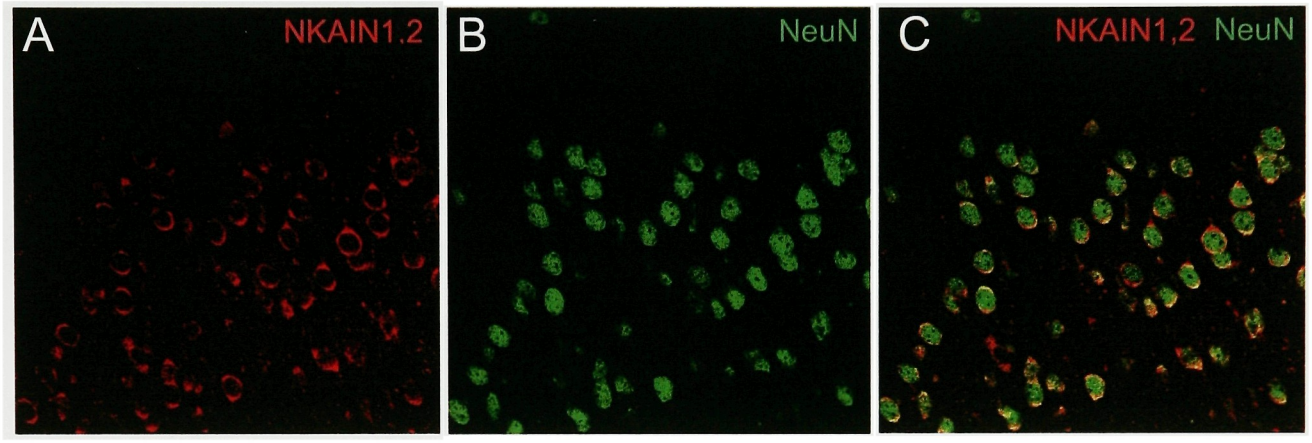


Figure 3-10. NKAIN1 and NKAIN2 are specific to neurons.
A. NKAIN1/2cterm antibody was used to label cells expressing NKAIN1 and NKAIN2 (red). B. Neurons were stained using anti-NeuN antibody (green). C. NKAIN1 and 2 labeling (red) is present on cells positive for neuron-specific marker NeuN (green). A section of cerebral cortex is represented in these images. Similar staining was observed in the other brain areas.

prediction programs such as HMMTOP (Tusnady and Simon, 2001), TMpred (Hofmann, 1993), SOSUI (Hirokawa et al., 1998), TMHMM 2.0 (Krogh et al., 2001). The first two transmembrane domains lie in 33-85aa region of NKAIN proteins and are predicted to be just 6-10 amino acids apart. Interestingly, this is the most conserved region of the protein. Sequence similarity between *Drosophila* and mammalian NKAINs is as high as 70-74% across the first two transmembrane domains (TM1 and TM2). The third transmembrane (TM3) domain is located at approximately 150-170aa position. Even though majority of prediction programs recognized this region as transmembrane, prediction scores in this area were generally lower comparing to the first two transmembrane regions. Only two first transmembrane domains are predicted for alternatively spliced form NKAIN2b. Similarly, TM3 segment is absent from NKAIN2c, NKAIN3b, NKAIN3c and NKAIN4c splice variants (Figure 3-2B). Thus, only the first two transmembrane domains are present in all NKAIN proteins, with rather uncertain prediction for the third transmembrane domain.

In order to confirm the results of membrane prediction programs and to determine membrane topology of NKAIN1, two different NKAIN1 specific antibodies were used for immunohistochemistry under permeabilizing and non- permeabilizing conditions. NKAIN1loop antibody recognizes the region after TM2, while NKAIN1,2cterm antibody is directed against the C-terminal tail of the protein. Both antibodies were used either with or without Triton X-100, a detergent permeabilizing cell membranes and allowing the antibody to enter cells and stain intracellular structures. Both antibodies labeled transfected cell membranes under either condition, thus suggesting that C-terminus and the loop after TM2 are extracellular (Figure 3-11A).

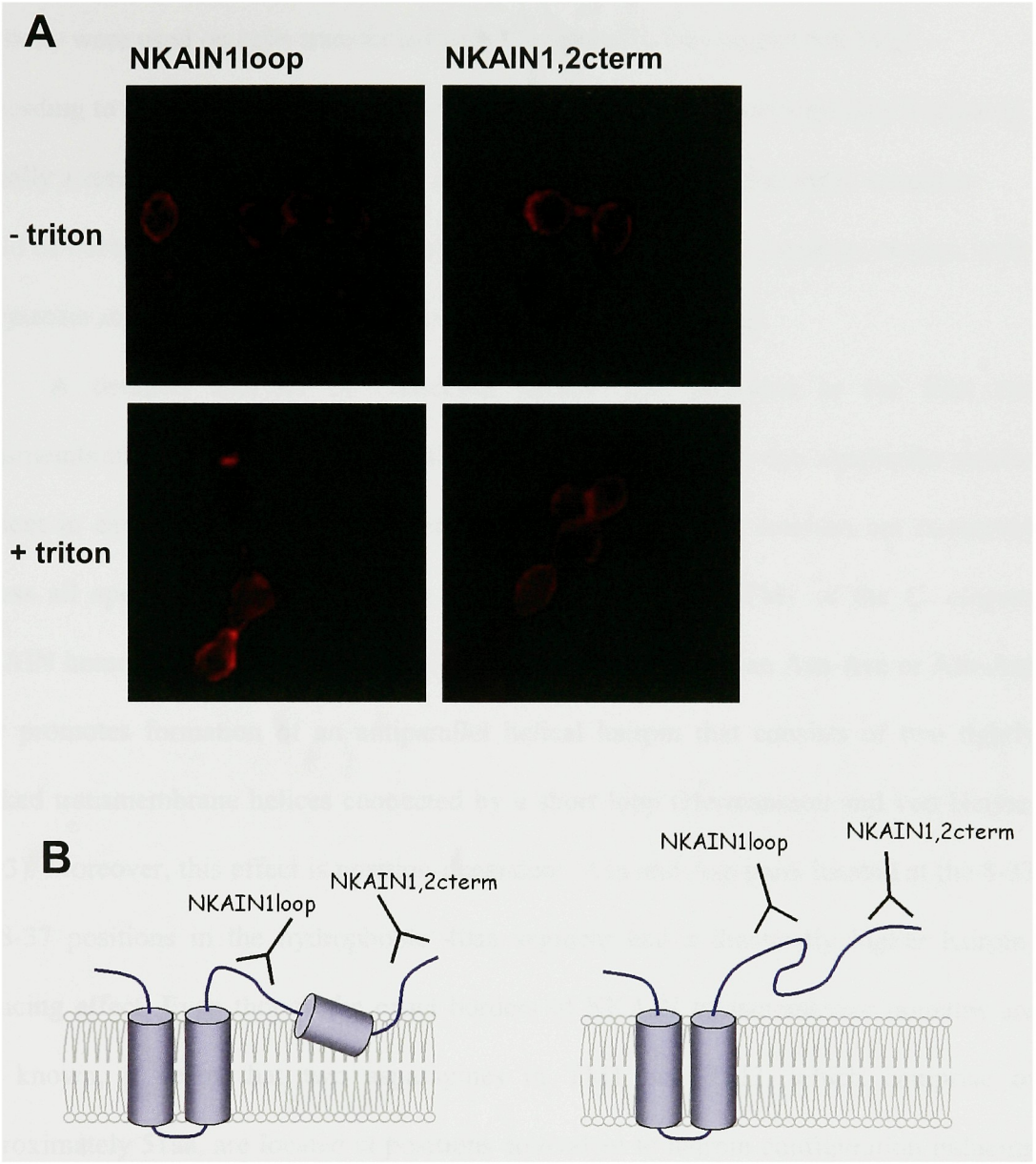


Figure 3-11. Membrane topology of NKAIN proteins.
A. Epitopes for the antibodies specific to the C-terminal domain (NKAIN1/2cterm) and to the TM2-TM3 loop (NKAIN1loop) are located extracellularly. HEK293T cells were transfected with NKAIN1 and labeled with both antibodies either with or without permealyzing cell with Triton X-100. B. Possible models of NKAIN membrane topology. Putative transmembrane region TM3 could be a re-entrant loop or entirely extracellular.

The same results were obtained when NKAIN1loop antibody and anti-flag antibody were used on cells transfected with C-terminally flag-tagged NKAIN1. According to these results, it is possible that the third putative transmembrane domain is actually a reentrant loop. Alternatively, the region between TM2 and the C-terminus could be completely extracellular (Figure 3-11B). Further detailed topology studies, such as cysteine accessibility method or introduction of tags, are needed.

A detailed analysis of conserved amino acid positions in the first two transmembrane domains reveals intriguing patterns. First, there is one asparagine residue present in each of the first two transmembrane domains. Both residues are conserved across all species, except for an Asn to Asp substitution in TM1 of the *C. elegans* NKAIN homolog. It has been previously shown that presence of an Asn-Asn or Asn-Asp pair promotes formation of an antiparallel helical hairpin that consists of two tightly packed transmembrane helices connected by a short loop (Hermansson and von Heijne, 2003). Moreover, this effect is position-dependent: Asn and Asp pairs located at the 8-33 or 8-37 positions in the hydrophobic 40aa segment had a drastically higher hairpin-inducing effect. Even though the exact borders of NKAIN transmembrane domains are not known, it seem that two asparagines in TM1 and TM2, which comprise of approximately 51aa, are located at positions equivalent to hairpin configuration inducing positions. Thus, it is likely that TM1 and TM2 of NKAIN proteins form two closely spaced transmembrane helices with Asparagine residues oriented towards each other (Figure 3-12).

Extremely high amino acid conservation between all vertebrate and *Drosophila* NKAINs makes it hard to pinpoint residues that might be important for NKAIN function.

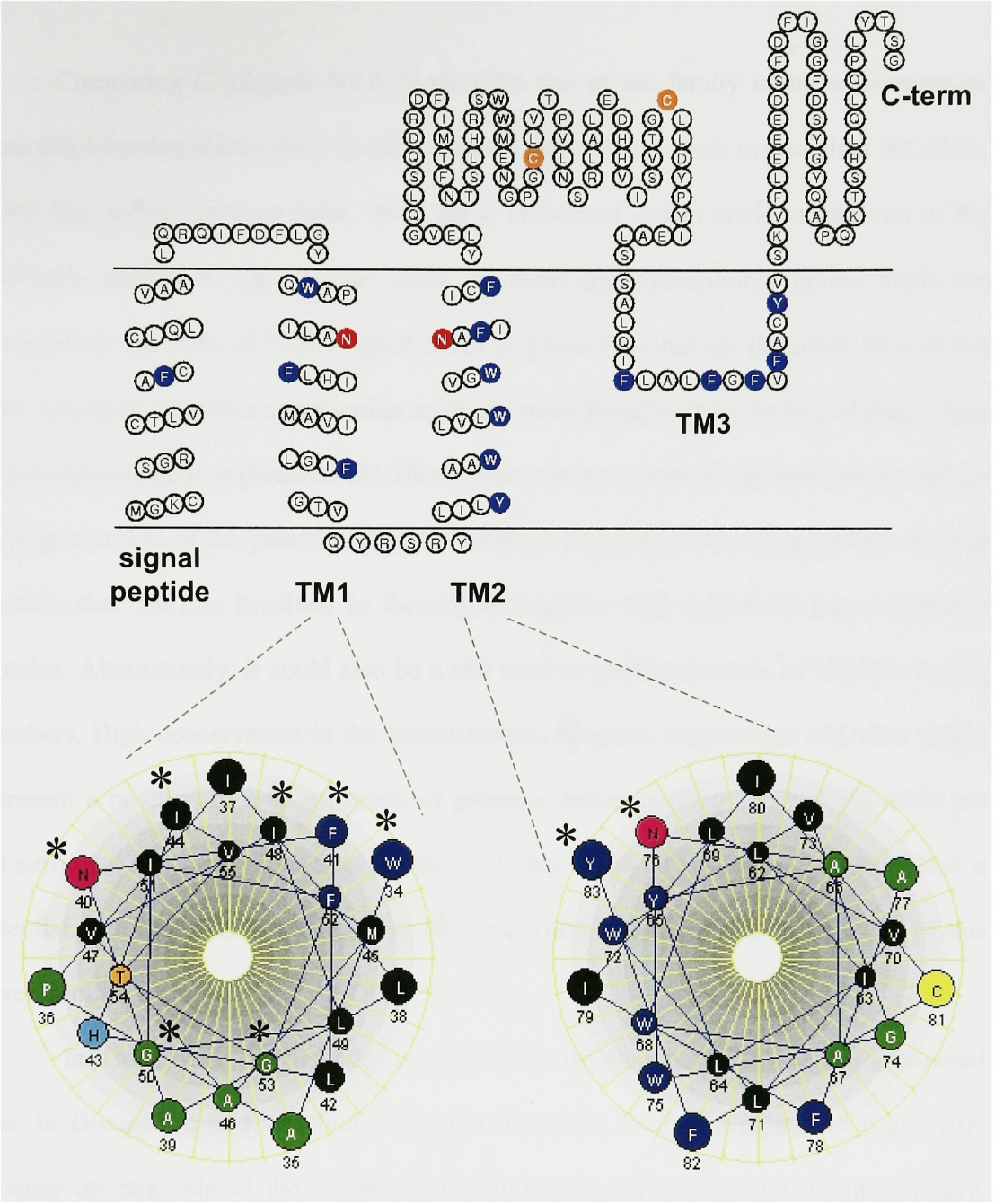


Figure 3-12. Sequence conservation of NKAIN transmembrane domains.
Asn residues that are likely to induce a helical hairpin formation between TM1 and TM2 are shown in red. Conserved cysteines are shown in orange. TM1 and TM2 are represented in helical-wheel models with the residues conserved between *C. elegans* and vertebrates marked by asterisks. Most of the conserved residues in TM1 are found on one side of the helix. Since the opposite side contains two conserved glycines as well as a number of small hydrophobic amino acids, it is likely to interact with another transmembrane helix. In vertebrate and *Drosophila* TM2, a number of aromatic amino acids (blue) are clustered on one side of the helix.

Comparing *C. elegans* NKAIN with the rest of the family is more informative, since this homolog shares only 43-45% amino acid similarity with mammalian NKAINs. In the first transmembrane helix, there are 6 conserved amino acids in addition to the previously discussed Asp residue. Four aromatic and hydrophobic amino acids are clustered on one side of TM1 (Figure 3-12) and two Glycines on the other face of the helix. Glycines and other small amino acids are often found at the interface of interacting transmembrane helices (Senes et al., 2004). Since these conserved glycines are located on the opposite side of the putative TM1-TM2 helical interaction site discussed above, it is possible that TM1 is involved in forming a complex with additional transmembrane proteins. Alternatively, it could also be a site used in multimerization of NKAIN family members. High conservation in the transmembrane regions suggest that NKAINs might represent a novel pore-forming group of proteins, however the individual NKAINs are not sufficient to form a channel, since they are only about 200 amino acids long. Thus, a potential multimerization site could be very important in determining the function of this novel family.

In TM2, there is only one conserved Tyr residue in addition to the conserved Asn. In *Drosophila* and vertebrate NKAINs, however, there is a cluster of 6 aromatic residues on one side of the second transmembrane helix (Figure 3-12). Interestingly, regions that are almost identical between vertebrate and *Drosophila* homologs lie in TM1 and TM2 segments proximal to the extracellular side of the membrane, as well as immediately adjacent to the membrane (Figures 3-1 and 3-12). This remarkable amino acid conservation between such distant organisms as *Drosophila* and mouse suggests that

regions critical for NKAIN protein function are located in transmembrane and perimembrane segments of NKAIN proteins.

Finally, there are two cysteines conserved across all species that might form a disulfide bond (Figures 3-1 and 3-12). Overall, the highest protein similarity between *C. elegans* NKAIN and other family members is found in the first transmembrane domain and adjacent N-terminal region, while high conservation between vertebrate and insect NKAINs extends throughout the protein sequence. It is possible that *Drosophila* NKAIN properties are closer to that of the vertebrate family members. This conservation pattern also suggests that functions common to NKAINs from all species are performed by the N-terminal part of the protein.

Analysis of gene-trap insertions at the *NKAIN1* locus

Gene-trap technology is based on integration of a specially designed gene-trap vector within genomic DNA, which creates fusion transcripts with endogenous genes and disrupts their normal expression (Skarnes et al., 1992). Since screens for the insertion sites can be performed in mouse embryonic stem cell lines without creating transgenic animals, a large number of genes in the mouse genome has been targeted and made available through International Gene Trap Consortium. One gene-trap insertion line RRE356 (BayGenomics) and three lines S19-12D1, S18-12D1 and S10-8E (FHRC) appeared to have transcript fusions between the reporter gene in the gene-trap vector and *NKAIN1* mRNA. The insertion sites of the RRE356 and S10-8E vectors were determined. Surprisingly, it became apparent that both of the gene-trap vectors were inserted in the opposite direction from *NKAIN1* transcription (Figure 3-13A).

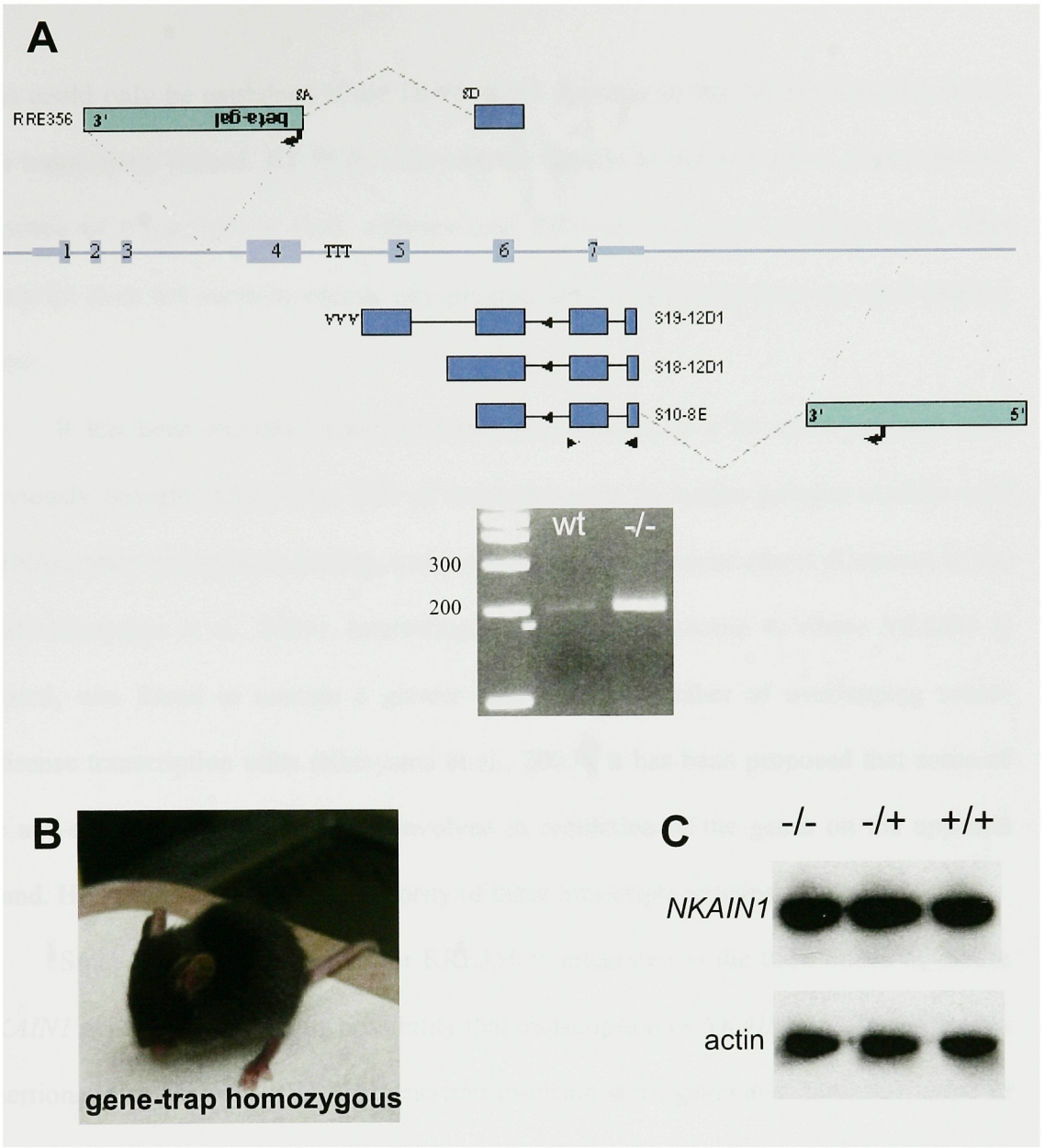


Figure 3-13. Gene-trap insertions in *NKAIN1* gene.
A. Gene-traps RRE356 and S10-8E (green) are inserted in the direction opposite to *NKAIN1* transcription. Fusion transcripts generated from the gene-trap insertions are shown in dark blue. Transcription from the antisense strand is confirmed by RT-PCR in wildtype and gene-trap mice. B. A very small number of mice homozygous for the gene-trap insertion were smaller then their littermates and had abnormal posture and gait. C. NKAIN1 expression is not affected by the gene-trap insertion. Extracts from mouse brains were used for Western blotting and stained with NKAIN1 specific antibody as well as anti-actin antibody as a loading control.

This could only be explained if the DNA strand opposite to the *NKAIN1* coding strand was transcribed. Indeed, RT-PCR with primers specific to the anti-sense strand showed presence of transcripts in both wildtype and RRE356 gene-trap carrying mice. This transcript does not seem to encode any protein, since it does not have an open reading frame.

It has been recently shown that this phenomenon is a lot more common than previously thought. More than 72% of transcript units in mouse genome overlap with mRNAs, often non-protein-coding, transcribed from the opposite strand (Carninci et al., 2005; Katayama et al., 2005). Interestingly, mouse chromosome 4, where *NKAIN1* is located, was found to contain a greater than average number of overlapping sense-antisense transcription units (Katayama et al., 2005). It has been proposed that some of the anti-sense transcripts might be involved in regulation of the genes on the opposite strand. However, function of the majority of these transcripts remains unknown.

Since a large gene-trap vector RRE356 is integrated in the third intron of mouse *NKAIN1* gene, there was still a possibility that transcription of *NKAIN1* is affected by this insertion. Mice carrying RRE356 gene-trap insertion were generated; however, most of the animals homozygous for the gene-trap insertion were not distinguishable from the wildtype. Two animals out of more than 25 homozygous mice were smaller than their littermates and appeared to have abnormal posture and gait (Figure 3-13B). However, brain extracts from the homozygous mice showed no difference in NKAIN1 expression on Western blot (Figure 3-13C). It is still possible that antisense transcripts affect NKAIN1 expression in a subset of cells, making it difficult to detect this change in expression in whole-brain extracts. Still, because of low penetrance and subtlety of the

phenotype, along with seemingly unaffected NKAIN1 expression in gene-trap homozygous animals, study of these mice was not pursued further.

NKAINs are targeted to intracellular structures in a subset of transfected cells

Expression of a construct containing the C-terminal 71 amino acid region of NKAIN1 N-terminally fused to a signal sequence and a yellow fluorescent protein Venus led to formation of large intracellular structures in HEK293T cells. Initial expression of this construct was targeted to plasma membrane, but within 24-48hr most of transfected cells became round with vesicular intracellular aggregates present (Figure 3-14A). It is unlikely that these structures are an artifact of a protein overexpression, since cells transfected in parallel with a similar Venus-tagged neurexin construct did not have the same morphology. These structures were also present in a small subset of cells transfected with full-length NKAINs, in addition to the previously described plasma membrane localization (Figure 3-14B).

Interestingly, formation of similar structures has been previously reported when Shaker potassium channels were co-expressed with SAP97 (Kim and Sheng, 1996) or after co-transfection of *Drosophila* Slowpoke calcium-dependent potassium channel with its interacting protein dSlob (Schopperle et al., 1998). These large intracellular membrane vesicles are ER derived (Tiffany et al., 2000) and represent a dynamic system moving within cells and undergoing divisions into smaller structures (Schopperle et al., 1998). Perhaps, similarly to potassium channels, NKAINs are also targeted to the same structures.

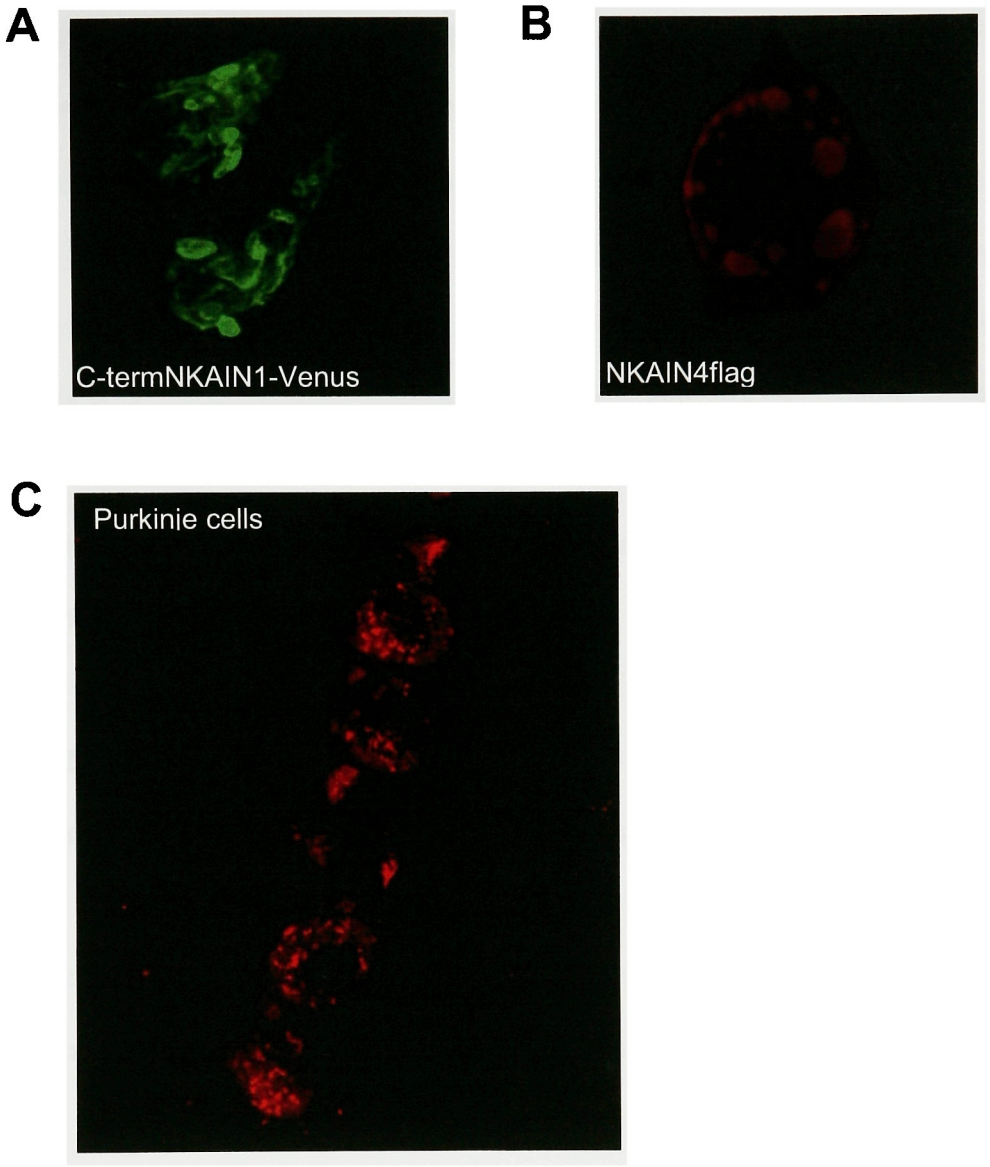


Figure 3-14. NKAINs localize to subcellular structures in some of transfected cells. A. C-terminal tail of NKAIN1 fused to YFP variant Venus localizes to large vesicular structures in transfected cells. A similar construct neurexin-Venus did not have this localization pattern. B. A small number of cells transfected with full-length NKAIN proteins contain the intracellular aggregates in addition to the membrane localization shown in the Figure 3-11. HEK293T cells were transfected with flag-tagged NKAIN4 and stained with anti-flag antibody. C. High magnification of Purkinje cells labeled with anti-NKAIN1 antibody reveals a pattern similar to the structures observed in transfected cells.

This possibility is especially intriguing since NKAINs might function as novel channels due to striking amino acid conservation of transmembrane regions between NKAIN family members. It would be interesting to see if any of the known proteins that affect the distribution of channels in heterologous cells, such as SAP97 or dSlob, would also induce redistribution of NKAIN proteins. Functional neuronal equivalent of these large intracellular structures is not known Strikingly, Purkinje cells stained with anti-NKAIN1 antibody appear to have similar vesicles in Purkinje cell bodies (Figure 3-14C). Internalization of receptors and ion channels is a well-known phenomenon involved in such important functions as synapse plasticity and drug desensitization. Perhaps a similar mechanism could regulate NKAIN function.

DISCUSSION

In this chapter, cloning and characterization of a novel family of four proteins NKAIN1, 2, 3 and 4 is presented. This family was selected based on its novelty, membrane localization, brain specificity and striking evolutionary conservation.

A remarkable sequence conservation of the first two transmembrane domains suggests that NKAIN proteins function within membrane bilayer. For example, they could modulate other membrane proteins. This hypothesis is supported by the fact that the first transmembrane domain contains two conserved glycines, which are often found at contact sites between transmembrane helices (Senes et al., 2004). This putative interaction site could participate in binding to other membrane proteins. Alternatively, NKAINs could form channel or pore structures. In this case, the second putative helix interaction site could be responsible for multimerization of NKAIN family members,

since individual NKAIN proteins are too short to form a complete pore. It is difficult to predict the stoichiometry of these complexes or if NKAINs would form homo- or heteromultimers. If these complexes exist, however, it is likely that NKAIN4 would be able to form homomultimers since it is the only isoform expressed in non-neuronal tissues.

All four NKAINs are expressed in a similar pattern in brain that consists of numerous neurons scattered across different brain regions. However, it is clear that expression of NKAINs is not ubiquitous. For example, NKAIN3 is expressed in pyramidal neurons in layers 2 and 5, while there are very few NKAIN3 expressing cells in other cortical layers. Although it is likely that many neurons might express several NKAIN proteins, some regions have a clear differential expression. For example, NKAIN2 is not expressed in hippocampal neurons or Purkinje cells, while other family members are prominently present in these cell types.

The diversity of NKAIN proteins expressed in a particular neuron is further enhanced by presence of splice variants. Except for NKAIN1, all other family members have several isoforms. A few interesting findings are evident from analysis of *NKAIN* exon-intron structure. First, the C-terminal region of the protein is alternatively spliced in most of the family members. Perhaps the N-terminal part of NKAIN proteins is most important for function, while the C-terminal tail is involved in their regulation and fine-tuning. Second, the signal sequence coding first exon is alternatively spliced in human *NKAIN2* and *NKAIN4*. It is possible that splice forms that have different signal sequence will be targeted to different regions of plasma membrane. Third, while *NKAIN4a* is expressed ubiquitously, the six exon form *NKAIN4b* is specific to brain and testis.

Existence of brain-specific alternatively spliced cassettes has been previously reported. There is a number of proteins that have neuron-specific splice variants such as c-src, clathrin light chain B, GABA-A and glycine receptors (Black, 1992; Jensen et al., 2000; Stamm et al., 1992). In several family members, an alternatively spliced form is suggested by presence of ESTs in humans, but is absent in mice. This is not uncommon, since less abundant splice forms have been previously found to be species-specific (Modrek and Lee, 2003). It is not clear at this point if these rare splice forms of genes are functional or represent aberrant splicing evens. For example, it has been suggested that low abundance splice forms are an evolutionary dead-end since they encode unstable proteins (Homma et al., 2004). On the other hand, they might have a function specific for a narrow developmental time period or expresses in a very small subset of cells, thus appearing to express at a much lower level than the major splice form.

A mouse mutant with a low seizure threshold (*Szt1*) carrying a 400kb deletion that includes *NKAIN4* gene has been published (Yang et al., 2003). Homozygous mice die shortly after birth, suggesting that *NKAIN4* is not critical for early embryonic development. Interpretation of *Szt1* mouse seizure phenotype is complicated, since the deleted region includes two other genes (nicotinic acetylcholine receptor subunit, *Chrna4* and potassium channel, *Kcnq2*) previously implicated in convulsions and epilepsy (Singh et al., 1998; Steinlein et al., 1995). Overall phenotype of *Szt1* mice most closely resembles *Kcnq2* knockout mice. However, in addition to the *Kcnq2* phenotype, these mice also have a significant reduction in the number of hippocampal but not cortical neurons (Yang et al., 2003). Since *NKAIN4* is highly expressed in hippocampus, it is possible that the hippocampal defect in *Szt1* mice is caused by *NKAIN4* deletion.

NKAIN2 truncations are associated with neurological defects

NKAIN2 has been previously named *TCBA1* (T-cell lymphoma breakpoint associated target I) since it was identified as a gene disrupted by a 6q22 chromosomal breakpoint in two T-cell lymphoma/Leukemia cell lines (Tagawa et al., 2002). Chromosomal breaks in the *NKAIN2* gene were located after exon 2 and exon 3 in cell lines *HT-1* and *ATN-1* respectively. There are also two clinical cases that report *NKAIN2* disruption by a chromosomal translocation (Bocciardi et al., 2005; Yue et al., 2005). One patient, a male with developmental delays, difficulties in motor skills and expressive speech, recurrent infections, cryptorchidism, facial dysmorphism and hair hypopigmentation was found to have a balanced de novo t(1;6)(q32.2;q22.3) translocation. Chromosomal translocation in this patient truncates *NKAIN2* after the third exon (Yue et al., 2005). Another clinical report describes a patient with severe febrile and afebrile seizures, muscle weakness, psychomotor retardation, hypogonadism, microcephaly, hair hypopigmentation as well as face and hand dymorphisms. The patient also had cerebral atrophy of periventricular white matter (Bocciardi et al., 2005). The chromosomal translocation breakpoint t(2;6)(q24.3;q22.31) was also located in *NKAIN2*, truncating it after exon 4. No other genes were disrupted by these two translocations, suggesting that the phenotype of these disorders is due to *NKAIN2* truncation (Bocciardi et al., 2005; Yue et al., 2005). A severe neurological involvement found in both cases argues that NKAIN2 protein is critical for brain function. It is possible that the truncated *NKAIN2* transcripts are not stable, thus leading to NKAIN2 haploinsuficiency. Alternatively, the truncated form could also function as a dominant negative.

In addition to clustering of several chromosomal breakpoints in the *NKAIN2* gene, the copy number of intron 1 region has been shown to vary among unrelated healthy individuals (Iafrate et al., 2004). Taken together, these findings suggest that the genomic region of *NKAIN2* is highly unstable and might be predisposed to chromosomal rearrangements.

Several human disorders are linked to the *NKAIN1*, *2* and *3* loci

Three human disorders have been mapped to the chromosomal regions where *NKAIN* genes are located. First, a dominant intermediate form of Charcot-Marie-Tooth Type C maps to *NKAIN1* region at chromosome 1p35 (Jordanova et al., 2003). Charcot-Marie-Tooth disorder (CMT) is a hereditary neuropathy, characterized by atrophy and progressive weakness of limb muscles and sensory abnormalities. It was mapped in two large families to a 6.3cM linkage interval flanked by markers D1S2787 and D1S2830, with the maximum LOD score at the marker D1S233, which is close to *NKAIN1* (Figure 2-15A). Twelve genes in this region have been excluded by sequencing, making *NKAIN1* a good candidate for gene mutated in Charcot-Marie-Tooth disease. However, no mutations were found in the protein-coding region of *NKAIN1* in affected patients (Dr. Timmerman, personal communication). It is still possible that defects in the regulatory or splicing elements of *NKAIN1* are responsible for CMT disease phenotype. Therefore, until mutations are found in another gene in the linkage region, possibility of NKAIN1 dysfunction causing Charcot-Marie-Tooth disorder cannot be excluded.

Multiple linkage studies reported mapping of neuropsychiatric diseases, such as bipolar disorder and schizophrenia, to chromosome 6q.

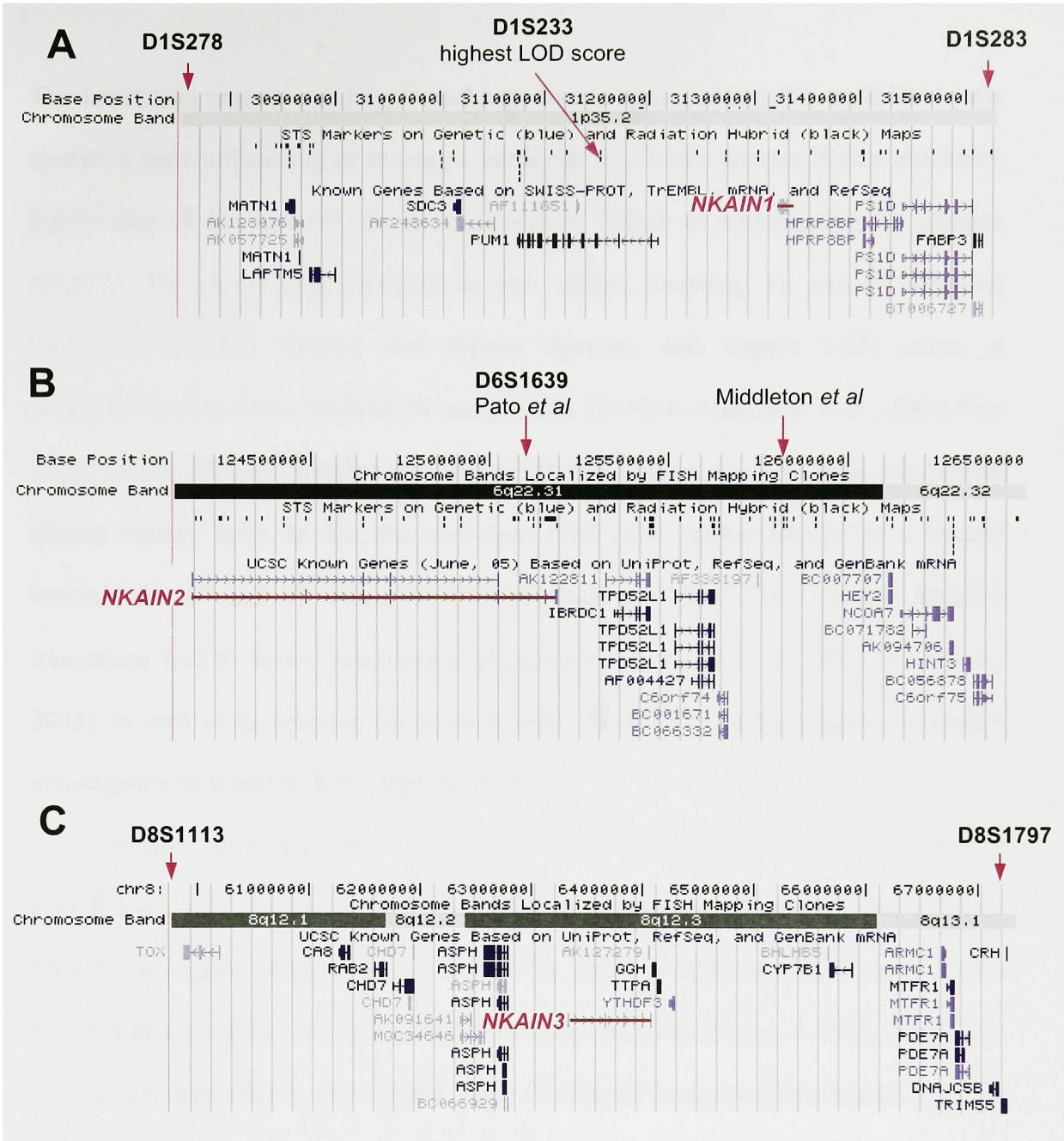


Figure 3-15. Several human diseases are linked to NKAIN1, 2 and 3 loci.
A. Dominant intermediate Charcot-Marie-Tooth disease was linked to 1p35 where NKAIN1 is located (Jordanova et al., 2003). B. Bipolar disease was linked to NKAIN2 locus at 6q22 in two studies involving multiple large Portuguese families (Middleton et al., 2004; Pato et al., 2004). C. Idyopathic torsion dystonia (DYT6) was mapped to the region containing NKAIN3 in two families (Almasy et al., 1997). Linkage regions as well as the highest LOD score markers are shown with arrows.

The implicated regions can be grouped into five loci, suggesting that each locus may contain a gene influencing or causing a particular psychiatric disease (Kohn and Lerer, 2005). One of the loci (F3) located at position 126Mb of chromosome 6q contains *NKAIN2*. This locus was identified in two studies involving 16 and 25 extended Portuguese families affected with bipolar disorder, with highest LOD scores at positions125Mb (marker D6S16339) and position 125.8Mb (Middleton et al., 2004; Pato et al., 2004). *NKAIN2*, which spans 124.2-125.2Mb region, overlaps with this bipolar disease linkage locus. In fact, the maximum LOD score marker D6S16339 is located between the 4^{th} and 5^{th} exons of *NKAIN2* (Figure 3-15B). The fact that *NKAIN2* truncations lead to severe neurological phenotypes (Bocciardi et al., 2005; Yue et al., 2005), as well as its neuronal localization make *NKAIN2* a good candidate for further investigating its possible role in bipolar disease.

Finally, idiopathic torsion dystonia (DYT6), characterized by sustained muscle contractions, was mapped in two large families to a region of chromosome 8 where *NKAIN3* is located (Almasy et al., 1997). The reported maximum LOD score region between markers D8S1113 and D8S1797 includes *NKAIN3* (Figure 3-15C), though the linkage region is actually much bigger, since all affected family members had an identical haplotype spanning 40cM region.

NKAIN2 truncation in patients with neurological dysfunction, possible linkage of *NKAIN* genes in several neuropsychiatric diseases along with neuronal expression of NKAIN family members strongly suggest that NKAIN proteins might be critical for neuronal function.

Chapter 4. NKAINs interact with the β subunit of the Na,K-ATPase

INTRODUCTION

Since NKAINs neither resemble any known proteins in the genome nor contain any known domains, their function cannot be predicted based on their protein sequence alone. Identifying interacting partners of a novel protein can often provide clues about biological pathways involving that protein. A search for proteins interacting with NKAINs revealed that they bind to the β subunit of the Na,K-ATPase.

Na,K-ATPase is composed of α and β subunits

Na,K-ATPase is a plasma membrane enzyme that is responsible for maintaining electrochemical gradient across the membrane by transporting two K^+ in and three Na^+ out of the cell in an ATP-dependent mechanism. Na,K-ATPase belongs to a family of P-type ATPases, of which only Na,K-ATPase and H,K-ATPase are heteromeric, that is requiring association of α and β subunits in order to form functionally active enzymes. The catalytic α subunit has 10 membrane spanning regions (Hu and Kaplan, 2000) and contains sites for ion, ATP and phosphate binding, as well as for drugs such as cardiac glycosides. The β subunit has a single transmembrane domain, short intracellular N-terminal and a large ectodomain containing N-linked glycosylation sites and three disulfide bridges. β2 also contains oligomannosidic glycans (Schmitz et al., 1993). The β subunit is responsible for structural and functional maturation of the enzyme (Ackermann and Geering, 1990; Geering et al., 1989). Numerous studies showed that β is required for

delivery and stabilization of the α subunit in the plasma membrane, since when expressed by itself, the α subunit is rapidly degraded in endoplasmic reticulum before reaching plasma membrane (Gatto et al., 2001; Geering et al., 1996; Jaunin et al., 1992). Interestingly, neither disulfide bridges nor glycosylation of the β subunit seem to have an effect on plasma membrane targeting or subunit assembly (Laughery et al., 2003; Tamkun and Fambrough, 1986). In addition to this stabilizing function, the β subunit has also been shown to affect enzyme properties, such as K^+ apparent affinity (Eakle et al., 1994; Geering et al., 1996; Jaisser et al., 1992a; Jaisser et al., 1994; Jaunin et al., 1993; Lutsenko and Kaplan, 1993; Schmalzing et al., 1992; Shainskaya and Karlish, 1996).

M7-M8 loop of α subunit interacts with the β subunit

The interaction of α and β subunits has been studied extensively. Using chimeric enzymes containing regions from α subunits of the Na,K-ATPase and non-heteromeric P-type ATPase SERCA1 (sarcoplasmic/endoplasmic reticulum Ca-ATPase), it was determined that 26 amino acids (NDVEDSYGQQWTYEQRKIVEFTCHTA) located in the extracellular loop between M7 and M8 transmembrane domains were sufficient for the interaction with the β subunit (Lemas et al., 1994a). The same extracellular loop was shown to interact with the β-subunit by yeast-two-hybrid. This interaction was abolished when four amino acids SYGQ within this 26 amino acid region were changed to alanines (Colonna et al., 1997). In another study, the overlapping four highly conserved amino acids DSYG (893-896) were shown to play a critical role in heterodimer assembly as well as enzyme activity, stability and interaction with K^+ ions (Becker et al., 2004). It was also shown that β interaction with the M7-8 loop is necessary for membrane insertion of the

M7 and M8 transmembrane domains of the α subunit (Beguin et al., 1998). Binding of the β subunit to this extracellular loop stabilizes and prevents the α subunit from degradation, possibly by masking the degradation signal located in the same region (Beguin et al., 2000). This 26 amino acid region, specifically the DSYG peptide, is highly conserved between all Na,K-ATPase and H,K-ATPase α subunits, which are the only P-type ATPase α subunits that form heterodimers with β subunits. This is consistent with results of multiple studies showing that H,K-ATPase β subunits can form dimers with Na,K-ATPase α subunits (Eakle et al., 1994; Horisberger et al., 1991; Noguchi et al., 1992). Indeed, both Na and H,K-ATPase β subunits bind to α subunits at the same 26aa region located in the loop between M7 and M8 transmembrane domains (Lemas et al., 1994b).

Several regions of the β subunit might interact with the α subunit

In contrast the α subunit, defining the exact interaction domain on β is more difficult, since several regions of the β subunit have been shown to be important for heterodimer formation. Initial studies involving cytoplasmic and transmembrane domain deletions as well as chimeras with dipeptidyl peptidase IV demonstrated that the ectodomain of the β subunit is necessary and sufficient for assembly with α subunit (Hamrick et al., 1993; Renaud et al., 1991). C-terminal truncations leaving just 96 amino acids of the extracellular domain weakened but did not disrupt this association (Hamrick et al., 1993). The interaction domain was further narrowed down to 63 amino acids just outside of the membrane, based on both yeast-two-hybrid and co-immunoprecipitation experiments (Colonna et al., 1997; Laughery et al., 2003). However, other regions of the

β subunit have also been implicated in the α-β assembly. For example, hydrophobic residues in the most C-terminal 10 amino acid segment or a conserved YYPYYG motif (Geering et al., 1993; Okamoto et al., 2000) were shown to participate in the heterodimer assembly (Beggah et al., 1993). The cytoplasmic N-terminus was also shown to interact, though this interaction was not critical for maturation of α subunits (Geering et al., 1996; Hasler et al., 1998). Finally, the transmembrane domain might also be in contact with the M8 of the α subunit, based on crosslinking studies (Ivanov et al., 2000; Or et al., 1999) and experiments involving substitution of conserved tyrosines that affected transport properties of the Na,K-ATPase (Hasler et al., 2001). Transmembrane region involvement is also supported by studies of truncated β subunits containing just 16 extracellular amino acids adjacent to cell membrane, which were sufficient for α and β interaction (Laughery et al., 2003). Taken together, results of these experiments demonstrate that while different regions of the β subunit contribute to association between subunits, the extracellular domain most proximal to the membrane is the most important region for the heterodimer stabilization.

Expression and properties of the Na,K-ATPase isozymes

There are four α and three β subunits. Each α subunit can assemble with any β subunit, thus creating a number of functionally distinct Na,K-ATPase isozymes (Blanco et al., 1995a; Blanco et al., 1995b; Schmalzing et al., 1997). Interestingly, isozymes containing identical catalytic α subunits but different β subunits also have different properties, since β subunit modulates K^+ and Na^+ affinity of the enzyme (Blanco et al., 1995a; Blanco et al., 1995b; Eakle et al., 1994; Eakle et al., 1995; Jaisser et al., 1992b).

For example, α2β2 had a slightly higher Na^+ affinity than α2β1, while sensitivity to ouabain, K^+ and ATP affinity and turnover rates remained the same (Blanco et al., 1995a). Similar results were obtained using α3β1 and α3β2 dimers (Blanco et al., 1995b). In addition to the diversity due to combination of different α and β subunits, there is evidence of additional isozymes created by alternative splicing. For example, a 554 amino acid truncated form of α is expressed in vascular smooth muscle (Medford et al., 1991). Truncated transcripts of α1 and β1 were also detected in human retina (Ruiz et al., 1995). Finally, it has been also shown that Na,K-ATPase forms tetramers in plasma membrane (Blanco et al., 1994; Norby and Jensen, 1989). Whereas physiological significance of it is not known, it is likely that multimerization of subunits will add to complexity of the sodium pump function.

While multiple combinations of α and β subunits are possible, the actual number of different Na,K-ATPases present in a particular cell is limited by expression pattern of its subunits. The most common isozyme is α1β1, which is believed to be a housekeeping enzyme responsible for maintaining ion gradient across the cell membrane. It is present in nearly all tissues, while the other forms of the sodium pump play tissue-specific roles (Blanco and Mercer, 1998). α2 is expressed in kidney, muscle, heart, adipocytes and brain (Herrera et al., 1994; Lavoie et al., 1997; Lucking et al., 1996; Lytton et al., 1985; McGrail et al., 1991; Zahler et al., 1992), while α3 is present in kidney, heart and is highly abundant in nervous tissues (Emanuel et al., 1987; Hieber et al., 1991; Lucking et al., 1996; McGrail et al., 1991; Orlowski and Lingrel, 1988; Wang et al., 1996). α4 isoform is testis-specific (Shamraj and Lingrel, 1994) and is involved in sperm motility (Woo et al., 2000). The β2 isoform is present in skeletal muscle and nervous tissues

(Lavoie et al., 1997; Peng et al., 1997; Shyjan et al., 1990; Watts et al., 1991), and β3 is expressed in retina, liver, stomach, small intestine, colon, testis, spleen, and lung (Malik et al., 1996).

Expression pattern of the Na,K-ATPase subunits in brain is highly complex: each subunit has a different but overlapping pattern of expression, with many cells and regions expressing more than one isoform (Herrera et al., 1994; McGrail et al., 1991). However, it has been shown that α3β1 isozyme is enriched in neurons, while glial cells preferentially express α2β2 (Brines and Robbins, 1993; Cameron et al., 1994; Watts et al., 1991).

In addition to differential expression between cell types, specific Na,K-ATPase isoforms might have different subcellular localization. It has been shown that while α1 is uniformly distributed over the surface of the plasma membrane, α2 and α3 are restricted to areas of the plasma membrane that come in close proximity to the endoplasmic or sarcoplasmic reticulum. This expression pattern corresponds to the distribution of plasma membrane Na/Ca exchanger (Juhaszova and Blaustein, 1997; Lencesova et al., 2004; Moore et al., 1993). These results suggest that α2 or α3 isoforms might regulate Na^+ and, indirectly, Ca^+ concentration in restricted sub-domains of the cell. Another example of differential subcellular localization is the redistribution of α3 and β2 from the body of photoreceptors at birth to inner segments and synaptic terminals later in development (Wetzel et al., 1999). Finally, targeting of P-type ATPases to a specific subdomain of the cell membrane is also demonstrated by specific localization of the Na,K-ATPase to the basolateral side of polarized epithelial cells, while the H,K-ATPases are targeted to the apical side (Dunbar and Caplan, 2001).

Pharmacological properties of different Na,K-ATPase isoforms from several species were studied extensively. In summary, the apparent affinity for Na^+ range in the following order $\alpha2\beta2 > \alpha2\beta1 > \alpha1\beta1 = \alpha3\beta2 > \alpha3\beta1$ and in the following way for the K^+ affinity: $\alpha1\beta1 > \alpha2\beta1 = \alpha2\beta2 > \alpha3\beta1 = \alpha3\beta2$ (Blanco and Mercer, 1998). Also, ATP affinity of α2 and α3 are higher than that of α1 (Blanco and Mercer, 1998). The results of these studies made it clear that the subtle differences between cation and ATP affinities of the sodium pump isoforms are essential for tailoring functions of the enzyme to specific physiological requirements. For example, since α1 and α2 have higher apparent affinity for cations comparing to α3 isoforms, α3 gets activated only when the ion concentrations are increased above resting state. This property of α3 is critical for neuronal function, where this subunit is especially abundant. When ion concentrations are increased dramatically after repeated firing of a neuron, α3 gets activated and helps restore the membrane potential, while α1 and α2 isoforms are working at saturation (Blanco and Mercer, 1998). The higher affinity for ATP also allows α3 to work in conditions of low ATP levels, induced by a prolonged neuronal activity. On the other hand, consistent with its housekeeping role, α1 should work at optimum rates under physiological conditions, but would be unable to increase its activity if physiological demands are raised (Crambert et al., 2000).

Na,K-ATPase subunits might have other functions besides ion transport

In addition to ion transport, some components of the Na,K-ATPase might also have other functions. For example, AMOG (adhesion molecule on glia), first described as a recognition molecule involved in neuron-astrocyte interaction in vitro (Antonicek et al.,

1987), was later found to be the β2 subunit of the Na,K-ATPase (Gloor et al., 1990). Adhesion functions of the β2 subunit are independent of the pump activity, since they were not blocked either by ouabain or by reducing temperature to 4°C (Gloor et al., 1990). At least one binding partner for β2 has been identified as basigin (Heller et al., 2003), a member of the Ig superfamily of recognition molecules shown to be important in learning and memory (Naruhashi et al., 1997), blood-brain barrier formation (Risau et al., 1986) and more recently found to be a component of the γ-secretase complex involved in Alzheimer's disease (Zhou et al., 2005). Initially, only β2 was though to possess adhesion function, since β2 and not β1 induced neurite outgrowth of cerebellar neurons in culture (Muller-Husmann et al., 1993). However, in a recent report, β1 was shown to bind β-N-acetylglucosamine (GlcNAc)-terminating oligosaccharides in a K^+-dependent manner as well as participate in neural cell aggregation (Kitamura et al., 2005). Thus, it is possible that all β subunits have some role in cell adhesion and recognition. It is still not clear, however, if this role is completely unrelated to the sodium pump function, or if the interaction of β subunits with other molecules is used to target Na,K-ATPase to a particular subcellular compartment.

It has also been suggested that Na,K-ATPase may play a role in unconventional secretion of FGF-2. FGF-2 is exported out of cells in ER/Golgi independent process that can be selectively inhibited by sodium pump blockers (Dahl et al., 2000; Florkiewicz et al., 1998). This process is independent of Na^+ and K^+ transport, since low concentration of external K^+ inhibits ion transport but not FGF-2 secretion (Florkiewicz et al., 1998). It has also been shown that FGF2 gets transported directly through the membrane without

unfolding, though the actual transport machinery that is required for this unconventional secretion is not known (Backhaus et al., 2004; Schafer et al., 2004).

Mouse knockouts of the Na,K-ATPase subunits

In an attempt to better understand the function of the Na,K-ATPase as well as to identify the function of different isoforms, several mice models lacking one of the components of the sodium pump have been generated to date. Mice deficient in β2 subunit appeared normal until postnatal day 15, when they began to exhibit motor coordination deficits, tremors and limb paralysis. As motor incoordination progressed, these null mice were not able to feed or drink and died within 2-3 days after onset of symptoms (Magyar et al., 1994). Histological analysis of these mice showed vacuoles and swollen cellular processes in brain stem, thalamus, striatum and spinal cord, but showed no abnormalities in cerebellum and hippocampus, even though these areas express β2 at high levels in wildtype brains (Magyar et al., 1994). In addition, massive apoptotic photoreceptor cell death was observed in retina at postnatal day 16 (Magyar et al., 1994; Molthagen et al., 1996). It was suggested that vacuolar structures appear as a result of reduced sodium pump activity and osmotic imbalance, though the ATPase activity of whole homozygous brain homogenates appeared to be normal (Magyar et al., 1994). The deficits in motor coordination, lethality and vacuolar abnormalities, but not photoreceptor degeneration, were rescued when β1 was knocked-in in the β2-null mice (Weber et al., 1998). Thus, β1 subunit can for the most part substitute for the β2 functions if expressed in the appropriate cell types.

α1 null mice are not viable, and α1 +/- mice appear normal but have hypocontractile heart (James et al., 1999). In contrast, hearts from the heterozygous α2 mutants were hypercontractile, suggesting that α2 is a regulator of calcium in the heart (James et al., 1999). Homozygous α2 null mice died shortly after birth and were unable to breath (Ikeda et al., 2003; Moseley et al., 2003). In one study, this breathing defect was explained by abnormal rhythm activity of neurons in the respiratory center, since brain and lung development, muscle contractility and neuromuscular transmission of the α2 mutant mice appeared to be normal (Moseley et al., 2003). In an independent set of experiments, it was shown that neurons in the respiratory center are dysfunctional due to increased intracellular Cl^- concentration. It was suggested that α2 plays a role in Cl^- homeostasis in neurons, since Na,K-ATPase α2 immunoprecipitated with neuron-specific K-Cl transporter (KCC2) (Ikeda et al., 2004). Another study showed neuronal cell death in amygdala and piriform cortex of the α2 null mice brains (Ikeda et al., 2003). Accordingly, heterozygous α2 mutants showed increased fear and anxiety behaviors. The ouabain-sensitive uptake of glutamic acid and GABA into crude synaptosomal fraction was impaired, while the same activity was not affected in astrocytes from the homozygous mutants. Based on these results, Ikeda et al. proposed that the α2 subunit in neurons contributes to clearance of neurotransmitters. In summary, disruption of the sodium pump function affects a variety of important physiological functions, making it difficult to determine which process is mainly responsible for phenotypes the Na,K-ATPase mouse mutants.

Human diseases cause by the Na,K-ATPase α subunit mutations

A number of mutations in the α subunits of the Na,K-ATPase were found to cause disease in humans. Recently, two missense mutations (L764P, W887R) in α2 were found in patients affected with familial hemiplegic migraine type 2 (FHM2), which is characterized by hemiparesis before the onset of migraine (De Fusco et al., 2003). Since then, 22 different missense mutations and 2 deletions were identified in a large number of families, making the *ATP1A2* the second major cause of familial hemiplegic migraine aside from *CACNA1A* which causes FHM1 (Jurkat-Rott et al., 2004; Kaunisto et al., 2004; Riant et al., 2005; Todt et al., 2005). The results of these studies also suggested a common pathogenesis in several similar disorders. For example, T348N mutation was found in two families with alternating hemiplegia of childhood, which is similar to FHM (Bassi et al., 2004; Swoboda et al., 2004). One of the missense mutations (G301A) caused seizures, coma and cerebellar signs such as ataxia, nystagmus, and dysarthria in addition to FHM (Spadaro et al., 2004). Another mutation (R689Q) was identified in the family in which FHM partially co-segregated with benign familial infantile convulsions (BFIC) (Vanmolkot et al., 2003). BFIC is a dominant form of benign childhood epilepsy that begins at age 3 to 12 and disappear within the first year of life (Vigevano, 2005). These findings suggest that at least some of the forms of epilepsy might be caused by mutations in the components of Na,K-ATPase.

α3 is also mutated in human disease. In a recent report, six missense mutations were identified in the *ATP1A3* gene in seven unrelated families affected with Rapid-onset Dystonia Parkinsonism (RDP, DYT12) (de Carvalho Aguiar et al., 2004). RDP is an autosomal-dominant disease, characterized by a dystonia and parkinsonism with a sudden

onset, often following a physical or emotional stress such as fever, exposure to heat, prolonged exercises, emotional stress or childbirth (Dobyns et al., 1993).

Regulation of the Na,K-ATPase

Majority of the previously identified proteins interacting with the Na,K-ATPase bind to the intracellular side of the enzyme. These include cytoskeletal proteins, such as spectrin, actin, adducin, pasin and ankyrin (Therien and Blostein, 2000), which target the sodium pump to the appropriate membrane compartment. Na,K-ATPase activity is regulated by a number of hormones, such as corticosteroids, catecholamines and peptide hormones, which act through signaling mechanisms involving PKA and PKC (Therien and Blostein, 2000). According to numerous studies in transfected cells, PKA phosphorylates the α subunit at the Ser938 and either increases or decreases the activity of the pump. However, a more recent study based on the predicted structure of the Na,K-ATPase argues that this phosphorylation site is inaccessible to PKA *in vivo* (Sweadner and Feschenko, 2001).

A family of seven single transmembrane domain proteins FXYD1-7, named for the conserved amino acid motif, has been recently identified. They exhibit tissue-specific distribution and alter transport properties of the Na,K-ATPase by changing the affinities for Na^+ and K^+ ions (Garty and Karlish, 2005). The single transmembrane domain of FXYD proteins fits in the groove formed by the M2, M6 and M9 transmembrane domains of the α subunit (Li et al., 2004).

Recently, the first extracellular modulator of the Na,K-ATPase was discovered. This novel protein, MONaKA, binds to the extracellular domain of the β subunit of the

sodium pump (Mao et al., 2005). MONaKA was initially identified as a mammalian homolog of the *Drosophila* protein dSlob, a slowpoke calcium-dependent potassium channel binding protein. However, MONaKA did not bind mammalian Slowpoke channels; instead, it bound Na,K-ATPase β subunits and was found to modulate sodium pump activity (Mao et al., 2005). Activity of the Na,K-ATPase was slightly decreased when it was co-expressed with MONaKA in transfected cells, though more reliable electrophysiological assays are needed to confirm this effect (Mao et al., 2005).

RESULTS

NKAIN1 interacts with the Na,K-ATPase β1 subunit by yeast-two-hybrid assay

In order to find proteins interacting with NKAIN family members, the C-terminal tail of NKAIN1 was used as a bait to screen a mouse brain cDNA library (Figure 4-1A). Out of 170,000 clones screened, six independent clones corresponded to the sequence of Na,K-ATPase β1 subunit. All six clones encoded different C-terminal fragments of β1, starting from positions 181, 189, 190, 193, 194 and 249 (Figure 4-1B). The shortest interacting clone of the β1 consisted of just 56 most C-terminal amino acids of this 304 amino acid protein. These interacting constructs were then retested by co-transformation with NKAIN1 bait in yeast cells. The C-terminal tails of NKAIN2 and 4 also interacted with the β1 constructs. NKAIN3 as well as the alternatively spliced form of NKAIN4 were not tested since they do not contain this C-terminal domain.

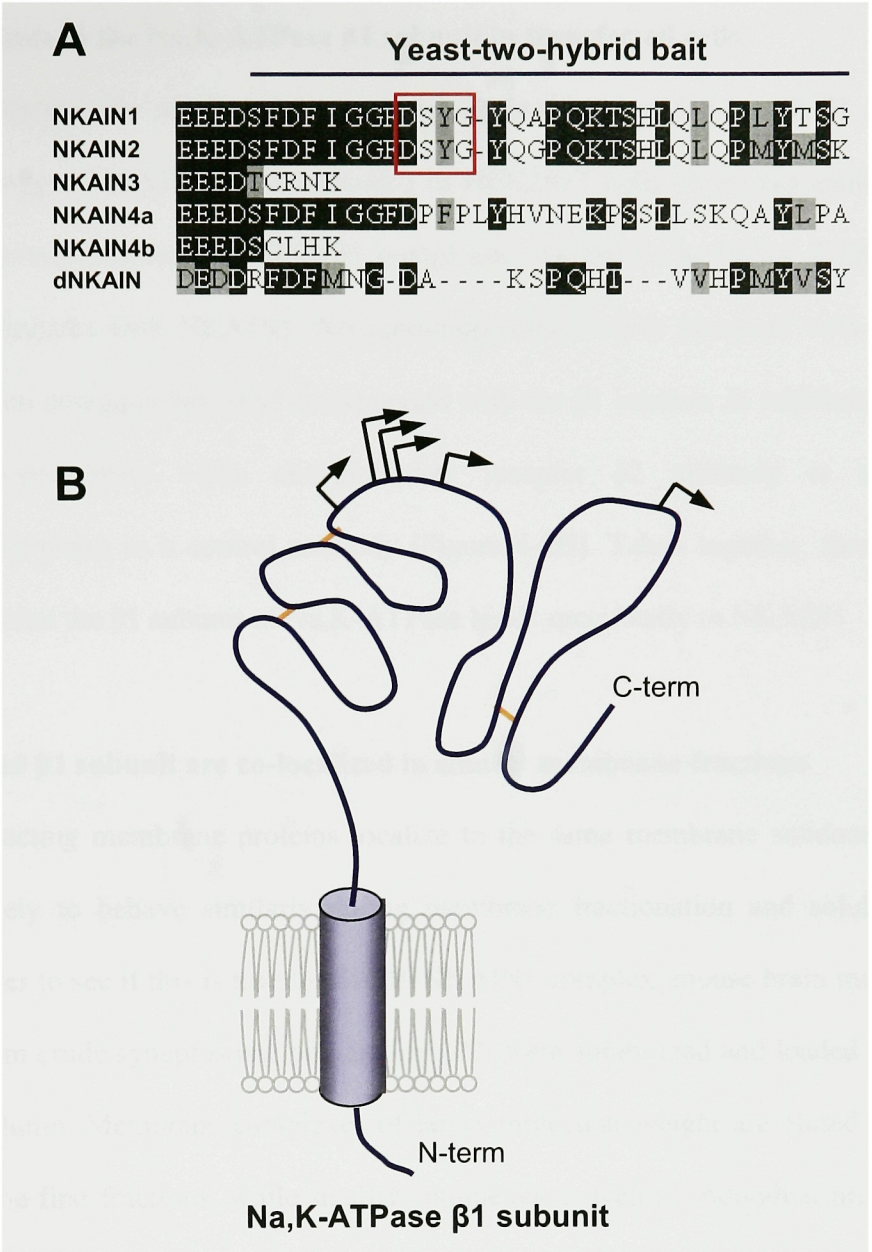


Figure 4-1. NKAIN1 interacts with the Na,K-ATPase β1 subunit by yeast-two-hybrid.

A. Multiple alignment of the C-terminal regions of mouse NKAINs. The C-terminal tail (177-207aa) of NKAIN1 was used to screen a mouse brain library. DSYG peptide, identical to the motif present in M7-M8 loop of the Na,K-ATPase α subunit, is boxed in red. 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 marked with arrows. The shortest interacting construct contained the last 56 amino acids of the β1 subunit. Three cysteine bridges are shown in yellow.

NKAIN1 binds to the Na,K-ATPase β1 subunit in transfected cells

To confirm the results of the yeast-two-hybrid screen, full-length β1 and full-length flag-tagged NKAIN1 were expressed in HEK293T cells. Anti-flag antibody was used to immunoprecipitate NKAIN1-β1 complexes. As shown in Figure 4-2A, β1 co-immunoprecipitates with NKAIN1. No immunoprecipitation is observed with anti-flag antibody when untagged NKAIN1 is expressed with the β1 subunit. In addition, β1 does not immunoprecipitate when anti-glutamate receptor δ2 antibody is used for immunoprecipitation as a control antibody (Figure 4-2B). Taken together, these results demonstrate that the β1 subunit of Na,K-ATPase binds specifically to NKAIN1.

NKAIN1 and β1 subunit are co-localized in similar membrane fractions

Interacting membrane proteins localize to the same membrane subdomains and thus are likely to behave similarly during membrane fractionation and solubilization steps. In order to see if this is true for the β1-NKAIN1 complex, mouse brain membranes obtained from crude synaptosome preparation (P2) were solubilized and loaded on a gel-filtration column. Membrane complexes of larger molecular weight are eluted from the column in the first fractions, while smaller complexes as well as individual proteins are found in the later fractions. Western blot membrane with the column fractions and solubilization steps (kindly provided by Dr. Selimi) was blotted with anti-NKAIN1 and anti-β1 antibody. As seen from Figure 4-2C, both NKAIN1 and β1 are distributed in the

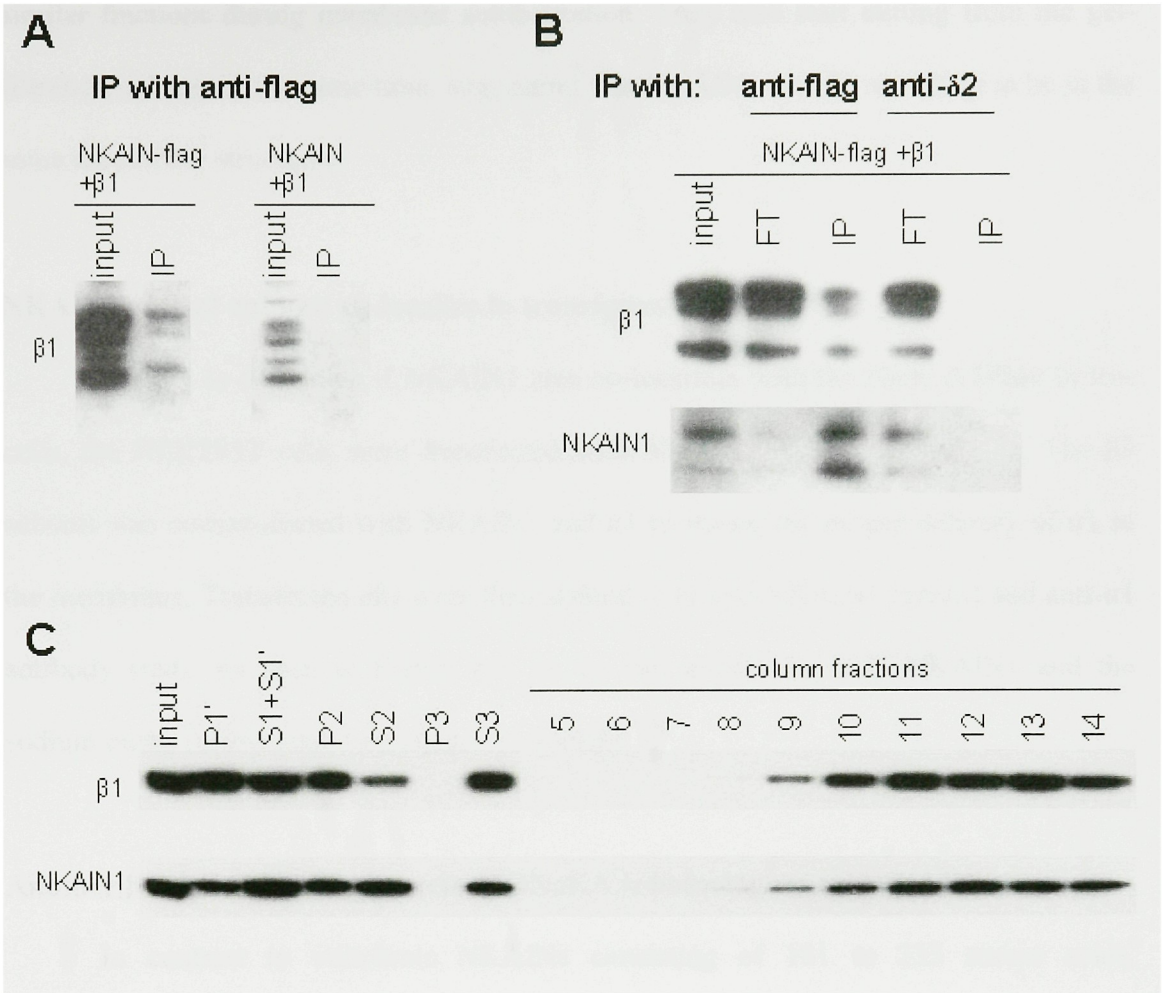


Figure 4-2. NKAIN1 interacts with the Na,K-ATPase β1 subunit in transfected cells. A. When the β1 subunit and flag-tagged NKAIN1 are expressed in HEK293T cells, β1 can be immunoprecipitated with anti-flag antibody. No immunoprecipitation was observed when non-tagged NKAIN1 is expressed with β1 in transfected cells. B. β1 immunoprecipitated together with NKAIN1flag when anti-flag antibody is 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. C. NKAIN1 and β1 localize to the same fractions after size-fractionation of mouse brain membranes. P2 fraction of crude synaptosome preparation was solubilized with Triton X-100 and loaded on a gel-filtration column. The Western blot membrane with the solubilization steps and column fractions was kindly provided by Dr. Selimi.

similar fractions during membrane solubilization. They also start eluting from the gel-filtration column at the same time, suggesting that NKAIN1 and β1 are likely to be in the same membrane structures.

NKAIN1 and α1 subunit co-localize in transfected cells

Finally, to determine if NKAIN1 also co-localizes with the Na,K-ATPase in live cells, the HEK293T cells were transfected with NKAIN, α1 and β1 subunits. The β1 subunit was co-transfected with NKAIN1 and α1 to insure the proper delivery of α1 to the membrane. Transfected ells were then stained with anti-NKAIN1 (green) and anti-α1 antibody (red). As seen in Figure 4-3, in the cell expressing both NKAIN1 and the sodium pump (arrowhead) two signals co-localize.

Another β subunit binding protein MONaKA is homologous to dNKAIN

In contrast to vertebrate NKAINs consisting of 181 to 233 amino acids, *Drosophila* homolog (dNKAIN) is 658 amino acid long. Existence of a 458aa C-terminal tail in addition to the first 200 amino acids of NKAIN homology region raised a possibility that the function of this tail domain is performed by another protein in vertebrates. However, no homology to any known proteins could be detected in the C-terminal 458aa part by using BLAST, PHI- or PSI-BLAST search tools. Except for proline-rich stretches of amino acids, which are often involved in protein-protein interactions (Rath et al., 2005), this region did not contain any characterized domains. However, mystery of a missing protein part in vertebrates was suddenly solved, when a novel modulator of the plasma

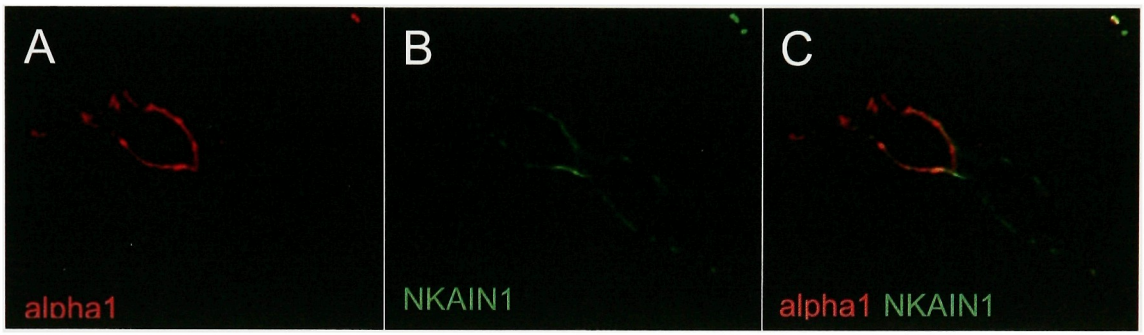


Figure 4-3. NKAIN1 co-localizes with α1 in transfected cells.
A,B. HEK293T cells transfected with NKAIN1, α1 and β1 subunits of the Na,K-ATPase were co-immunostained with anti-α1 antibody (red) and anti-NKAIN1antibody (green). C. In the cell expressing both α1 and NKAIN1 (arrowhead) both signals co-localize.

membrane Na,K-ATPase (MONaKA) interacting with the β subunit was discovered (Mao et al., 2005).

Alignment of MONaKa with the C-terminal tail of fly NKAIN using DIALIGN program revealed several stretches of homology with up to 33% amino acid identity (Figure 4-4). DIALIGN algorithm is designed to detect local protein similarities even if global sequences are not closely related (Morgenstern et al., 1998). Because of this feature, it performs best in detecting distant evolutionary related proteins or protein family members (Lassmann and Sonnhammer, 2002). Similarly to the C-terminal part of fly NKAIN, MONaKA is also proline-rich. One has to be careful in determining the significance of protein homology if the compared proteins contain an increased number of a certain amino acid, since high alignment scores could be an overestimation due to aligning of this common amino acid. However, in the case of MONaKa and dNKAIN, the amino acid identity in homologous regions was mostly due to amino acids other than proline.

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

Based on the yeast-two-hybrid results, the β subunit of the Na,K-ATPase is likely to interact with MONaKA and NKAIN using domains located in distinct regions of the ectodomain (Figure 4-5A). Thus, it is possible that MONaKA and mouse NKAIN bind β subunits together to form a complex functionally equivalent to *Drosophila* NKAIN. To test this hypothesis, HEK293T cell were transfected with myc-tagged MONaKA, NKAIN1 and β1 subunit of the sodium pump. Using anti-myc antibody, both β1 and

Figure 4-4. Alignment of MONaKA and C-terminal domain of *Drosophila* NKAIN. Regions of similarity between MONaKA and dNKAIN range from 27% to 33% amino acid identity. MONaKA region that contains the putative kinase domain and shows the highest similarity to dSlob (blue line) does not align with dNKAIN. DIALIGN program was used to produce this alignment.


MONaKA MAFMEKPPAGKVLLDDTVPLTAAVEASQSLQSHTEYIIRVQRGISAENSWQIVRRYSDFDLLNNSLQITGLSLPLPPKKLIGNMDREFIAERQRGLQNYLNVIMANHVL
dNKAIN T SIPTTSASATMQSNKHLQLQHQQPQ QNSLKLYHHQQQQQPKLHHFNKNYQLSGSNNNTLNNNNLHQRAPALLPPNTTNNRSASF
MONaKA SNCELLKKFLDPNNYSANYTEIALQQVSMFFRSEPKWEVVEPLKDIGWRIRKKYFLMKIKNQP
dNKAIN QTQSHPSNNHVTQRTGGEGSNCSSLRRHRQHHSKALVSPSPMSPQTTPSLSYASLQNSSPYLAGNSLSNSNYSIFQSPDSLQGSSHFARIHHKPK
Slob homology region
MONaKA KERLV VDVHCFGHLLYEMTYGRPPDSVPVDSFPPASSLAVVAVLESTLSCEACKNGMPTVSRLLQMPLFSDVLLTTSEKPQFKIPTKLKEALRIAKECIEKRLTEEQKQIHQHRRLT
dNKAIN PPKSDYPVSGEFNPGGNISSPVRPLDRLSRSLEDDEDNFSLQKFAPGEHGVTYVPFQSPTPNSLFLGENN
MONaKA RAQSHHGSEEERKRRKILARKKSKRSAVENSEEQPVKHSNSNNSGSGASSPLTSPSSPTPPSTAGLSSAL PPPPPPPPPPP
dNKAIN NSQPHLVFHTNSRSSPNNNAYPYDQSGLPSSLRMGSNSNARRPTHIPLPTVPMHNCQEVENDEDADGESEQDHDQMLTPPPPPLVRPHIH
MONaKA PPPAGPSPTSATEMPAPFLPQPVNGVNRGALLSSIQNFQKGTLRKAQTCDHSAPKIG
dNKAIN QRLGQAPYLDLSPEVAERYAIPSKLGPSLPIQVPLPVPHGSPMVRRSNRRPRPSNPVNFCDQIRATPPGYVVRAQSDDRLMEQVEADAAPHVNRRSGRGGSGQKTRPRSFCNSIVGVQG

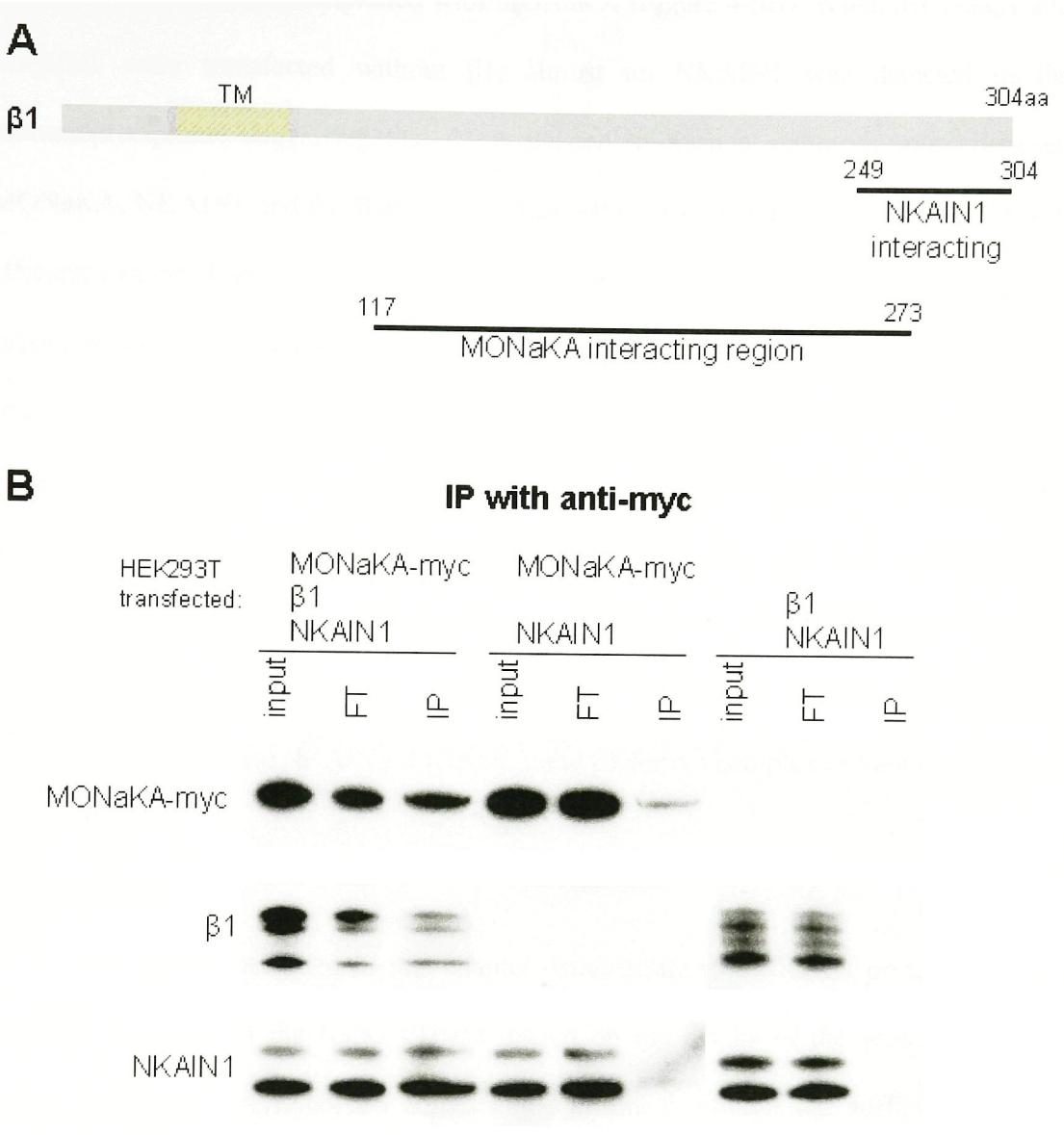


Figure 4-5. MONaKA, NKAIN1 and β1 form a complex in transfected cells.
A. Regions of the β subunit containing the NKAIN and MONaKA interacting domains only partially overlap. Amino acid positions of the interacting domains are marked. The numbering corresponds to mouse β1 subunit. B. MONaKA, NKAIN1 and 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. Very little 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.

NKAIN1 were immunoprecipitated with MONaKA (Figure 4-5B). 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 in experiments. It cannot be explained by lower transfection levels or the antibody coupling efficiencies, since the antibody-coupled beads are identical in both experiments and transfection level is equal as seen in the input and flow-through lanes. Thus, it is possible that binding of MONaKA to β1 induces a conformation change that make the myc epitope more accessible for the antibody used for immunoprecipitation. 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.

DISCUSSION

The results presented in this chapter demonstrate that NKAIN proteins interact with the β subunit of the Na,K-ATPase. Based on the results of the yeast-two-hybrid screen, the most C-terminal 56 amino acids of the β subunit are sufficient for the interaction with NKAIN1. Even though several regions of the β subunit have been implicated in the interaction with α subunit, most studies suggest that the region following the transmembrane domain of the β subunit is the major region contributing to α-β interaction. Thus, NKAIN1 and the α subunit interact with the β subunit at distinct sites (Figure 4-6).

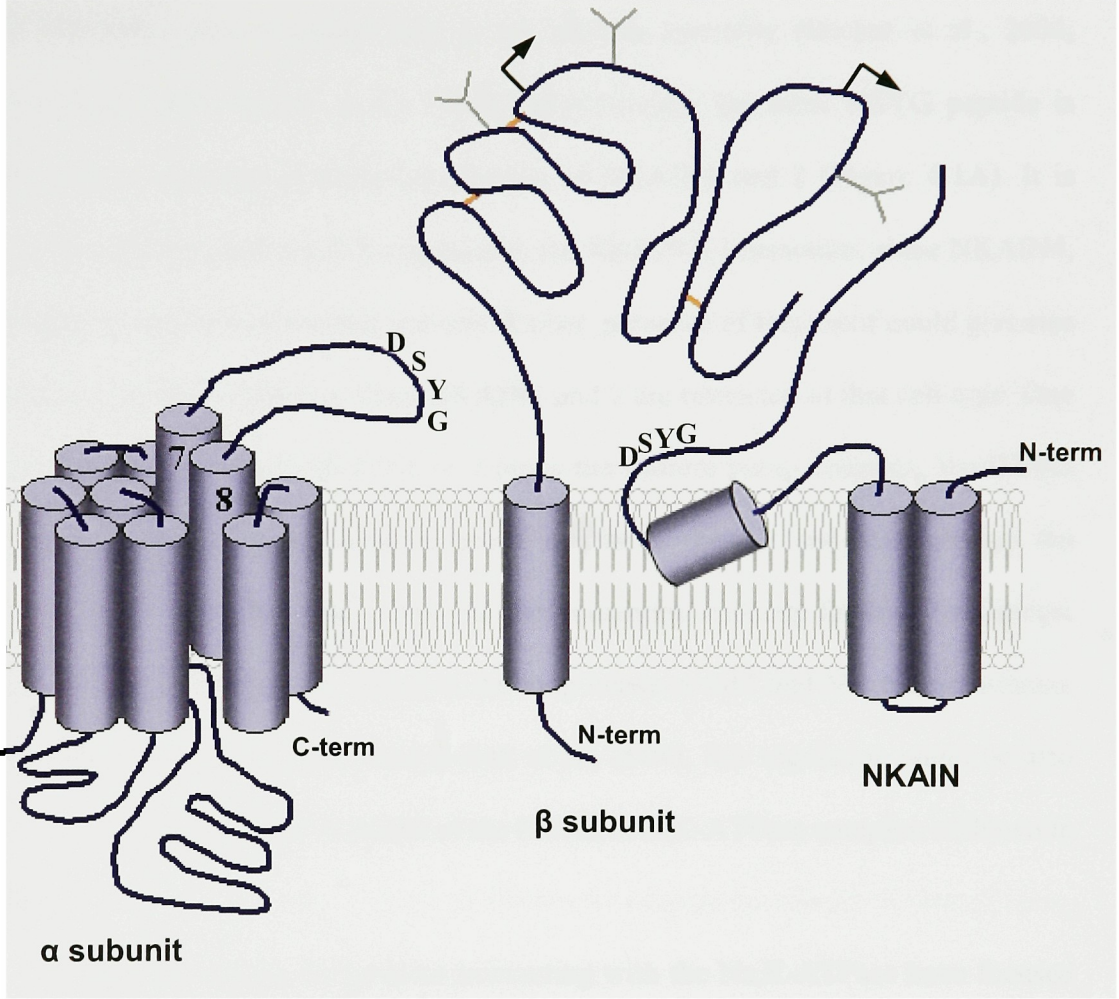


Figure 4-6. A model of the Na,K-ATPase and NKAIN complex.
C-terminal region of NKAINs interacts with the C-terminal domain of the β subunit of the Na,K-ATPase. The shortest and the longest NKAIN1 interacting c-terminal fragments of the β subunit identified in the yeast-two-hybrid screen are marked by 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.

The region of the α subunit responsible for the interaction with β subunits is more clearly defined. A stretch of 26 amino acids on the extracellular loop between M7 and M8 transmembrane domains of the α subunit, specifically highly conserved amino acids DSYG (893-896), play a critical role in the subunit assembly (Becker et al., 2004; Colonna et al., 1997; Lemas et al., 1994a). Interestingly, the same DSYG peptide is present in the C-terminal β-interacting domain of NKAIN1 and 2 (Figure 4-1A). It is unlikely that DSYG motif itself is responsible for NKAIN-β interaction, since NKAIN4, which lacks it, still interacts with β subunit. Rather, presence of this motif could give rise to functions specific to neurons, since NKAIN1 and 2 are restricted to that cell type. One could speculated that when NKAIN1 or 2 binds the sodium pump complex, the DSYG motif could compete with analogous motif on the α subunit and thus change the properties of the enzyme. Since two of the mutations causing familial hemiplegic migraine (W887R, E902K) are found in the loop between tM7 and M8 transmembrane domains (De Fusco et al., 2003; Jurkat-Rott et al., 2004), this regulation could be also important for human disease. A model of the NKAIN-Na,K-ATPase complex is shown in Figure 4-6.

Most of the screens for proteins interacting with the Na,K-ATPase have focused on the intracellular domains of the α subunit (Pagel et al., 2003). In fact, the only example of a protein binding to the sodium pump extracellularly is the recently discovered novel protein MONaKA, which interacts with the ectodomain of the β subunit (Mao et al., 2005). Remarkably, MONaKA protein sequence appears to have regions of similarity to the C-terminal domain of *Drosophila* NKAIN. MONaKA contains a truncated putative protein kinase domain in the central portion of the protein that is the

region of the highest similarity between MONaKA and dSlob. The dNKAIN homology regions, however, lie outside of this domain (Figure 4-4). Sequence similarity between the C-terminal domain of dNKAIN and MONaKA is about 30%, which is much lower than the sequence conservation between the N-terminal domain of dNKAIN and vertebrate NKAIN family members. Domain families sharing similar function typically have 30-40% sequence identity between their members (Aloy et al., 2003). However, it has been recently shown that the number of amino acids responsible for the formation of similar protein folds in otherwise unrelated sequences is even lower than previously thought (Socolich et al., 2005). Since MONaKA and *Drosophila* NKAIN share stretches of 27-33% amino acid identity, the number of identical residues between MONaKA and dNKAIN would be more than sufficient to induce formation of a protein fold that could carry out similar functions in flies and mammals. Prediction that the C-terminal part of *Drosophila* NKAIN is functionally related to MONaKA was further confirmed by immunoprecipitation experiment showing that mammalian NKAIN, MONaKA and the β1 subunit form a complex in transfected cells. These results are also supported by the fact that NKAINs and MONaKA are likely to interact with the β1 subunit at distinct sites, since the 156aa region of the β1 subunit containing putative MONaKA-interacting domain only partially overlaps with the 56aa NKAIN-interacting region (Figure 3-5A). Since the N-terminal part of dNKAIN is highly homologous to mammalian NKAINs, while the C-terminus is similar to MONaKA, it is likely that dNKAIN function is performed by two separate proteins in mammals – mammalian NKAIN and MONaKA (Figure 4-7).

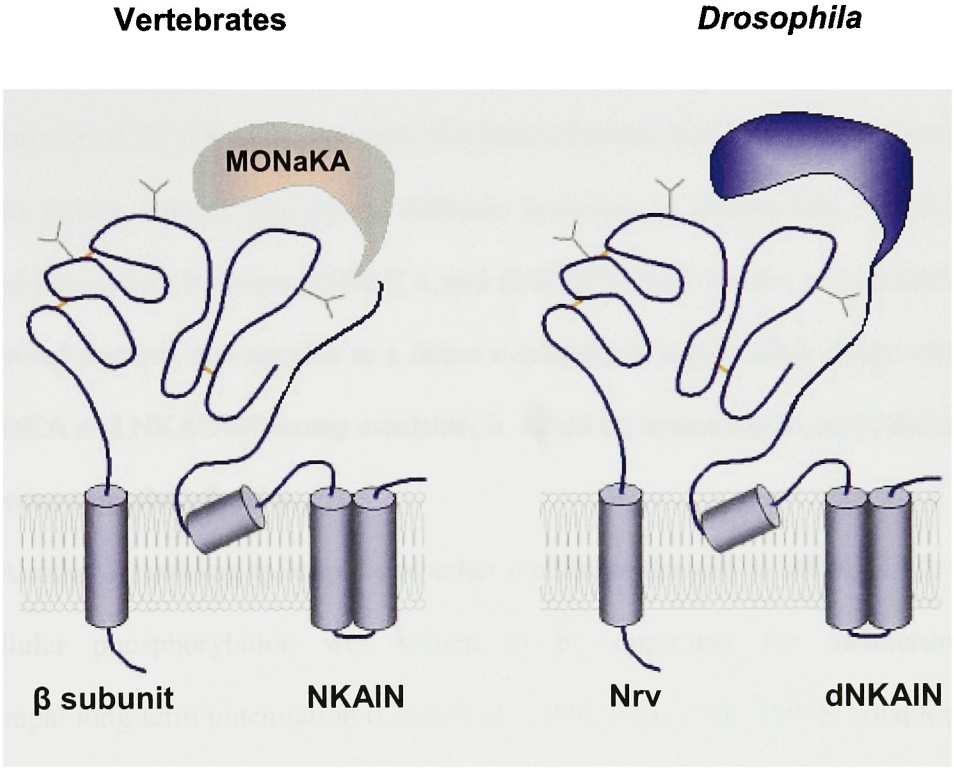


Figure 4-7. Model of the NKAIN-β complex in *Drosophila* and in mammals.
MONaKA binds to a distinct region on the β subunit of the Na,K-ATPase and is similar to the C-terminal domain of dNKAIN. Functions of MONaKA and mammalian NKAINs could be performed by a single protein dNKAIN that combines both domains.

There are many examples of a function carried out by two proteins containing separate functional domains in some species, while the equivalent function in another organism belongs to a protein that has both of these domains fused. For example, two structural domains of a eukaryotic enzyme imidazole glycerophosphate synthase are encoded in separate genes in archea (Aloy et al., 2003). These proteins are usually the result of gene fusion or gene fission events during evolution (Snel et al., 2000). It is not clear, however, if fly NKAIN represents this kind of event, since MONaKA contains an additional kinase domain and has a different homolog in *Drosophila.* Alternatively, amino acid similarity between MONaKA and dNKAIN could be due to presence of yet undiscovered domain, and not due to a direct evolutionary relationship. When structures of MONaKA and NKAINs become available, it would be interesting to see if this domain is also present in other proteins.

Another interesting question is whether the kinase domain in MONaKA is active. Extracellular phosphorylation was shown to be important for maintenance of hippocampal long-term potentiation (Chen et al., 1996; Fujii et al., 1995), synaptogenesis (Muramoto et al., 1994) and neurite outgrowth (Nagashima et al., 1991). Several candidate kinases have been proposed to carry out extracellular phosphorylation, such as CK1 and CK2 (Walter et al., 2000) and ecto-PKC (Wirkner et al., 2005); however, it is possible that other extracellular kinases exist. Perhaps MONaKA can phosphorylate Na,K-ATPase subunits, NKAINs or other proteins present in the sodium pump complex. Alternatively, the kinase domain in the central region of MONaKA might be inactive, since it is missing about 40% of the putative kinase domain. In this case, the dead-kinase part of the protein could serve as a scaffold to support the β1 subunit interacting domain.

This possibility is more likely, since the kinase domain is absent from the C-terminal part of the *Drosophila* NKAIN homolog.

MONaKA has been shown to modulate the Na,K-ATPase activity. However, the functional effect of MONaKA was rather minor, since there was only 19% reduction in the $^{86}Rb^{+}$ uptake in transfected cells and 30-38% inhibition of the Na,K-ATPase activity in *in vitro* assays (Mao et al., 2005). It is possible that results obtained in these experiments differ from *in vivo* sodium pump modulation properties since NKAIN was absent from these essays. To test this hypothesis, electrophysiological recordings from oocytes expressing either individual components or full sodium pump complex are currently under way in collaboration with Professor Kaethi Geering at the University of Lausanne, Switzerland. These experiment would also provide information about functional effect of NKAINs and MONaKA on Na,K-ATPase properties, such as ion affinities and ouabain binding.

Chapter 5. Drosophila NKAIN affects membrane excitability

INTRODUCTION

Results in the previous chapter show that NKAINs interact with the Na,K-ATPase, but the functional effect of this interaction is not known. There are several uncharacterized *Drosophila* mutants with P-elements inserted in *Drosophila NKAIN* (*dNKAIN*) gene available from public stock centers. Analyzing these mutants might provide insight into function of mammalian NKAINs, since dNKAIN is highly homologous to vertebrate NKAIN family members. The phenotype of fly mutants with P-element insertions in *dNKAIN* gene along with the phenotype of flies expressing dNKAIN in all neurons suggest that dNKAIN plays a role in membrane excitability, possibly by stimulating the activity of the *Drosophila* Na,K-ATPase.

Na,K-ATPases in *Drosophila*

The *Drosophila* homolog of the α subunit of the Na,K-ATPase is ATPalpha (Lebovitz et al., 1989). According to *Drosophila* Exon Database (Lee et al., 2004), *ATPalpha* gene has 3 alternative transcription start sites, two alternative termination exons and 6 exon cassettes, which leads to a number of alternatively spliced forms of this gene. Sequencing of *Drosophila* genome allowed identification of all P-type ATPases in this organism using a bioinformatic approach (Okamura et al., 2003). In addition to the *ATPalpha*, *Drosophila* also contains two more alpha subunits belonging to the Na,K-ATPase and H,K-ATPase family (Okamura et al., 2003). Similarly to mammalian α subunits, these subunits contain the conserved β interacting motif, the phosphorylation

domain in the cytoplasmic loop and the ouabain-binding M1-M2 loop. However, transport properties of these two α subunits remain unknown.

There are six β subunits in *Drosophila* genome. Dmβ-1, Dmβ-2 and Dmβ-3 are most closely related to mammalian β subunits and were previously identified as Nrv1, Nrv2 (Sun and Salvaterra, 1995a; Sun and Salvaterra, 1995b) and Nrv3 (Paul et al., 2003). Dmβ-4, Dmβ-5 and Dmβ-6 remain uncharacterized (Okamura et al., 2003).

Expression of ATPalpha was detected in the muscle, nervous tissue, malpighian tubules and salivary gland (Lebovitz et al., 1989; Schubiger et al., 1994). Nrv1 is expressed in muscle, digestive system, Malpighian tubules as well as nervous tissues. Nrv2 had a much stronger expression in brain and thoracic ganglia, and was suggested to be the neuronal isoform (Sun et al., 1998; Xu et al., 1999). However, recent reports showed Nrv2 is specifically involved in epithelial structure formation (Genova and Fehon, 2003; Paul et al., 2003). Nrv3 expression has not been described.

Na,K-ATPase *Drosophila* mutants

Several reports showed that insertion of P-elements in the *ATPalpha* gene leads to bang-sensitivity, that is paralysis after a short vortex stimulus (Feng et al., 1997; Schubiger et al., 1994). Interestingly, none of the reported P-elements disrupt the open reading frame of the gene, since they insert in the first intron of the *ATPalpha* gene. However, P-element insertion in the intron leads to decreased expression of the protein, as shown for $ATPalpha^{2206}$ flies. These homozygous flies expressed only 30% of ATPalpha protein comparing to wildtype flies and showed bang-sensitive paralysis as well as increased sensitivity to ouabain (Schubiger et al., 1994). The stress-sensitive

phenotype is most likely to occur due to decrease in Na,K-ATPase function, since injecting wildtype flies with a sodium pump inhibitor ouabain phenocopied the *ATPalpha*^{2206} mutation (Schubiger et al., 1994)

Recently, two more Na,K-ATPase α subunit mutants *ATPalpha*^{DTS1} (*DTS1*) and *ATPalpha*^{DTS2} (*DTS2*) were shown to have point mutations in the *ATPalpha* gene, both causing amino acid changes at adjacent positions in M10 transmembrane segment of the α subunit (Palladino et al., 2003). Surprisingly, these mutants were temperature-sensitive and showed bang-sensitivity only when they were maintained at 28°C. In addition to temperature-sensitive paralysis, heterozygous flies had reduced lifespan, age dependant neurodegeneration and severe neuronal hyperexcitability. The dominant nature of these mutations was further confirmed by the fact that the phenotype revertants, generated by mutagenizing *DTS* flies, had deletions in the *ATPalpha* gene (Palladino et al., 2003). Even though hyperexcitability of *DTS* flies is consistent with inhibition of the sodium pump, it is not clear if the activity of the Na,K-ATPase is reduced in these flies. The mechanism by which point mutations in the 10th transmembrane segment could lead to decrease in Na,K-ATPase function is not known. The possibility of these mutations affecting trafficking of the enzyme was ruled out, since expression and localization of sodium pumps was normal in *DTS* flies (Fergestad et al., 2006). Since the phenotype of *DTS1* and *DTS2* flies is more severe than the null phenotype, it was proposed that these point mutations cause a dominant negative or gain-of-function phenotype, perhaps by incorporating mutant proteins into complexes with the normal pumps (Palladino et al., 2003).

In addition to the excitability phenotype described above, mutations in the *Drosophila* Na,K-ATPase components also cause defects in septate junctions of multiple ectodermally derived epithelia, such as trachea, salivary glands and epidermis (Genova and Fehon, 2003; Paul et al., 2003). Embryos with P-element insertions in *Nrv2* and *ATPalpha*, as well as *Nrv2*-null mutants had pronounced defects in septate junctions (Genova and Fehon, 2003; Paul et al., 2003). Only Nrv2 seems to be important for causing this phenotype, since Nrv1 or Nrv3 could not rescue the septate junction defect (Paul et al., 2003). Adult homozygous Nrv2 mutant flies were not viable. In order to form septate junctions, Na,K-ATPase forms a complex with previously identified junction components, such as Coracle, Neurexin, Gliotactin and Neuroglian (Genova and Fehon, 2003; Paul et al., 2003). Invertebrate septate junctions are molecularly and structurally homologous to vertebrate paranodal junctions, which are essential for axonal propagation of action potentials (Tepass et al., 2001). The results of these studies together with experiments showing mammalian β subunit involvement in cell adhesion (Antonicek et al., 1987; Gloor et al., 1990; Kitamura et al., 2005) suggest a role for the Na,K-ATPase in neuron-glia interaction. It is not clear at this point if these processes are dependant on ion transport by the Na,K-ATPase.

Temperature-sensitive fly mutants

Temperature-sensitive paralysis in *Drosophila* can be caused by several different mechanisms. First, decrease in sodium conductance leads to neuronal hypoexcitability affecting propagation of action potentials and thus causing paralysis of flies (Wu, 1992). Indeed, one of the first characterized temperature-sensitive mutants (*para*) had mutations

in the sodium channel (Loughney et al., 1989). A similar phenotype is also caused by mutations in proteins that affect expression and localization of sodium channels, such as *nap*^{ts}, *tipE*, *khx* or *axo* (reviewed in (Ganetzky, 2000). Firing of neurons could also be affected by defective synapse or neurotransmitter release machinery. For example, mutations in SNAP-25, syntaxin, NSF and dynamin lead to temperature-sensitive phenotype (Littleton et al., 1998; Pallanck et al., 1995; Rao et al., 2001; van der Bliek and Meyerowitz, 1991). Finally, mutations increasing membrane excitability also cause paralysis preceded by seizures and uncoordination when flies are exposed to higher temperatures. *Drosophila* temperature-sensitive mutants *sei* and *slo* are caused by mutation in HERG type and BK type potassium channels respectively (Atkinson et al., 1991; Wang et al., 1997).

RESULTS

Anti-dNKAIN antibody characterization

A polyclonal peptide antibody was developed against residues 183-197 of the *Drosophila* NKAIN homolog. The antibody was tested on HEK293T cells transfected with full-length dNKAIN. Similarly to mammalian homologs, dNKAIN is localized to plasma cell membrane in transfected cells, as shown in Figure 5-1A.

When used for Western blotting on extracts from HEK293T cells transfected with full-length dNKAIN, the antibody recognized a 75kD band that is the predicted size of *Drosophila* NKAIN. The same band was also detected in fly head extracts, though there were many background bands present (Figure 4-1B). To confirm the specificity of the antibody, a peptide competition assay was carried out. When anti-dNKAIN antibody

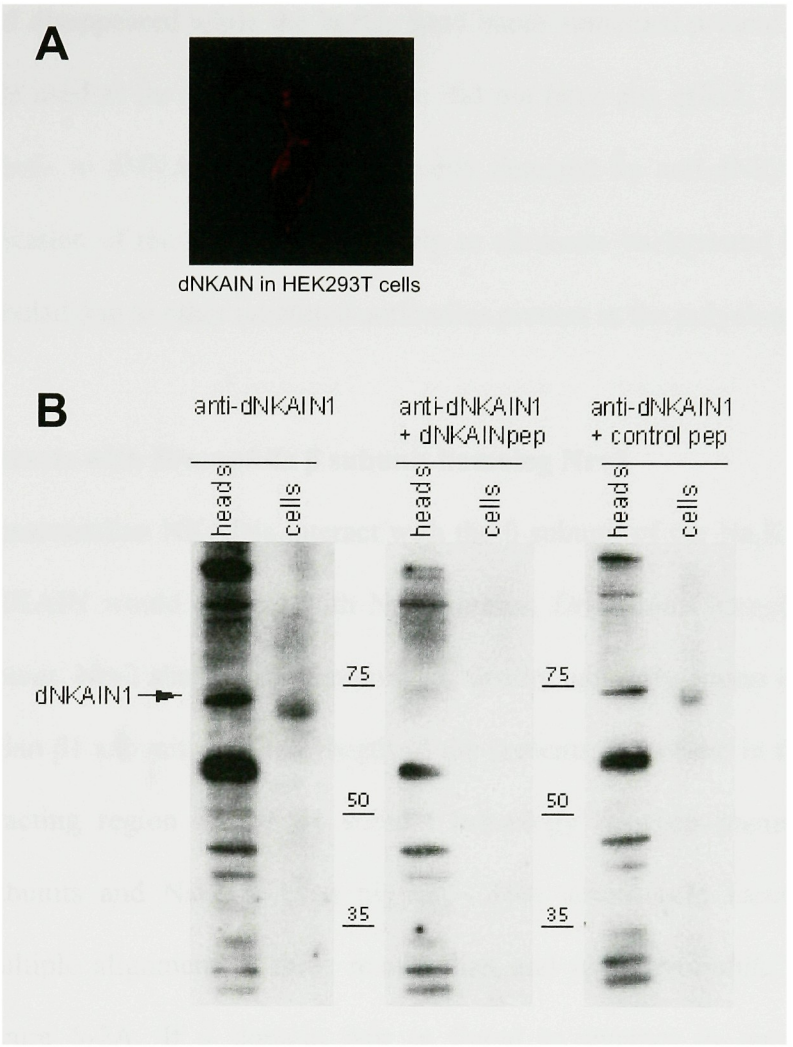


Figure 5-1. Anti-dNKAIN antibody characterization.
A. Anti-dNKAIN labels plasma cell membrane of HEK293T cells transfected with Drosophila NKAIN. B. A band of expected size (75kD) is detected in extracts from dNKAIN transfected cells. A band of similar size is also found in fly head extracts. Peptide competition assay shows that the 75kD bands disappear when anti-dNKAIN antibody is pre-incubated with dNKAIN peptide but not when anti-dNKAIN is pre-incubated with an unrelated control peptide. Other bands in fly head extracts are not specific, since they are still detected by anti-dNKAIN antibody pre-incubated with dNKAIN peptide.

was pre-incubated with the peptide used to raise this antibody before Western blotting, the 75kD band disappeared while the background bands remained present. An unrelated control peptide used at the same concentration did not have any effect. Thus, the 75kD band corresponds to dNKAIN and is specifically detected by anti-dNKAIN antibody. Affinity purification of the antibody might help to eliminate background bands that are likely to be labeled due to other unrelated antibodies present in the polyclonal serum.

dNKAIN interacts with *Drosophila* β subunit homolog Nrv2

Since mammalian NKAINs interact with the β subunit of the Na,K-ATPase, it is likely that dNKAIN would interact with Nrv proteins, *Drosophila* homologs of Na,K-ATPase β subunit. Nrv2 shares 28% amino acid identity and 44% amino acid similarity with mammalian β1 subunit over full length of the proteins. However, in the C-terminal NKAIN interacting region of the β1 subunit homology between mammalian Na,K-ATPase β subunits and Nrv2 is even higher – 38% amino acid identity and 48% similarity. Multiple alignment of three mammalian and two *Drosophila* β subunits is shown in Figure 5-2A. If a domain pair is found to interact in one organism, a homologous domain pair with sequence identity as low as 30-40% is also likely to interact (Aloy et al., 2003). In order to test the interaction between dNKAIN and Nrv proteins, flag-tagged dNKAIN was co-transfected with Nrv2. Immunoprecipitation with anti-flag antibody pulled down Nrv2 protein along with flag-tagged dNKAIN (Figure 5-2B). When Nrv2 was transfected with untagged dNKAIN as a control, no Nrv2 was present in the immunoprecipitate fraction. Thus, dNKAIN specifically interacts with Nrv2, a *Drosophila* homolog of the β subunit of the Na,K-ATPase .

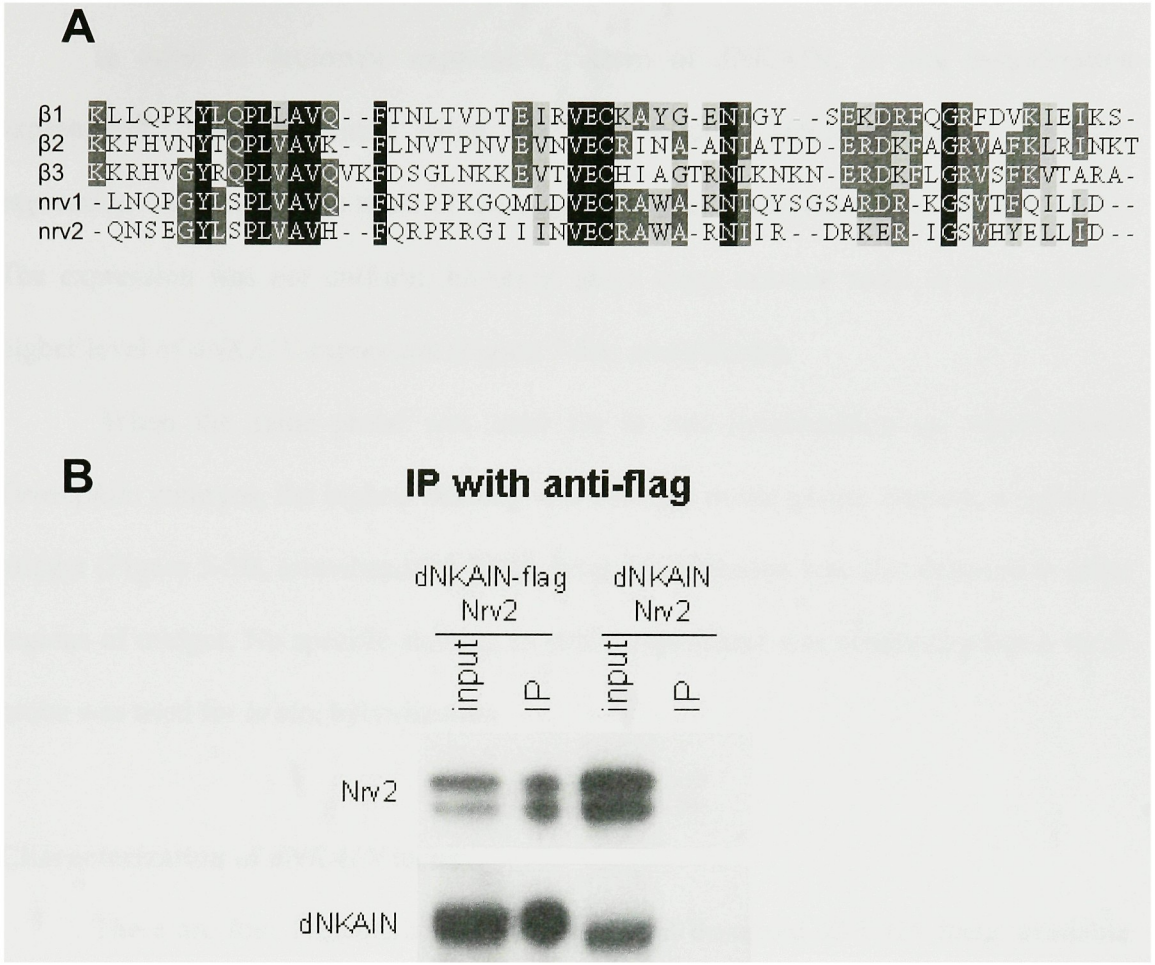


Figure 5-2. dNKAIN interacts with a Nrv2.
A. Conserved regions revealed by multiple alignment of the C-termini of three mouse and two *Drosophila* β subunits (Nrv) of the Na,K-ATPase suggest that Nrv proteins might also contain the NKAIN interacting domain. B. dNKAIN co-immunoprecipitates with Nrv2. HEK293T cells were co-transfected with Nrv2 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-dNKAIN interaction is specific, since Nrv2 immunoprecipitates with the flag-tagged dNKAIN but not with the untagged dNKAIN.

dNKAIN is expressed in adult *Drosophila* brain

In order to determine expression pattern of dNKAIN, *in situ* hybridization experiments were performed using *dNKAIN* specific anti-sense probe. *dNKAIN* expression was detected in most of the regions of the *Drosophila* brain (Figure 5-3A). The expression was not uniform, however, since some neurons seem to have a much higher level of *dNKAIN* expression (Figure 5-3A, arrowheads).

When the same probe was used for *in situ* hybridization on whole-mount *Drosophila* embryos, the highest staining was detected in the gastric caecum, a region of midgut (Figure 5-3B, arrowhead). A lower level of expression was also detected in other regions of midgut. No specific staining in either experiment was observed when a sense probe was used for *in situ* hybridization.

Characterization of *dNKAIN* locus

There are four P-element insertion lines with disrupted *dNKAIN* locus available from public stock centers: *l(2)k03205* (Spradling et al., 1999), *EP2274*, *EP560*, *EP618* (Rorth, 1996) and *l(2)SH1452* (Oh et al., 2003). In all four stocks, a P-element is inserted in the first exon of *dNKAIN* (Figure 5-4). This exon is alternatively spliced, but the insertion site is located in the exonal region common to all three splice forms. Alternative splicing of the first exon does not affect open reading frame of *dNKAIN* gene, since translation initiation site is located in the second exon. Existence of several 5'UTR splice variants suggests that the first exon might contain important regulatory elements, which could be affected by P-element insertions. All P-element insertion sites were confirmed by PCR.

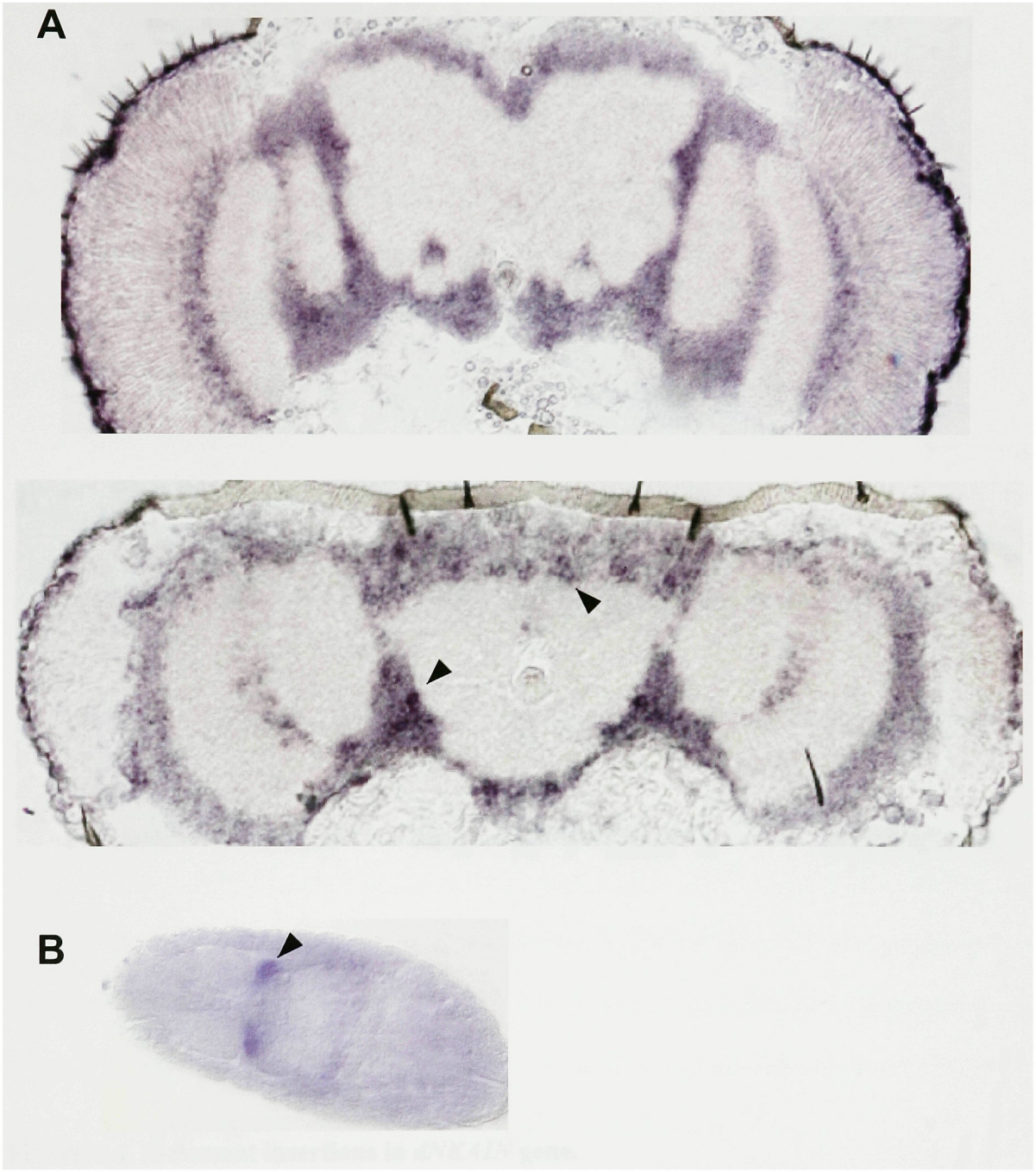


Figure 5-3. Expression of *dNKAIN* in Drosophila adult brains and embryos.
A. *dNKAIN* is expressed in multiple neurons in adult fly brain. The level of *dNKAIN* expression is higher in some cells (arrowheads). B. *dNKAIN* is expressed in region of the midgut that later becomes gastric caecum (arrowhead). The same probe containing the 3' UTR of *dNKAIN* gene was used for *in situ* hybridization in both experiments. Adult brains and embryos did not have any staining when sense probe was used as a control.

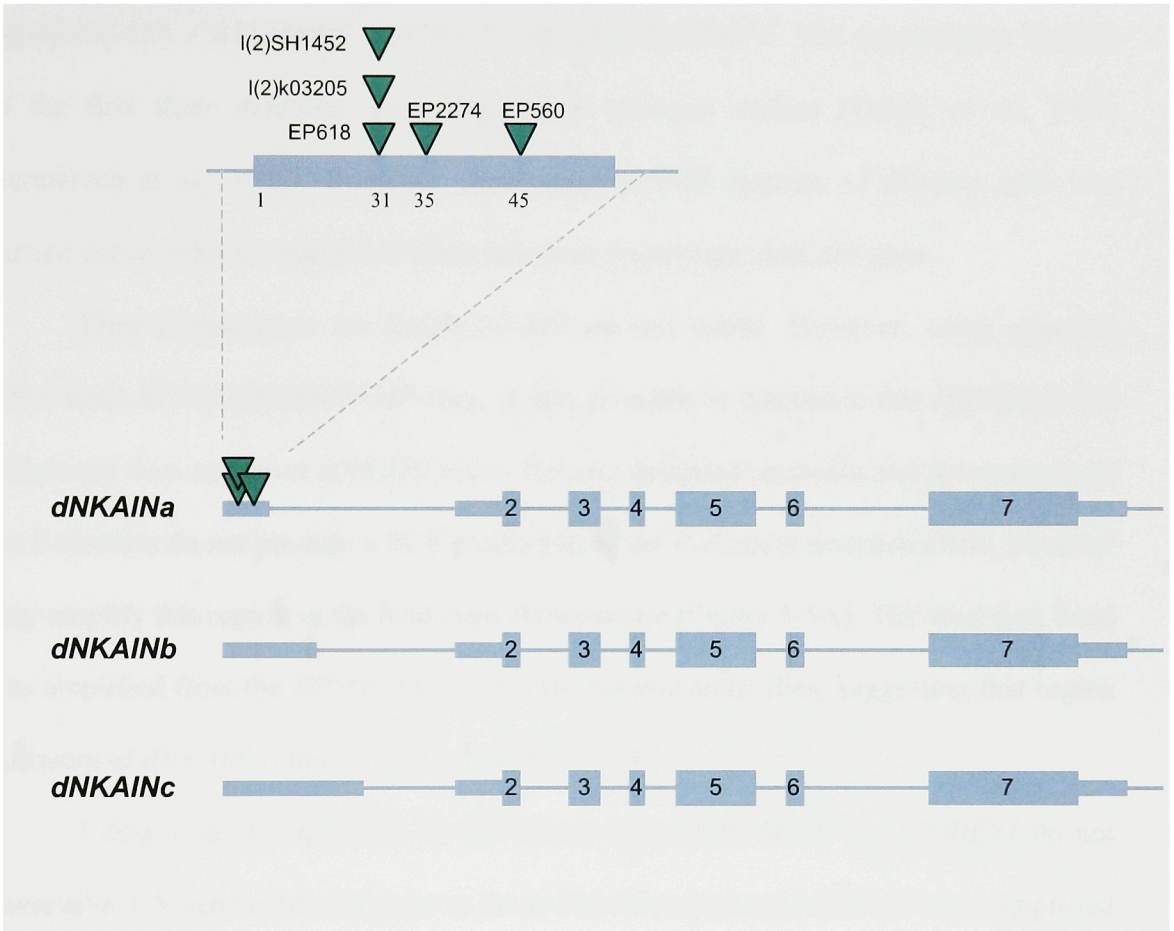


Figure 5-4. P-element insertions in *dNKAIN* gene.
The exon-intron structure of *dNKAIN* is similar to that of mammalian *NKAINs*. In all four p-element insertion lines, p-elements are inserted in the first exon of the gene. Nucleotide positions of the insertions (triangles) are shown. Insertion sites of the P-elements were confirmed by PCR. P-element insertions region is common between three splice variants of *dNKAIN* gene. The start codon is located in the second exon.

Deficiency chromosomes with large deletions at *dNKAIN* locus are useful for confirming phenotypes caused by P-element insertion. Thus, several *Drosophila* deficiency lines with deletions that map to *dNKAIN* chromosomal region were analyzed: *Df(2R)Dll-MP*, *Df(2R)M60E*, *Df(2R)ES1* and *Df(2R)ED4071*. The approximate borders of the first three deletions were mapped in previous reports (Cohen et al., 1989; Germeraad et al., 1992). However, more detailed PCR analysis of deletion ends was carried out in order to establish if these deletions remove the *dNKAIN* gene.

Flies homozygous for *Df(2R)Dll-MP* are not viable. However, using genomic DNA from *EP560/Df(2R)Dll-MP* flies, it was possible to determine that *Df(2R)Dll-MP* deficiency does not cover d*NKAIN* locus. Primers designed upstream and downstream of the P-element do not produce a PCR product from the P-element insertion allele, however they amplify this region in the wild-type chromosome (Figure 5-5A). The wild-type band was amplified from the *EP560/ Df(2R)Dll-MP* heterozygous flies, suggesting that region upstream of *dNKAIN* is not deleted in *Df(2R)Dll-MP*.

Using a similar approach, it was shown that *Df(2R)M60E* or *Df(2R)ES1* do not cover *dNKAIN* gene either. Regions as far as 3kb downstream of *dNKAIN* were amplified by primers (1F-10R) that can only produce a PCR product from a wildtype chromosome (Figure 5-5A). The 5' end of *Df(2R)M60E* was found to lie in a downstream gene, *DSC1* (*NaCP60E*), specifically in the region containing 7^{th} and 8^{th} exons.

Finally, *Df(2R)ED4071* deficiency was found to delete *dNKAIN* gene. This deletion is available from DrosDel, a collection of deletions obtained by rearrangements between pairs of engineered P-elements (Ryder et al., 2004). This technique allows the

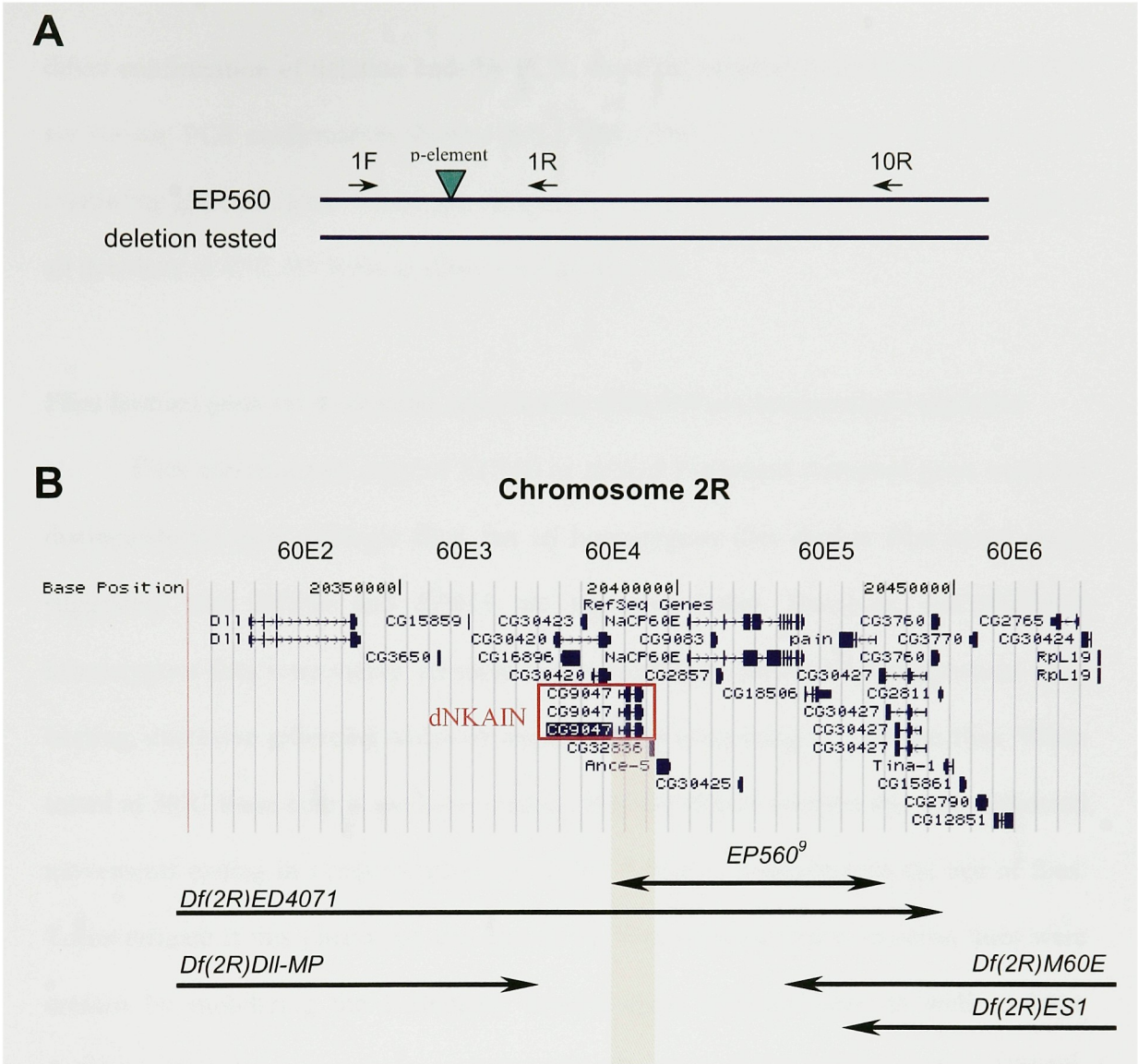


Figure 5-5. Mapping the deficiencies at the *dNKAIN* locus.
A. A strategy to determine if a deletion covers *dNKAIN* gene. If the tested deficiency does not delete *dNKAIN* gene, the 1F-R primer pair would produce a PCR product. 10R is located 3kb downstream of *dNKAIN* gene. B. Of the four deficiencies located in the *dNKAIN* region, only *Df(2R)ED4071* deletes the *dNKAIN* gene. *EP5609* is a deletion generated by imprecise excision of *EP560* P-element. *Df(2R)Dll-MP* and *Df(2R)ES1* were excluded based on the strategy presented in A. The ends of the *Df(2R)ED4071* and *EP5609* were confirmed by PCR. The 5' end of the *Df(2R)M60E* deletion is located in the region of 7-8th exons of *DSC1 (NaCP60E)* gene, as determined by PCR.

direct confirmation of deletion ends by PCR, since the original P-element insertion sites are known. PCR confirmation showed that *Df(2R)ED4071* indeed deletes a 540kb region containing *dNKAIN* gene. Thus, this deficiency was used in further experiments. Map of all deletions at *dNKAIN* locus is shown in Figure 5-5B.

Flies homozygous for P-element insertion in *dNKAIN* are temperature-sensitive

Flies carrying one copy of *EP560* or *EP618* P-element disrupted gene were not distinguishable from wildtype flies, but all homozygous flies died at first larval stage, suggesting that *EP560* and *EP618* are recessive lethal. However, *EP560/EP618* heterozygous flies were viable. At room temperature these flies exhibited increased wing beating, excessive grooming and were more sluggish comparing to wildtype flies. When tested at 38°C these compound heterozygous flies developed seizures and uncoordinated movements ending in complete paralysis within 5-30min, depending on the age of flies. To investigate if this phenotype was due to the P-element insertion, excision lines were created by mobilizing the P-element. Several clean excision lines as well as large deletions of the locus were generated. Surprisingly, clean excision lines, such as $EP560^2$, were still recessive lethal. This suggested that there was another recessive lethal mutation outside of *dNKAIN* gene, killing $EP560^2/EP560^2$ homozygous flies even though *dNKAIN* gene was no longer mutated.

In order to remove the putative lethal mutation, *EP560* flies were outcrossed to *yw* wildtype flies. 10% of the resulting lines were viable as homozygotes, suggesting that in these flies the putative second site lethal mutation was eliminated. *EP560/EP560* homozygous flies displayed excessive grooming and a temperature-sensitive phenotype

identical to the phenotype of *EP560/EP618* flies. These results strongly suggest that temperature sensitivity, wing beating and excessive grooming is the phenotype caused by P-element insertion in *dNKAIN* gene, while recessive lethality observed in the original stock is not. The new "clean" stock of *EP560* was used in further experiments.

When placed at 38°C, homozygous *EP560* flies become severely uncoordinated within 10 minutes (Figure 5-6A). Some flies also display seizure-like movements lasting up to several seconds. When older flies are tested in the same conditions, they become completely paralyzed and fall on the side. In contrast, *EP560*/CyO or wild-type flies do not show such change in behavior. When shifted back to room temperature, homozygous flies return to normal within 10 to 15 minutes. However, prolonged exposure to high temperature often causes lethality in older flies.

If temperature-sensitive phenotype of *EP560* flies is due to P-element insertion in *dNKAIN* gene, flies carrying *EP560* P-element and the deficiency chromosome deleting *dNKAIN* gene would still be temperature sensitive. *EP560* flies were crossed to *Df(2R)ED4071*, which is the only available deficiency with *dNKAIN* gene deleted. *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 ($EP560^{9}$) were not viable. Thus, complete deletion of *dNKAIN* leads to lethality. However, when a clean P-element excision line $EP560^{2}$ was crossed to the deletion, heterozygous flies were viable and not temperature sensitive.

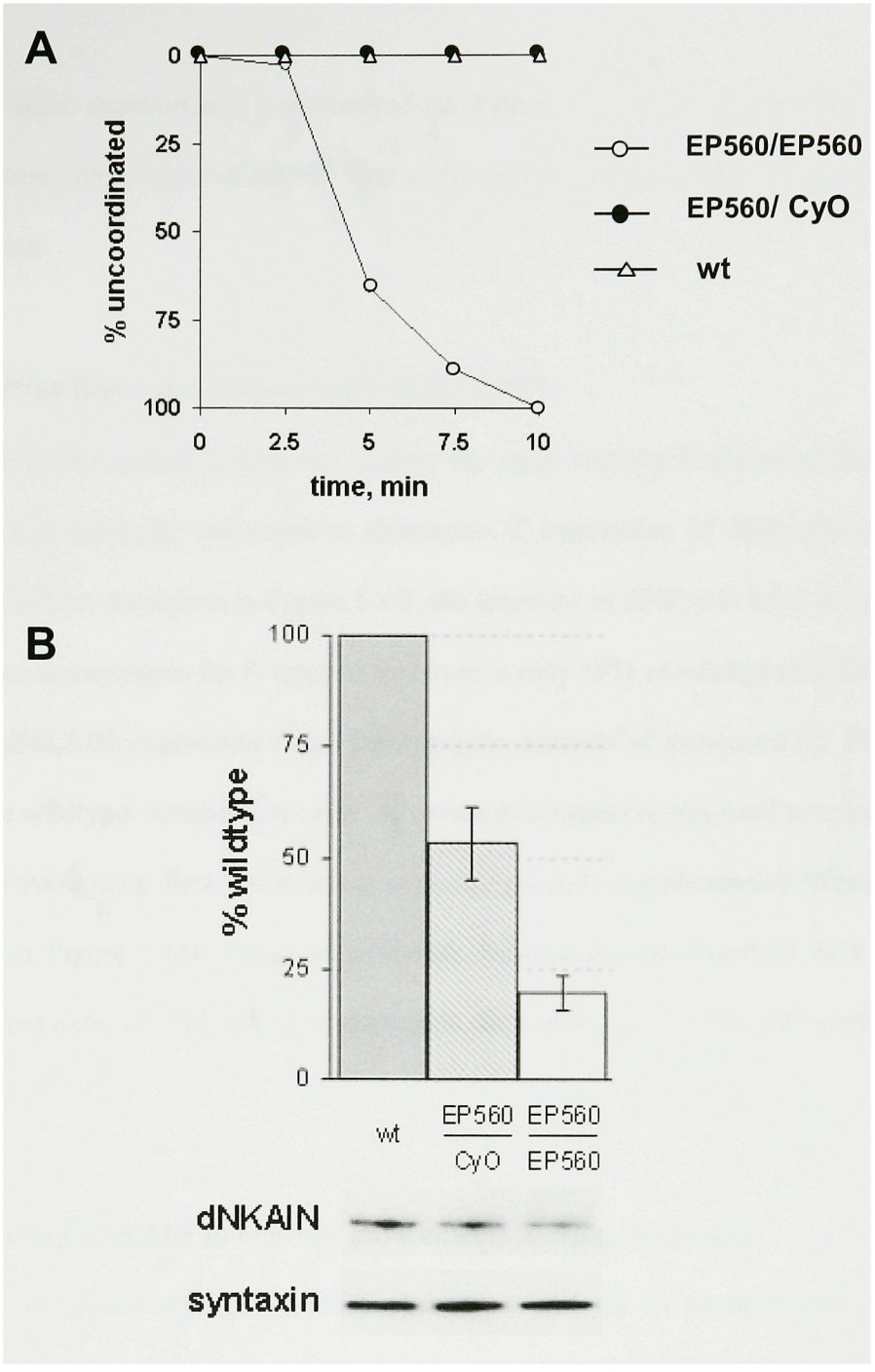


Figure 5-6. Flies expressing lower level of dNKAIN are temperature sensitive.
A. *EP560/EP560* flies become severely uncoordinated and paralyzed when exposed to 38°C, while heterozygous and wild-type flies behave normally. 60 homozygous flies and 30 heterozygous and wild-type flies were used for this experiment. B. *EP560* homozygous flies express lower level of dNKAIN comparing to wildtype 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 phosophoimager, was normalized against the intensity of the loading control syntaxin bands. A representative western blot and the average of three experiments are shown.

The results of these experiments, summarized in Figure 5-7, demonstrate that the temperature sensitive phenotype of *EP560* flies is due to disruption of *dNKAIN* gene by P-element insertion.

Homozygous *EP560* flies express lower levels of dNKAIN

Since P-element insertion does not disrupt the open reading frame of *dNKAIN* gene, anti-dNKAIN antibody was used to determine if expression of dNKAIN was affected in *EP560* flies. As shown in Figure 5-6B, the intensity of dNKAIN band in head extracts from flies homozygous for P-element insertion is only 19% of wildtype dNKAIN band intensity. dNKAIN expression from heterozygous animals is decreased by 47%, comparing to the wildtype. 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 5-6B. These experiments demonstrate that insertion of a P-element in the first exon of *dNKAIN* gene decreases the expression of dNKAIN protein product.

Flies overexpressing dNKAIN in neurons have defects in wing expansion

In order to rescue *dNKAIN* mutant phenotype as well as to determine the phenotype of *dNKAIN* overexpression, 15 lines carrying *UAS-dNKAIN* constructs were created. 10 lines contained *UAS-dNKAIN* insertion on the third chromosome and 5 lines on the second chromosome. *UAS-dNKAIN* flies were first crossed to flies carrying *actin-GAL4* transgene, which leads to ubiquitous expression of *dNKAIN* in animals carrying both transgene. All 15 lines of *UAS-dNKAIN* flies expressing *dNKAIN* ubiquitously were

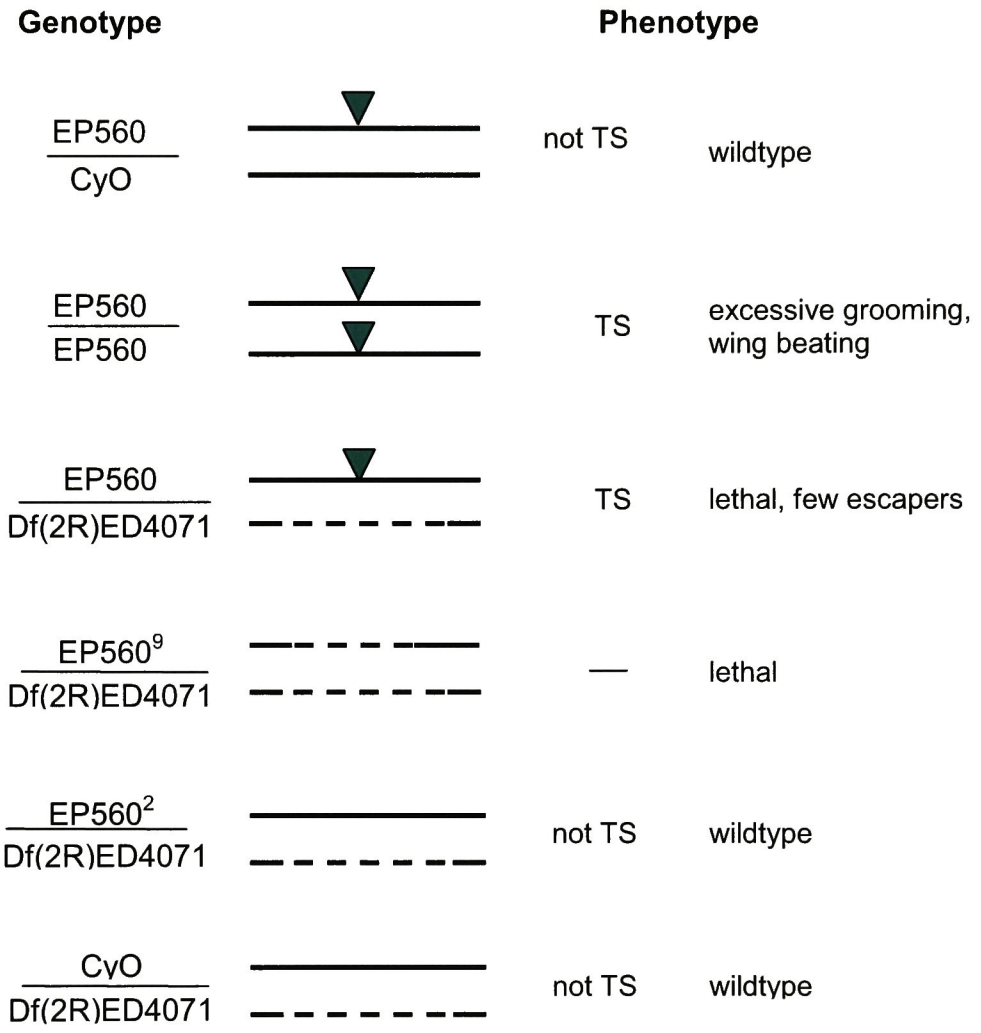


Figure 5-7. P-element insertion in *dNKAIN* gene causes temperature-sensitivity. Flies homozygous for *EP560* P-element insertion are temperature sensitive (TS). A deficiency deleting the *dNKAIN* gene does not complement the temperature-sensitive phenotype. Flies with clean excision of the P-element are no longer temperature-sensitive. *EP560* flies shown here have been outcrossed to *yw* flies and do not contain the recessive lethal mutation present in the original stock.

not viable. These results suggest that dNKAIN is expressed from UAS constructs and that dNKAIN expression in all cells of the organism is lethal.

Then, *dNKAIN* was expressed in neurons using *elav-GAL4* driver. *Elav-GAL/UAS-dNKAIN* flies were somewhat uncoordinated and had uninflated wings (Figure 5-8). In addition, hyperpigmented tridents were present on thoraces of these flies. The percentage of flies with unexpanded wings varied between *UAS-dNKAIN* lines, probably reflecting the copy number of *UAS-dNKAIN* constructs or presence of other enhancers at the construct integration site. For example, when *UAS-dNKAIN-7* line was crossed to neuronal driver *elav-GAL4*, 100% of flies expressing *dNKAIN* in neurons had defects in wing expansion, while very few flies with unexpanded wings were observed in *UAS-dNKAIN-9* line. Wing expansion phenotype in flies overexpressing dNKAIN is identical to the phenotype observed in flies expressing the "electrical knockout" potassium channel (EKO) that leads to inhibition of neuronal excitability (White et al., 2001). These results suggest that overexpression of dNKAIN leads to decrease in neuronal excitability.

Finally, *Nrv1-GAL4* and *Nrv2-GAL4* flies were crossed to *UAS-dNKAIN* animals. No obvious abnormality was present in *Nrv-GAL4/UAS-dNKAIN* flies. However, expressing *dNKAIN* under regulation of *Nrv1* and *Nrv2* promoters did not rescue temperature-sensitivity of *EP560* homozygous flies. These results suggest that a more fine-tuned regulation of *dNKAIN* is needed for the rescue. Alternatively, temperature sensitivity could be caused by *dNKAIN* defect in cells that do not express Nrv1 or Nrv2, and therefore is not rescued by expressing dNKAIN under the Nrv1 and Nrv2 promoters.

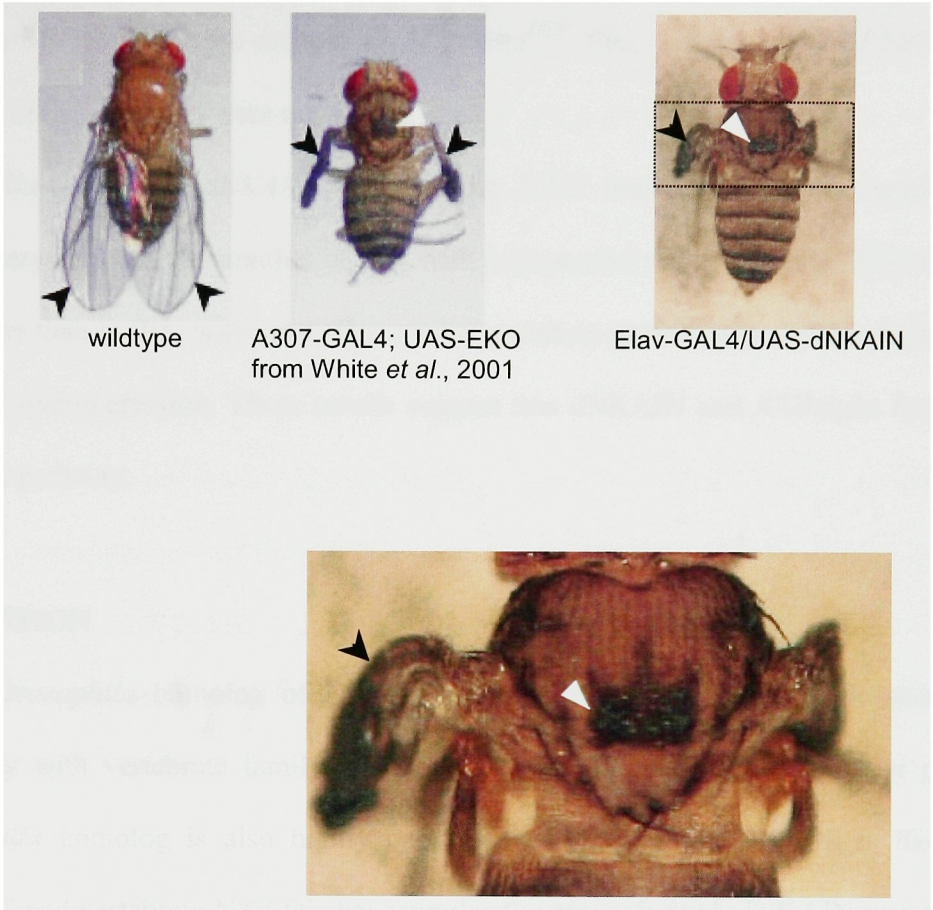


Figure 5-8. dNKAIN expression in neurons leads to wing-expansion defect.
Flies expressing dNKAIN in neurons using elav-GAL4 driver have unexpanded wings (black arrowhead) and a deeply pigmented thoracic trident (white arrowhead). Flies expressing "electrical-knockout" (EKO) channels in a subset of neurons under A307-GAL4 regulation have an identical phenotype. Enhancer trap line A307 expresses GAL4 in several identified neurons, such as giant fiber pathway, as well as numerous unidentified neurons (White *et al.*, 2001).

Genetic interaction between dNKAIN mutants and *ATPalpha^{DTS}* flies

To determine if Na,K-ATPase mutants genetically interact with the NKAIN mutants, *EP560* flies were crossed to *ATPalpha^{DTS1}* flies. Homozygous *EP560* animals carrying *DTS1* mutation were not viable.

Elav-Gal4/UAS-dNKAIN flies carrying *DTS1* mutation were also made. From preliminary analysis, the number of flies with unexpanded wings carrying *DTS1* mutation was lower than in flies without it. Thus, *DTS1* mutation partially rescues the phenotype of *dNKAIN* overexpression. These results suggest that dNKAIN and ATPalpha function in the same pathway.

DISCUSSION

Drosophila homolog of NKAIN proteins (dNKAIN) shares high amino acid similarity with vertebrate family members. Similarly to mammalian NKAIN proteins, *Drosophila* homolog is also highly expressed in adult brain. Thus, it is likely that dNKAIN and vertebrate NKAINs have similar functions. Indeed, dNKAIN was found to interact with the fly homolog of the Na,K-ATPase β subunit. Even though interaction between NKAIN family members and the sodium pump was established in mammals and flies as described in this and previous chapters, the functional effect of this interaction remained elusive until characterization of dNKAIN *Drosophila* mutants suggested that dNKAIN affects membrane excitability.

P-element insertion in the first exon of dNKAIN gene leads to decreased expression of dNKAIN protein in adult fly brain. 5' localization of the P-element insertion sites in dNKAIN gene is consistent with previous findings that majority of P-

elements insert in the 5' untranslated gene regions (Spradling et al., 1995). This preference for the 5' end of a transcription unit is generally suggested to be an evidence that the genes disrupted in this fashion are the primary targets affected by the P-element insertion (Spradling et al., 1995). It is also common for P-elements to affect the expression of genes without disrupting their open reading frame. For example, a P-element insertion in the intron of *ATPalpha*^{2206} mutant decreases *ATPalpha* expression to 30% comparing to wildtype flies (100%) and causes a stress-sensitive phenotype (Schubiger et al., 1994).

Flies with decreased expression of dNKAIN protein caused by P-element insertion 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 is due to P-element insertion in *dNKAIN* gene. First, when 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 the downstream putative sodium channel DSC1, since mutations and deletions in *DSC1* gene do not cause temperature-sensitivity, but an odor guidance phenotype (Kulkarni et al., 2002; Sakai et al., 1989).

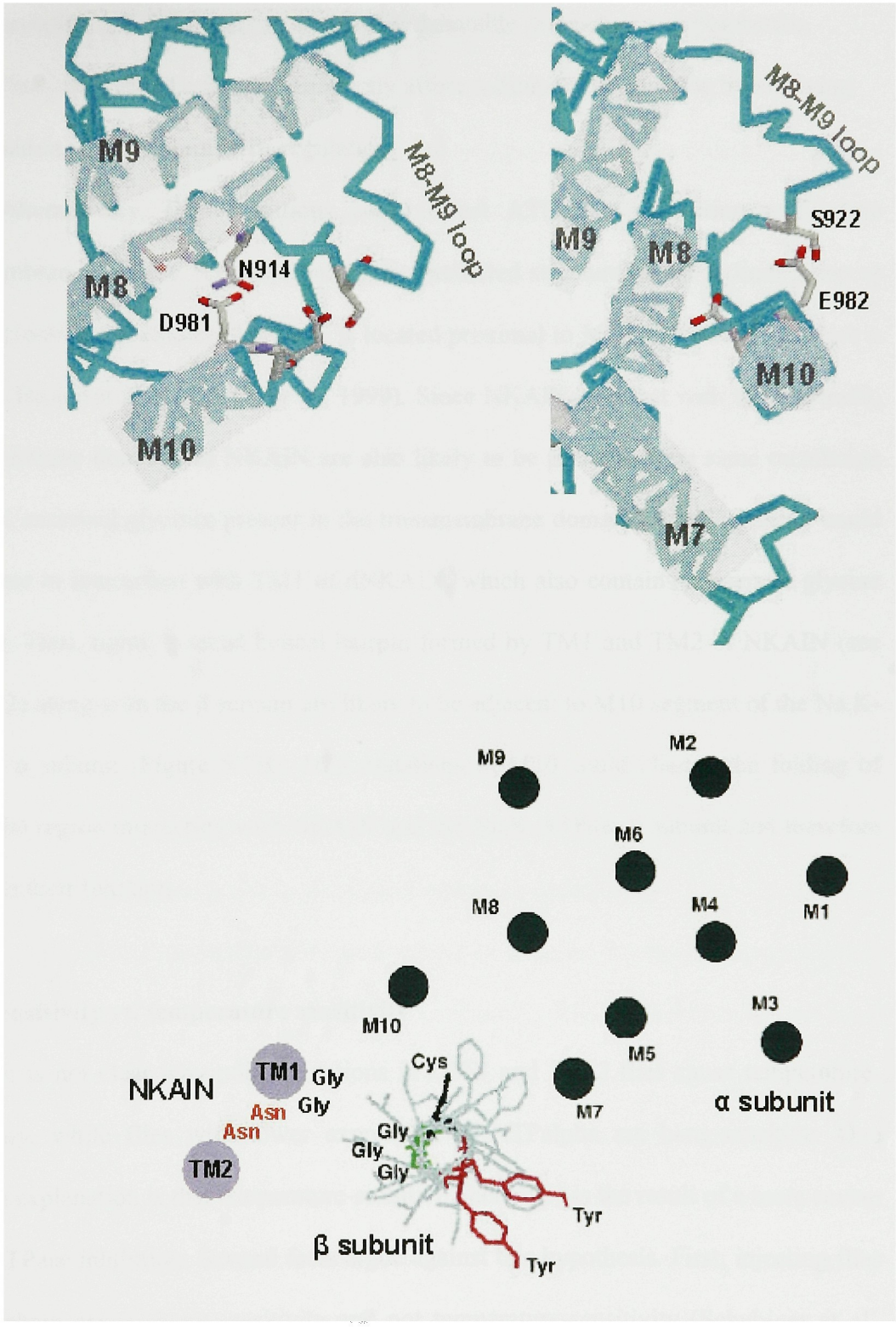
One of the uncharacterized temperature sensitive mutations *TipD* maps to the chromosomal region of *dNKAIN* (Kulkarni, 1982), and therefore could be caused by a mutation in the *dNKAIN* gene. When *TipD* flies were crossed to deletions *Df(2R)M60E*, *Df(2R)Dll-MP* and *Df(2R)ES1*, *TipD* phenotype was not affected (Germeraad et al.,

1992). Since these deletions were found to lie outside of *dNKAIN* locus, these findings are consistent with the possibility of *dNKAIN* mutation in *TipD* flies.

Flies with mutations in the Na,K-ATPase α subunit (*ATPalpha*^{DTS1} and *ATPalpha*^{DTS2}) are temperature-sensitive due to increased neuronal excitability, based on bursting activity observed during electrophysiological recordings from dorsal flight muscles in these flies (Palladino et al., 2003). This phenotype is expected in the Na,K-ATPase mutants, since inhibiting sodium pump in hippocampal neurons lead to increase in neuronal excitability preceded by membrane depolarization (McCarren and Alger, 1987; Vaillend et al., 2002). Phenotypic similarity between dNKAIN and Na,K-ATPase mutants provides the first *in vivo* evidence of interaction between NKAINs and the sodium pump.

It is not clear how *DTS* mutations in *ATPalpha* can affect sodium pump function. Both amino acid changes in *DTS* flies are located in M10 transmembrane domain (Palladino et al., 2003), which is located on the outside of α subunit and is not directly involved in ion transport. By examining the structure of SERCA1a, a P-type ATPase similar to Na,K-ATPase (Sweadner and Donnet, 2001), it is clear that both amino acids mutated in *DTS* flies are oriented towards the inside of ATPalpha protein. *DTS2* mutation changes D981 residue interacting with M8 transmembrane domain, and thus is likely to affect packing of ATPalpha transmembrane helices (Figure 5-9A). The other *DTS* mutation (E982) changes the residue interacting with S922 on the cytoplasmic M8-M9 loop into a positively charged amino acid lysine (Figure 5-9A). M8-M9 loop is likely to be important for sodium pump function, since mutations in the same region of human α2 subunit of the Na,K-ATPase cause familial hemiplegic migraine (Riant et al., 2005).

Figure 5-9. Model of Na,K-ATPase region interacting with NKAIN.
A. Residues mutated in *ATPalpha*^{DTS} flies orient towards the inside of ATPalpha protein. Amino acid positions were predicted from structure of the corresponding region of SERCA1a. D981, present in the *DTS1* flies is oriented towards the M8 helix; the most proximal residue on SERCA1a structure is N914. N914 is not conserved between SERCA1a and ATPalpha, making it difficult to predict the possible interacting residue on ATPalpha protein. E982 (mutated in *DTS2* flies) is likely to interact with S922 in the M8-M9 intracellular loop. S922 is conserved between P-type ATPases and is believed to be the site of PKA phosphorylation. B. A model of membrane arrangement of transmembrane helices of the Na,K-ATPase (adapted from Hasler et al., 2001) and NKAIN. The Asn residues in the TM1 and TM2 are likely to induce helical hairpin formation between these segments. Conserved glycines in the TM1 are oriented towards putative transmembrane helix interaction domain (three Gly) on the β subunit. Mutations of the Tyr shown in red have been previously shown to affect transport kinetics of the sodium pump (Hasler et al., 2001). Cys on the β subunit crosslinks to M8 of the α subunit (Ivanov et al., 2000; Or et al., 1999).


M9
M8-M9 loop
N914
M8
D981
M10
M8-M9 loop
S922
M9
M8
E982
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M7
M9
M2
M6
M1
M8
M4
M3
M10
M5
M7
α subunit
NKAIN
TM1
Gly
Cys
Gly
Asn
Asn
Gly
Gly
TM2
Tyr
β subunit
Tyr

Ser922 (Ser938 in mammals) is the conserved PKA phosphorylation site, though whether phosphorylation at S938 occurs *in vivo* is still debatable (Sweadner and Feschenko, 2001). Thus, *DTS* mutations could indirectly affect sodium pump function by changing conformation of the protein or its regulation.

Alternatively, *DTS* mutations could affect ATPalpha interaction with other transmembrane proteins. As determined from predicted structure of the sodium pump as well as crosslinking studies, β subunit is located proximal to M10 of the Na,K-ATPase α subunit (Hasler et al., 2001; Or et al., 1999). Since NKAINs interact with the β subunit, transmembrane domains of NKAIN are also likely to be present in the same membrane region. Conserved glycines present in the transmembrane domain of the β subunit could participate in interaction with TM1 of dNKAIN, which also contains conserved glycine residues. Thus, tightly packed helical hairpin formed by TM1 and TM2 of NKAIN (see chapter 2) along with the β subunit are likely to be adjacent to M10 segment of the Na,K-ATPase α subunit (Figure 5-7B). *DTS* mutations in M10 could change the folding of ATPalpha region interacting with NKAIN and the Na,K-ATPase β subunit and therefore influence their function.

Bang-sensitivity vs. temperature sensitivity

It is not clear why point mutations in *DTS1* and *DTS2* flies cause temperature-sensitivity, while flies with lower expression of ATPalpha are bang-sensitive. One possible explanation is that temperature-sensitive phenotype is the result of a more severe Na,K-ATPase inhibition. Several facts argue against this hypothesis. First, injecting flies with ouabain causes bang-sensitivity and not temperature-sensitivity (Schubiger et al.,

1994). Second, if temperature-sensitivity is a more severe form of the Na,K-ATPase inhibition phenotype, one would still expect bang sensitivity to be present in *ATPalpha DTS* mutants.

Alternatively, *DTS* mutations could cause a novel gain of function in the sodium pump that would lead to temperature-sensitivity through a mechanism different from the bang-sensitivity causing process. The dominant nature of *DTS* mutations is consistent with this possibility.

Finally, *DTS* mutations could cause temperature-sensitivity by affecting other interacting membrane proteins, such as dNKAIN. Bang-sensitive phenotype would then correlate with pure ATPalpha dysfunction and decreased ion transport, while temperature-sensitive phenotype would be caused by the effect of mutant ATPalpha on dNKAIN.

Electrophysiology studies to determine if *DTS* mutant sodium pumps have abnormal transport properties would help to distinguish between these possibilities.

dNKAIN overexpression leads to decreased neuronal excitability

Inhibiting cellular excitability in a subset of neurons by expressing genetically modified Shaker K^+ channel ("electrical knockout", EKO) phenocopies neuronal overexpression of *dNKAIN*. EKO channels are designed to activate at –60mV and lack the inactivation domain. This leads to shunting of depolarizing currents in cells expressing EKO channels, effectively suppressing the electrical activity of these cells (White et al., 2001). Flies with three copies of EKO expressed in a subset of neurons by *A307-GAL4* driver had unexpanded wings. *Drosophila* wings normally expand shortly

after eclosion as the result of pumping hemolymph into wing veins by abdominal contractions and would fail to do so if neurons innervating these muscles were inactive. White *et al.* further demonstrated that the wing phenotype in *A307-EKO* flies is due to decreased neuronal excitability, since this phenotype could be modulated by levels of Na^+ and K^+ currents. For example, 79% of flies carrying $para^{ts1}$ mutation in addition to two copies of EKO channels had unexpanded wings, comparing to normal wing expansion of the flies carrying two copies of EKO channels in wildtype background (White et al., 2001). Thus, decreasing neuronal excitability by overexpressing EKO channels alone or in combination with decreased Na channel function, leads to defective wing expansion in *Drosophila*. An identical phenotype was also observed when *dNKAIN* was expressed in neurons using *elav-GAL4* driver, suggesting that *dNKAIN* overexpression decreases neuronal excitability. This result is in agreement with the fact that decrease of dNKAIN expression phenocopies hyperexcitability phenotype of *ATPalpha* mutants.

The fact that increasing dNKAIN expression leads to hypoexcitability, while the opposite effect is caused by decrease in dNKAIN expression suggests that dNKAIN stimulates the activity of the Na,K-ATPase. Sodium pump activity tends to shift the membrane potential in the negative direction, since it transports 3 Na^+ out and 2 K^+ into the cell. Thus, dNKAIN overexpression would stimulate the sodium pump and hyperpolarize the cell, silencing its activity. Indeed, the phenotype of flies with overexpressed dNKAIN is identical to that of flies with inhibited cellular excitability by expressing EKO channels. Another finding that supports this hypothesis is that flies over-expressing *ATPalpha* by *Nrv1-GAL4* driver also had uninflated wings, though the phenotype was much more severe (Sun et al., 2001). Na,K-ATPase stimulation by

NKAIN would be especially important for neuronal function, where activation of the sodium pump is critical for neuron recovery after prolonged firing.

Alternatively, NKAIN itself could function as a novel channel with hyperpolarizing function. Extremely high evolutionary conservation in the first two transmembrane domains supports this hypothesis.

Neurodegeneration and NKAIN *Drosophila* mutants

A number of temperature-sensitive *Drosophila* mutants, including *ATPalpha DTS* mutants, exhibit age-dependant neurodegeneration in the central nervous system (Palladino et al., 2003; Palladino et al., 2002). Neurodegeneration has been found in both hypo- and hyperexcitable fly mutants, suggesting that excitotoxicity alone is not sufficient to explain the phenotype (Fergestad et al., 2006). Decreased viability of *dNKAIN* mutant flies was observed after they were exposed to high temperature for a longer period of time. Interestingly, out of all temperature-sensitive mutants, only *ATPalpha DTS* mutants have been reported to show similar phenotype – when flies are exposed to 38°C for an extended period of time, only 40% survive the treatment (Fergestad et al., 2006). Thus, it is possible that similar to *DTS* mutant flies, *dNKAIN* mutants also develop neurodegeneration.

Chapter 6. Conclusion

In this study, cloning and characterization of a novel protein family are presented. NKAIN family members are evolutionary conserved, with the highest protein sequence conservation found in the transmembrane domains of these proteins. They are also highly expressed in brain, specifically in neurons. Several mammalian NKAINs, as well as the *Drosophila* homolog dNKAIN, were found to interact with the Na,K-ATPase β subunit. Analysis of *Drosophila* NKAIN mutants suggested that NKAINs are likely to affect membrane excitability.

Based on the results presented in previous chapters, two models of NKAIN function can be proposed (Figures 6-1 and 6-2). In the first model, NKAIN proteins could stimulate the activity of the Na,K-ATPase (Figure 6-1A). Since Na,K-ATPase transports three Na^+ ions outside for every two K^+ transported inside of the cell, increase in sodium pump activity makes the cell membrane potential more negative. Similarly, decrease in the activity of the Na,K-ATPase would have the opposite effect on membrane potential, making cells more excitable. The temperature sensitive phenotype of flies with *dNKAIN* gene disrupted by a P-element insertion (*EP560*) could be explained by hyperexcitability of neurons due to decreased stimulation of Na,K-ATPase by dNKAIN (Figure 6-1B). This phenotype is similar to the phenotype of $ATPalpha^{DTS}$ flies, which have decreased Na,K-ATPase function due to point mutations in the *ATPalpha* gene (Figure 6-1C). According to the first model, overexpression of NKAIN would lead to increased stimulation of the sodium pump activity and hyperpolarization of cell membrane. Indeed, the phenotype of flies expressing dNKAIN in all neurons is identical to the

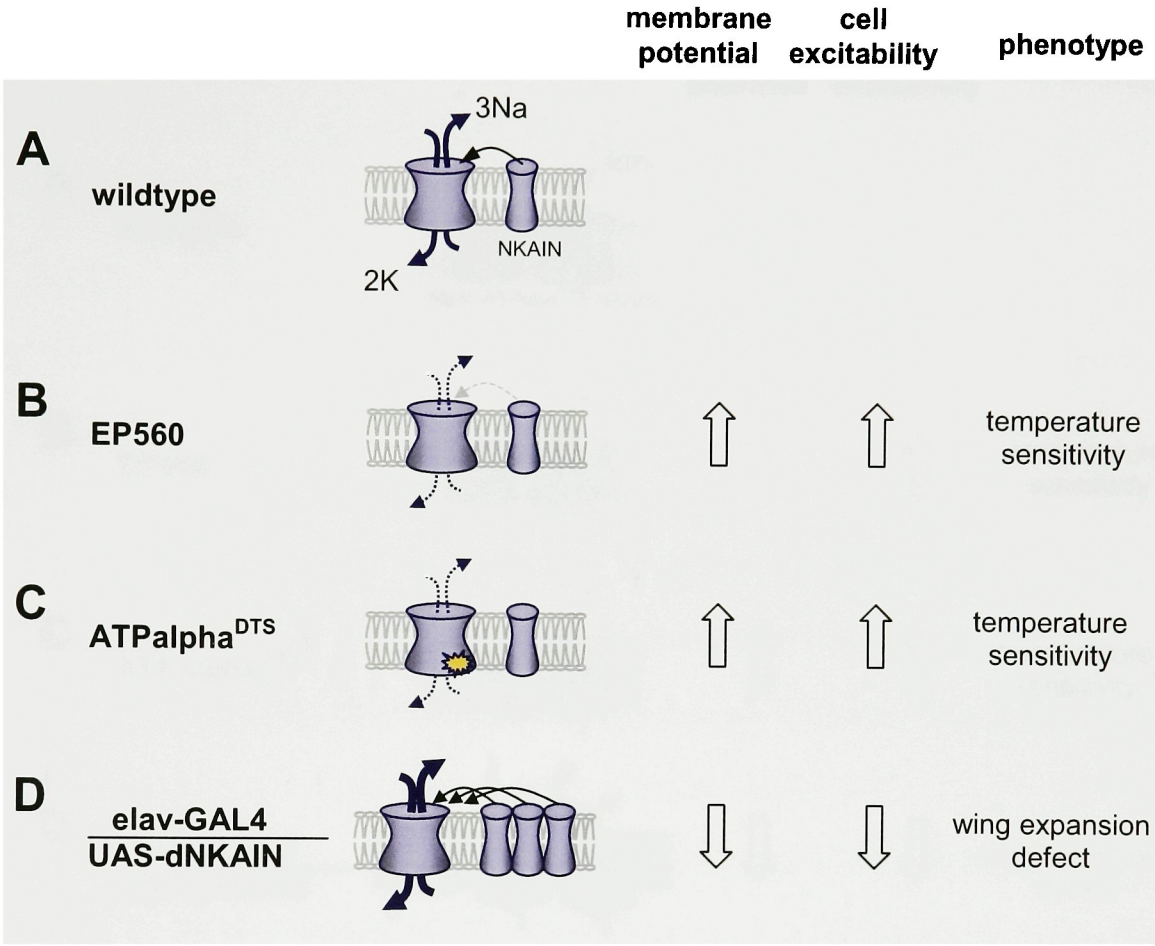

Figure 6-1. First model of NKAIN function.
A. NKAIN stimulates Na,K-ATPase activity. B. In *EP560* flies, lower level of NKAIN expression leads to decreased activity of Na,K-ATPase and therefore hyperexcitability. C. The same phenotype is caused by sodium pump mutations in *ATPalpha*^{DTS} flies. D. Overexpression of NKAIN in *elav-GAL4/UAS-dNKAIN* flies leads to increased stimulation of Na,K-ATPase and therefore hyperpolarization of cell membrane. Phenotype of these flies is similar to the phenotype of flies expressing "electrical-knockout" (EKO) potassium channels.

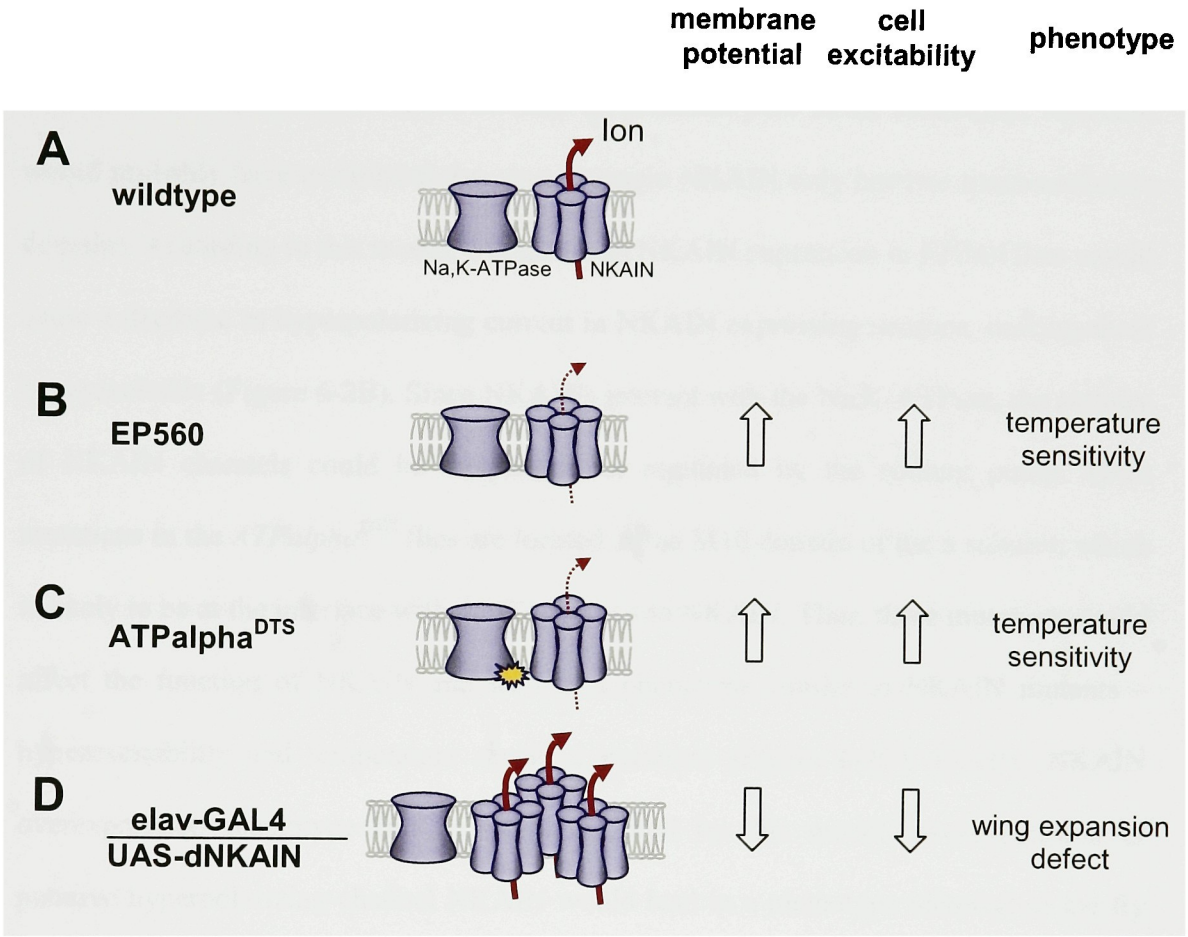

Figure 6-2. Second model of NKAIN function.
A. NKAINs form channels with hyperpolarizing activity. B. Neurons are hyperexcitable in *EP560* flies since membrane hyperpolarization by NKAIN is decreased. C. Mutations in ATPalpha protein (star) affect NKAIN function since they are located at the interface with the β subunit and possibly NKAIN. This leads to the same phenotype as NKAIN dysfunction of *EP560* flies. D. NKAIN overexpression leads to the same effect as overexpressing hyperpolarizing potassium channels EKO.

phenotype of flies expressing "electrical-knockout" channels that hyperpolarize cell membranes and inhibit neuronal activity (Figure 6-1D).

According to the second model, NKAIN proteins could themselves form channels with hyperpolarizing activity (Figure 6-2A). For example, they could transport K^+ ions outside or Cl^- ions inside of cells. To form a functional pore in the membrane, NKAINs would probably have to multimerize since a single NKAIN only has two transmembrane domains. According to this model, a decrease in NKAIN expression in *EP560* flies would cause a decrease in hyperpolarizing current in NKAIN expressing neurons, making them hyperexcitable (Figure 6-2B). Since NKAINs interact with the Na,K-ATPase, the activity of NKAIN channels could be coupled to or regulated by the sodium pump. Point mutations in the $ATPalpha^{DTS}$ flies are located in the M10 domain of the α subunit, which is likely to be at the interface with the β subunit and NKAIN. Thus, these mutations could affect the function of NKAIN and lead to a phenotype similar to NKAIN mutants – hyperexcitability and temperature sensitive paralysis (Figure 6-2C). Finally, NKAIN overexpression phenotype can be explained well by this model: overexpression of putative hyperpolarizing channel NKAIN would lead to a phenotype identical to the fly phenotype caused by overexpressing hyperpolarizing EKO channels (Figure 6-2D).

It is also possible that NKAIN function combines both sodium pump regulation and ion transport. In fact, there are reported examples of similar dual function for Na,K-ATPase modulating proteins. There is evidence that FXYD proteins might multimerize and form ionic channels in addition to their role in regulating ion affinities of the sodium pump (Garty and Karlish, 2005). Finally, NKAIN function could be completely

independent of the sodium pump activity, if the Na,K-ATPase β subunit could form a complex with NKAINs separate from the α subunits.

Several experiments might help to distinguish between these two proposed models. Electrophysiological analysis of the Na,K-ATPase from *ATPalpha*^{DTS} flies is needed to determine if the sodium pump activity is affected in these flies. If Na,K-ATPase activity is normal in flies carrying *DTS* mutation, then the second model is more likely, since it is not dependent on ion transport (Figure 6-2C).

Analysis of the revertants of *ATPalpha*^{DTS} flies can also help to determine mechanisms that lead to the temperature sensitive phenotype. Four out of five revertants of *DTS* flies have been reported to have reduced or absent expression of the sodium pump from the mutated allele (Fergestad et al., 2006; Palladino et al., 2003). Rescue of temperature sensitivity in these revertants is consistent with both models, since it would either remove dysfunctional pumps from the membrane (model 1) or eliminate the deleterious effect of mutated pumps on NKAIN function (model 2). One revertant (*ATPalpha*^{DTS2R3}), however, has two point mutations in the ATPalpha protein that also rescue the temperature sensitive phenotype. One explanation, is that similarly to other revertants, the number of sodium pumps present in the membrane is decreased by these mutations due to abnormal maturation and targeting of the Na,K-ATPase. In this case, both models are consistent with the phenotype rescue seen in *ATPalpha*^{DTS2R3} revertants. If sodium pumps from *ATPalpha*^{DTS2R3} flies expresses normally on cell membranes, but lacks ion transporting activity, then the first model is more likely. Since the original mutation (marked by a star in Figure 6-2C) is still present in *ATPalpha*^{DTS2R3} flies, inhibiting the ion transport would have no effect on NKAIN-sodium pump interaction. In

other words, the temperature-sensitive phenotype would be caused by gain-of-function *DTS* mutations dependent on ion transport and not NKAIN function.

While the exact mechanism of NKAIN action remains obscure, it is likely that NKAIN proteins decrease membrane excitability. This property is crucial for neuronal function: it could either stimulate the Na,K-ATPase to restore ion balance or could help keep the membrane from depolarizing after prolonged firing. Neuronal expression of NKAIN proteins is consistent with the proposed function.

NKAINs also play a role in human disease. There are two reported clinical case of 6q22 chromosomal translocations that truncate *NKAIN2* gene (Bocciardi et al., 2005; Yue et al., 2005). The most significant finding in both affected patients is a severe neurological dysfunction, though a variety of other symptoms are also present. Interestingly, in both reports, truncations of NKAIN2 delete the region that interacts with the Na,K-ATPase β subunit. Perhaps truncated NKAIN2 proteins are still integrated into sodium pump complexes but are unable to function. In this case, they would act as dominant negative forms by competing with wildtype NKAIN2 proteins. The best way to test this hypothesis is to generate transgenic mice expressing truncated NKAIN2 proteins.

It is also of interest that seizures, which are prominent in one of the patients carrying NKAIN2 truncation, are also present in several cases of familial hemiplegic migraine caused by mutations in the α2 subunit of the Na,K-ATPase (Spadaro et al., 2004; Vanmolkot et al., 2003). The similarity between clinical findings in human diseases caused by mutations in the sodium pump and in NKAIN2 is consistent with results demonstrating interaction between NKAINs and Na,K-ATPase presented in this study. Many human disorders involving seizures are caused by increased neuronal

excitability. For example, a number of mutations in potassium channels have been identified in familial epilepsies (Charlier et al., 1998; Du et al., 2005; Singh et al., 1998). It has also been proposed that neuronal hyperexcitability predisposes one to migraines (Bussone, 2004). NKAINs effect on membrane excitability makes them a good drug target for these disorders. Hyperexcitable *Drosophila* NKAIN mutants might also be useful for understanding and ultimately treatment of human epilepsy and migraine.

Another human disorder caused by mutations in the Na,K-ATPase α subunit is Rapid-onset Dystonia Parkinsonism (RDP, DYT12), characterized by an onset of symptoms following an emotional or physical stress, such as fever, prolong exercises, exposure to heat or childbirth (Dobyns et al., 1993). Since the *ATPalpha*^{DTS} and *dNKAIN* mutants also have a marked decrease of viability after exposure to 38°C, pathogenic mechanisms in these *Drosophila* mutants could be similar to RDP. Perhaps affected sodium pumps in RDP patients and in fly mutants function almost normally, until stress puts neurons out of equilibrium. Abnormal pumps would be unable to quickly restore ionic disbalance caused by stress or heat shock, triggering other damaging downstream processes.

Finally, another type of involuntary muscle contraction disorder, idiopathic torsion dystonia (DYT6), maps to the chromosomal region where *NKAIN3* is located (Almasy et al., 1997). Often mutations in proteins involved in the same physiological process cause human diseases with similar symptoms. Since mutations in α3 subunit of Na,K-ATPase cause Rapid-onset Dystonia Parkinsonism (DYT12), NKAIN3, a member of the Na,K-ATPase interacting protein family, is a good candidate gene for DYT6.

Truncation of NKAIN2 in patients with mental retardation and neurological dysfunction, neuronal expression of NKAINs and excitability phenotype of *Drosophila* NKAIN mutants strongly suggest that NKAINs are critical for neuronal function.

Materials and Methods.

Multiple alignment

Full-length cDNAs are available for mouse NKAINs: 2610200G18Rik (NKAIN1), 6330571D19Rik (NKAIN2), E130310K16Rik (NKAIN3) and C030019F02 (NKAIN4)

Xenopus tropicalis NKAIN1 and 3 were assembled from ESTs, while a dull-length cDNA sequence was available for NKAIN4. There are only two ESTs corresponding to *Xenopus tropicalis* NKAIN2, which contain exons 1-4. The last 3 exons were predicted from genomic sequence using GeneScan.

Galus galus NKAIN1 protein sequence was obtained mostly from the two ESTs available. A short missing part of exon 4 was predicted from the genomic sequence. Protein sequences of NKAIN2, 3 and 4 sequence was assembled from a number of ESTs and cDNAs available in the GenBank.

D. melanogaster and *C. elegans* NKAINs correspond to the uncharacterized genes CG9047 and T13H5.6 respectively.

RT-PCR

RNA was isolated from 9 different mouse tissues as well as 6 brain regions using Trizol® reagent (Life Technologies). Four sets of primers for RT-PCR were designed for each family member. 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 using Primer3 program (Rozen and Skaletsky, 2000). RT-PCR was

performed on each RNA sample with four sets of primers. Beta-actin primers were used to control for RNA quality.

cDNA constructs

The following constructs were made:

name	vector	restriction sites
NKAIN1-myc	pcDNA3.1	EcoRI/XbaI
NKAIN1-flag	CMV5.1	EcoRI/XbaI
NKAIN1	CMV5.1	HindIII/XbaI
NKAIN2-flag	CMV5.1	HindIII/XbaI
NKAIN4-flag	CMV5.1	HindIII/XbaI
beta1	CMV5.1	EcoRI/XbaI
beta1-flag	CMV5.1	EcoRI/XbaI
NKAIN1-cterm	PPV2	EcoRI/XbaI
NKAIN1	pGBKT7	NdeI/XmaI
NKAIN2	pGBKT7	NdeI/XmaI
NKAIN4	pGBKT7	NdeI/XmaI
beta2	pACT2	EcoRI/XhoI
beta3	pACT2	NcoI/XbaI
MONaKA-myc	pCDNA3.1A	EcoRi/XbaI
dNKAIN	pBSK	SpeI/HindIII
dNKAIN-flag	CMV5.1	BglII/XbaI
dNKAIN	CMV5.1	BglII/XbaI
dNKAIN	pUAST	BglII/XbaI
Nrv1	CMV5.1	HindIII/XbaI
Nrv2	CMV5.1	HindIII/XbaI

All of the cDNAs were amplified by RT-PCR from mouse or *Drosophila* RNA using SuperScript First-Strand Synthesis System (Invitrogen) and Platinum *Pfx* DNA polymerase according to manufacturer's instructions. All constructs were confirmed by sequencing. Mammalian expression vectors pFLAG-CMV5.1 (Sigma) and pcDNA3.1/Myc-His+A (Invitrogen) were used for flag- and myc- tagging respectively. PPV2 (kindly provided by Dr. Selimi) was used to clone the C-terminal tail of NKAIN1 fused to Venus. pGBKT7 and pACT2 (Clontech) were used for yeast-two-hybrid screen. pBSK (Stratagene) was used for the *in situ* probe generation. pUAST was used for the generation of UAS-dNKAIN flies.

Antibody generation and characterization

Two anti-mouse NKAIN1 polyclonal peptide antibodies were prepared by injecting rabbits with the peptides containing amino acids 116-136 (LVTPVLNSRLALEDHHVISVT) and 186-204 (SYGYQAPQKTSHLQLQPLY). The first antibody (NKAIN1loop) recognizes the region between the second and third transmembrane domains of NKAIN1. The second antibody (NKAIN1,2cterm) is specific to the c-terminal tail of both NKAIN1 and NKAIN2. The peptides were selected using ANTIGENIC program from EMBOSS (Kolaskar and Tongaonkar, 1990). Peptide synthesis and rabbit immunization were performed by Invitrogen. NKAIN1loop antibody was affinity purified using NKAIN1loop peptide coupled to cyanogen bromide-activated Sepharose 4B (Sigma) according to manufacturer's instructions.

Peptide competition assay was performed as follows. 1μg of NKAIN1loop antibody was incubated at 4 °C overnight in 1ml of 5% milk in PBS-0.1% Tween-20

(PBST) with either 5μg of loop peptide (116-136aa) or 5μg of unrelated peptide as a control. This solution was then diluted to 5ml with PBST and used for primary antibody incubation in the standard Western blot protocol. Monoclonal anti-β actin (Sigma) antibody was used as a loading control. Polyclonal peptide anti-dNKAIN was made using the peptide containing amino acids 183-197 (GDAKSPQHTVVHPMY) of the fly NKAIN protein.

Immunocytochemistry

Transfected HEK293T cells were washed in cold PBS, fixed in 4%PFA, washed in PBS and pre-blocked in 5% Normal Goat Serum (NGS) in 0.1% Triton X-100 in PBS. Cells were incubated in blocking solution for 1hr RT with the following primary antibodies: monoclonal M2 anti-flag (Sigma) 1:1000, anti-NKAIN1loop 1:1000, anti-NKAIN1cterm 1:1000, monoclonal anti-Na,K-ATPase α1 (Upstate, 05-369) 1:1000, polyclonal anti-Na,K-ATPase β1 (Upstate, 06-170) 1:1000, monoclonal anti-Na,K-ATPase β1 (Upstate, 05-382) 1:1000. After washing with PBS, the transfected cells were incubated with the corresponding fluorescent secondary antibodies (goat anti-rabbit, or goat anti-mouse Alexa Fluor 568 or 488) at 1:1000 dilution in 1%NGS in PBS for 1hr at RT. Confocal imaging of the stained cells was done on LSM 510 Axioplan (Zeiss).

Mouse brains were perfused with 4% PFA and postfixed overnight at 4°C. 70μm vibratome sections were immunostained using the same protocol as for the transfected cells.

Transfected cell extracts

HEK293T cells were transfected using standard calcium phosphate method. Extracts of HEK293T transfected cells were obtained by homogenizing the cells grown in 60mm dish in 1ml of homogenization buffer (150mM NaCl, 10mM Tris, 1mM EDTA, protease inhibitors (Sigma), pH 7.4). Homogenates were centrifuged for 5min at 14000g at 4°C, and the pellets were resuspended in 200µl of homogenization buffer. To solubilize the membrane proteins, 20µl of 0.3M DHPC (Avanti), 60µl 5% Na Deoxycholate and homogenization buffer were added to the final volume of 600µl (final concentration 10mM DHPC, 0.5% Na Deoxycholate). Extracts were then rotated at 4°C for 30min and then centrifuged for 10min at 8000g at 4°C. Supernatants were used for western blotting or immunoprecipitation. Brain extracts were obtained using the similar protocol, except using 0.1% triton X-100 for solubilization.

Immunoprecipitation

2mg of Dynabeads M270 (Dynal) were coupled with 10µl of M2 anti-flag or anti-myc antibodies (Sigma) according to the manufacturer's instructions. The coupled beads were washed 4 times with PBS and 4 times with the homogenization buffer. 200µl of cell extract were added per 1mg of beads and rotated at 4°C for 1 hour. The beads were then washed 5 times with the wash buffer (150mM NaCl, 10mM Tris, 1mM EDTA, protease inhibitors (Sigma), 0.5% Na Deoxycholate pH 7.4). To elute the immunoprecipitated proteins the beads were boiled for 5min in the sample loading buffer (Invitrogen).

Glycosylation

Deglycosylation of the brain extracts was done using the Enzymatic Protein Deglycosylation Kit (Sigma) according to the manufacturer's instructions.

Western blot

NuPAGE Bis-Tris 4-12% gels and the MOPS or MES running buffers were used for the Western Blot. The primary antibodies were used at the following dilutions: HRP-M2 (Sigma) 1:1000, HRP-myc (Santa Cruz) 1:1000, polyclonal anti-β1 (Upstate, 06-170) 1:5000, anti-NKAIN 1:2000, anti-dNKAIN 1:5000, monoclonal anti-Nrv (5F7, Developmental Studies Hybridoma Bank) 1:1000. The HRP-coupled secondary anti-rabbit and anti-mouse antibodies (Pierce) were used at 1:25000 dilution. The signal was then revealed using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer) or Supersignal Femto Maximum Sensitivity Substrate (Pierce), and photographed using BioMax MR film (KODAK).

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 University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

Brain membranes solubilization and size fractionation

Membrane containing the fractions from the size-fractionation column was generously provided by Dr. Selimi. anti-NKAIN and anti-β1 antibodies were used as described above.

Gene-trap insertion mapping

ES cells for *RRE356* gene-trap line was obtained from BayGenomics, *S10-8E* line from Fred Hutchinson Cancer Research Center. The genomic sequence upstream of the *RRE356* gene-trap vector integration site was determined using the Vectorette™ System (Sigma/Genosys). Genomic DNA from ES cells carrying a gene-trap was digested with several restriction enzymes and ligated into corresponding Vectorette Units according to the manufacturer's instructions. PCR amplification using gene-trap and vectorette-specific primers yielded a 700bp band from *Hpa*II Vectorette constructs that corresponded to the sequence from the third intron of *NKAIN1*.

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 a c-terminal domain of NKAIN1. C-terminal part of NKAIN1 encoding amino acids 177-207 was cloned into pGBKT7 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. The plasmids of the positive clones were isolated and sequenced. Clones containing the sequence fragments of the Na,K-ATPase β1 subunit, the only putative interacting partner corresponding to 6 different 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 β1 subunit of the Na,K-ATPase.

Fly stocks

All fly stocks were maintained on conventional cornmeal-agar-molasses medium under a 12 hr light:12 hr dark cycle at 18°C or 25°C. *EP(2)560* and *EP(2)618* stocks were obtained from Exelixis, South San Francisco, (Rorth, 1996). The position of the p-element insertions was confirmed by PCR and sequencing. The deficiency stocks *Df(2R)Dll-MP, Df(2R)M60E, Df(2R)ES1* were from the Bloomington *Drosophila* Stock Center. *Df(2R)ED4071*, which is a part of the DrosDel collection (Ryder et al., 2004), was also obtained from the Bloomington Stock Center. $ATP\alpha^{DTS1}$ strain was kindly provided by Barry Ganetzky (University of Wisconsin, Madison, WI) and *Nrv1-GAL4*, *Nrv2-GAL4* by Paul Salvaterra (City of Hope Graduate School of Biological Sciences, Duarte, CA).

P-element excision

Mobilization of the P(EP) element, which contains a mini-white gene, was achieved by mating *EP560*/CyO females to Bl/CyOΔ2-3[w+]; ry e ca/TM6B males, and then mating the individual dysgenic males *EP560*/CyOΔ2-3[w+] to Bl/CyO; TM2/TM6B females. The individual *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 excisions resulted in approximately 40kb deletions of the dNKAIN locus as

determined by PCR using a set of primers located upstream and downstream of the original P-element insertion. One of these dNKAIN-null lines, *EP560*^{9} , was further analyzed. Using the primers on both sides of this deletion, the fusion site was PCR amplified and subcloned into a plasmid. The exact deletion ends, however, could not be sequenced due to presence of repetitive sequence.

Outcrossing of *EP560* to *yellow-white* flies

In order to test for an unlinked lethal mutation on the *EP560* chromosome, *EP560/CyO* males were mated to *yw* females. Then *EP560*/+ F1 females, in which recombination between the second chromosomes could occur, were mated to *Bl/CyO* males. 100 individual *EP560/CyO* males were then crossed to *Bl/CyO* females to establish the *EP560c/CyO* stocks, which were then screened for presence of the homozygous *EP560*c animals. Ten homozygous viable stocks were established, and the *EP560c25/EP560c25* flies were used in subsequent tests.

cDNA rescue

Full-length *dNKAIN* was cloned in to a pUAST vector using a forward primer containing a *Drosophila* Kozak sequence 5'ggaagatctcaacatgGGCTCGTGCTCGTGTAC and the following reverse primer gctctagaGTGCAGCGGGACGTACTTAAG. *yw* flies were transformed using standard procedures. *UAS-dNKAIN* flies were then mated to *elav-GAL4*, *actin-GAL4*, *Nrv1-GAL4* and *Nrv2-GAL4* flies.

Adult fly brain in situ

Antisense digoxigenin riboprobes were made by cloning a fragment of the *dNKAIN* gene amplified by RT-PCR from *Drosophila* mRNA using primers 5'ggactagtTTCGACTTCCTCGGCTACAT and 5'cccaagcttTGGTGTAGTTTGGGTTGCTG into the pBSK vector and DIG labeled using the DIG DNA Labeling kit (Roche) according to manufacturer's instructions. The alkaline phosphatase visualized in situ hybridization was performed as previously described (Vosshall et al., 1999).

***Drosophila* embryos in situ**

Whole mount *Drosophila in situ* hybridization was carried out as previously described (Schmucker et al., 2000) using the same probe as for the adult brain *in situ*.

***Drosophila* behavioral testing**

Six 4-6 day old flies were placed in the glass vials and immersed in a 38°C water bath. The number of paralyzed or severely uncoordinated animals was counted every 2.5min for 10min. A total of 60 *EP560/EP560* flies were used, and 30 heterozygous and wildtype controls were examined.

Fly western blot and dNKAIN expression level

50 fly heads of each genotype were collected and washed in cold 0.1% triton X-100 in PBS. The heads were homogenized in 200µl of homogenization buffer (150mM NaCl, 10mM Tris, 5mM EDTA, 0.5% triton, protease inhibitors cocktail P8340, Sigma). The

homogenate was centrifuged for 15min at 16,000g at 4°C. The supernatant was collected and diluted 3x before adding the loading buffer (Invitrogen). NuPAGE Bis-Tris 4-12% gels and the MOPS running buffer were used for the Western Blot. Anti-dNKAIN antibody was used at 1:5000 dilution, and the anti-syntaxin (8C3, Hybridoma Bank) was used for control at 1:1000 dilution. After incubation with the HRP-coupled secondary antibodies and either Western lightning Chemiluminescence Reagent Plus (Perkin Elmer) or Supersignal Femto Maximum Sensitivity Substrate (Pierce), the membranes were scanned using the Typhoon 9200 Imager. The resulting images were analyzed using ImageQuant software. The dNKAIN band intensity values were normalized to the 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.

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