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Report

A Putative Cation Channel and Its Novel Regulator: Cross-Species Conservation of Effects on General Anesthesia

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Summary

Volatile anesthetics like halothane and enflurane are of interest to clinicians and neuroscientists because of their ability to preferentially disrupt higher functions that make up the conscious state. All volatiles were once thought to act identically; if so, they should be affected equally by genetic variants. However, mutations in two distinct genes, one in *Caenorhabditis* and one in *Drosophila*, have been reported to produce much larger effects on the response to halothane than enflurane [1, 2]. To see whether this anesthesia signature is adventitious or fundamental, we have identified orthologs of each gene and determined the mutant phenotype within each species. The fly gene, *narrow abdomen* (*na*), encodes a putative ion channel whose sequence places it in a unique family; the nematode gene, *unc-79*, is identified here as encoding a large cytosolic protein that lacks obvious motifs. In *Caenorhabditis*, mutations that inactivate both of the *na* orthologs produce an Unc-79 phenotype; in *Drosophila*, mutations that inactivate the *unc-79* ortholog produce an *na* phenotype. In each organism, studies of double mutants place the genes in the same pathway, and biochemical studies show that proteins of the UNC-79 family control NA protein levels by a posttranscriptional mechanism. Thus, the anesthetic signature reflects an evolutionarily conserved role for the *na* orthologs, implying its intimate involvement in drug action.

Results and Discussion

Volatile anesthetics have long had a prominent place in the practice of medicine, but there remains much uncertainty about the mechanism of action of these drugs [3, 4]. Most current studies examine the way volatile anesthetics affect biochemical and physiological processes [5, 6]. To provide a different perspective, we have used a pharmacogenetic approach with the invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster*, organisms that respond to volatiles in ways that are reminiscent of mammalian responses. Among the mutations identified in our studies, a few showed larger effects with one anesthetic than with another; this phenotype is of particular interest because it undermines the classical notion [7, 8] that all volatile agents work via the same mechanism and at the same targets.

The earliest report of agent-specific effects involved *unc-79* mutants [9, 10]. Three alleles of the *C. elegans* gene conferred strikingly increased sensitivity to the immobilizing action of some volatile anesthetics, such as halothane, but left sensitivity to other volatiles, such as enflurane, unaffected or even slightly lowered [1, 9]. We tested seven other alleles (see the Supplemental Data available online) and found that each conferred a similar phenotype. Remarkably, mutations that were subsequently mapped to the *narrow abdomen* (*na*) gene of *D. melanogaster* produce a closely related pattern of altered sensitivity, at least in assays of one anesthetic endpoint [2]. In addition to the common pattern of sensitivity, the mutants share a nonanesthetic phenotype in that they each display a pattern of locomotion characterized by periods of quiescence—"fainting" in *unc-79* [9–11] and "hesitant walking" in *na* mutants [12, 13]. To explore whether the similar phenotypes imply a significant connection between the genes, one needs to look at their effects in the same organism. Here, we use molecular analysis of *unc-79* and *na* to acquire orthologous mutations and determine whether the agent-specific phenotype reflects a conserved role for these genes in anesthesia.

The *na* gene of *Drosophila* has been shown to encode a polypeptide whose predicted topology resembles that of voltage-gated sodium and calcium channels [14]. Indeed, the aligned sequences of *na* and its invertebrate and vertebrate orthologs [15] clearly form a branch of this superfamily, designated the α1U branch [16], whose members share distinct intracellular domains and pore signatures. Although failure of heterologous expression has so far precluded a definitive demonstration of α1U channel activity, recent evidence for the importance of a presumptive pore-lining residue of NA [17] supports its assignment as an ion channel. The α1U family was first discerned in *C. elegans* [15], where it is represented by two genes, *nca-1* and *nca-2*. When deletion alleles of the putative-channel genes (Figure S1) are combined, the resulting *nca-2(gk5);nca-1(gk9)* double mutant is virtually identical to an *unc-79* mutant in sensitivity to various anesthetics (Figure 1A) and in displaying a "fainting" pattern of locomotion (Movie S1). In fact, all double-null-allele combinations of *nca-2;nca-1* are similar to *unc-79* in anesthetic sensitivity (Figure 1B) and move

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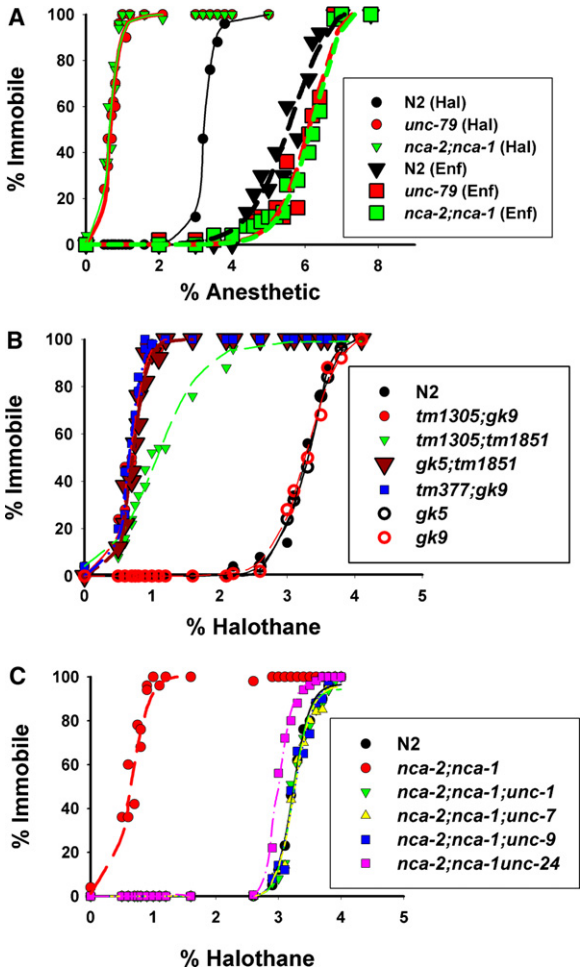

Figure 1. The Effect of *nca-1* and *nca-2* Mutations on Anesthetic Sensitivity in *C. elegans*

(A) Dose-response studies in two volatile anesthetics, halothane (Hal) and enflurane (Enf), for the wild-type N2 control nematode, *unc-79(ec1)*, and *nca-2(gk5);nca-1(gk9)*. The data points are fit to the standard concentration-response formula [23] with a slope constant of 18. Note that both mutant strains have a markedly increased sensitivity to halothane (solid lines, small symbols). In contrast, only a small difference in sensitivity, a mild resistance [1], is seen with enflurane (dashed lines, large symbols).

(B) By comparison with the N2 control strain, double mutants with the indicated combinations of independently generated null alleles of *nca-2* and *nca-1* are also hypersensitive to halothane, but single-mutant alleles, *nca-2(gk5)* and *nca-1(gk9)* are not. Concentration-response curves were fitted as in (A) except that the curves for *nca-2(tm1305);nca-1(tm1851)* and *nca-2(gk5);nca-1(tm1851)* used slope constants of 4 and 6, respectively. None of the mutants, tested either singly or in double-mutant combination, display increased sensitivity to enflurane (not shown).

(C) Null mutations [*unc-1(fc53)*, *unc-24(e138)*, *unc-7(e5)*, *unc-9(e101)*] in each of the four indicated genes that are known to suppress the halothane hypersensitivity of *unc-79* [1, 24] also suppress the *nca-2(gk5);nca-1(gk9)* double mutant. Note that the suppression is essentially complete in that the halothane sensitivity is restored to the level of the control strain, N2. The previously published effects of these suppressors on *unc-79* are not shown but are essentially identical to those for the *nca* double mutants.

in a fainting manner. In contrast, animals with mutations in *nca-1* or *nca-2* individually resemble the wild-type strain (N2) in both locomotion (not shown) and anesthetic sensitivity (Figure 1B). Because the mutations tested, *nca-1(gk9)* and *nca-2(gk5)*, are null alleles (Supplemental Experimental Procedures), the paralogs appear to function redundantly for the endpoints we have examined. This is confirmed by the ability of either an *nca-1* or an *nca-2* transgene to fully rescue both the fainting locomotion and the anesthetic hypersensitivity of the double mutant (Table S1). The *nca* genes not only yield a similar phenotype to *unc-79* but, as evidenced by genetic interaction studies, also appear to have a strong functional relationship with it. Four mutations have been shown to suppress the anesthetic phenotype of *unc-79* [1], and each of them suppresses the *nca-2;nca-1* double mutant (Figure 1C). Moreover, triple mutants with either *unc-79(ec1)* or *unc-79(e1068)* together with *nca-2(gk-5)* and *nca-1(gk9)* have a fainting phenotype and anesthetic-sensitivity profile that is identical to that of the single *unc-79* mutant (not shown).

In a parallel way, we wished to ask whether loss of UNC-79 function in *Drosophila* produced a phenotype similar to that showed by *na* mutants. The first, and most difficult, step was to molecularly identify the *C. elegans* gene associated with the *unc-79* mutations. After recombination mapping, the *unc-79* gene was localized by cosmid rescue of the mutant phenotype (Table S1). The gene occupies almost all of a 19 kb fragment that contains what had been predicted to be four separate genes (Figure 2A). That *unc-79* comprises all four predicted ORFs is demonstrated by both the location of sequence changes in various alleles and the existence of cDNAs that span them (Figure 2B). Although the predicted sequence for the gene product provides no hint as to the function of UNC-79, database searches show that flies, mice, and humans each contain a single close ortholog. The *Drosophila* ortholog is annotated as CG5237 [18], and from a public repository [19] we obtained a strain bearing a transposon that disrupts the gene (hereafter called *dunc79*). When assayed for the ability of anesthetics to interfere with reactive climbing [13], the *dunc79* mutant has a halothane sensitivity exactly like that of an *na* mutant, and an *na;dunc79* double-mutant strain has a halothane sensitivity no different than that of either single mutant (Figure 3A). In addition, like *na* mutant flies, *dunc79* flies elute more slowly than control flies during inebriometer tests of postural control [2] in response to halothane but not to enflurane (Figure 3B). Flies bearing mutations in *na* or *dunc79* also share nonanesthetic phenotypes, including altered circadian locomotor patterns ([14], Bridget Lear, personal communication), cylindrically shaped abdomen (not shown), and hesitant walking mode (Movie S2). In addition, both *na* and *dunc79* mutants display an oscillation that can be recorded electroretinographically (unpublished data) or visualized as periodic twitching in restrained animals [14].

Given the similar phenotype conferred by their inactivation within each organism (Table 1) plus the effect of mutant combinations described above, the genes we have studied appear to act in a common pathway. The simplest model for their relationship would thus be for one to control the expression of the other. Indeed, western blots and immunohistochemistry (Figure 4) reveal that levels of the putative channel are reduced to background in the absence of UNC-79/DUNC79 function.

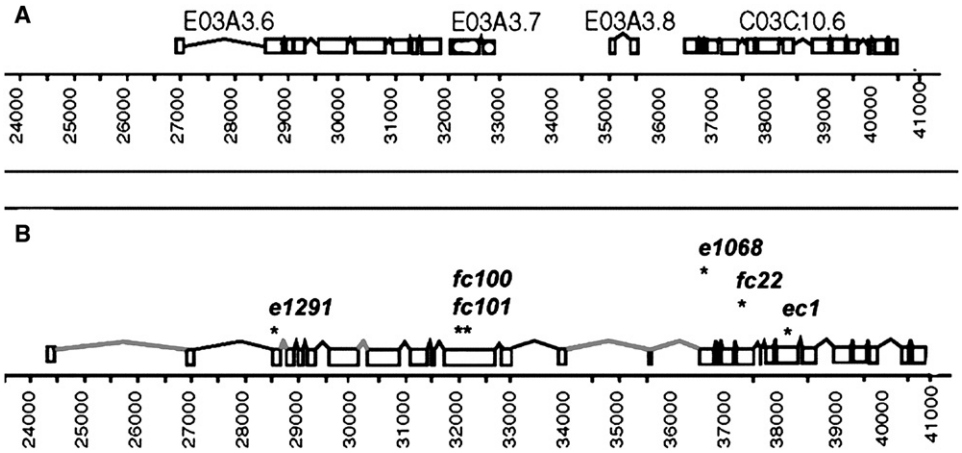

Figure 2. The Structure of the *C. elegans unc-79* Gene

(A) The original Genefinder annotation of predicted intron-exon boundaries for four separate *C. elegans* genes from the cosmid-E03A3 region that rescues Unc-79 phenotypes.

(B) The experimentally determined structure of the gene. A 6451 nt transcript, assembled from two overlapping RT-PCR products, is shown and encompasses all the predicted ORFs of (A). Successful transgenic rescue of the *unc-79* mutant phenotype requires a genomic fragment (bp 21858–40962 of cosmid E03A3) that includes all this material, and *unc-79* mutations (asterisks) are distributed throughout the region. Details of the cDNAs and the full-length ORF are provided in Supplemental Experimental Procedures, as is evidence that this gene frequently accumulates transcripts with retained introns.

Given the sensitivity of these assays, we cannot rule out that low levels of protein are present; such residual expression in flies might explain why some aspects of the *dunc79* mutant phenotype (e.g., Figure 3B) are weaker than those of *na* mutants. Nevertheless, the expression defect is at least 10-fold and is specific, because western blots revealed no change in level of other fly and nematode membrane proteins (not shown). Interestingly, in both organisms the UNC-79 ortholog appears to have little or no effect on transcription of the genes encoding the putative channel. Probing northern blots for the *nca-1* and *nca-2* transcripts revealed no effect of *unc-79(e1291)* on message size and amount (Figure S2A). Similarly, RT-PCR revealed that disruption of *dunc79* caused no perturbation in structure or gross level of *na* transcripts (Figure S2B). This implies that UNC-79/DUNC79 normally serves in the posttranscriptional processing of the putative channel, e.g., by affecting translation, protein modification, trafficking, protection against degradation, etc. The same may be true for the nematode *unc-80* gene, known to generate a mutant phenotype that precisely mimics the Unc-79 phenotype [9, 10], because the *e1272* mutant also lacks detectable NCA1/2 protein despite the presence of normal transcripts (not shown).

How directly do the UNC-79 orthologs control the expression of the putative channel? One way to explore this issue is to see whether the corresponding gene products are present in the same tissue at the same time. Toward this end, we examined GFP expression in *C. elegans* from constructs in which this reporter was linked to the promoter regions of *unc-79*, *nca-1*, or *nca-2*. Although each reporter shows conspicuous (but not exclusive) expression in the nervous system, the pattern of *unc-79* does not coincide with that of *nca-1* or *nca-2* or with their sum (Figure S3). This disagreement might merely reflect infidelity of a reporter pattern, but, taken at face value, it hints that the regulation is indirect, e.g., that UNC-79 might be used nonautonomously.

An intriguing feature of the genes studied in this paper is that inactivating them produces agent-specific effects on anesthesia. There are several implications of this phenomenon. First, differences between mutant effects on halothane and enflurane sensitivity make it unlikely that the affected genes are merely needed for vigorous neuromuscular function; if they were, inactivation would render either organism hypersusceptible to both agents. Further evidence against such a trivial model comes from the observation [20] that an *na* mutation increases the potency with which a volatile anesthetic alters local field potentials recorded directly from fly brains. To this background, as summarized in Table 1, our current work establishes that agent-specific effects of the *unc-79/dunc79* and *na/nca* genes are conserved between organisms. This evolutionary conservation is particularly incisive, arguing against many scenarios for indirect effects. According to such models, the putative channel is not in neurons that are affected directly by anesthetics but only influences sensitivity because neurons that depend on it for optimal performance are in communication with such target neurons. However, because the neuronal circuitry of the nematode is strikingly different from that of the fruit fly, it is hard to imagine that in each organism there are halothane-sensitive and enflurane-insensitive neurons that just by chance are connected to neurons containing the putative channel. Thus, indirect models for the effect of *na* and its orthologs on anesthesia are disfavored. It must be noted that, at least in fruit flies, some anesthetic endpoints do not show the same agent-specific effects described in Table 1. For example, in the distribution test *na* and *dunc79* mutants are hypersensitive to both halothane ([13, 21] and above) and enflurane ([21] and data not shown). Because these effects lack a distinctive signature, it is hard to rule out indirect models for their generation. Nevertheless, for

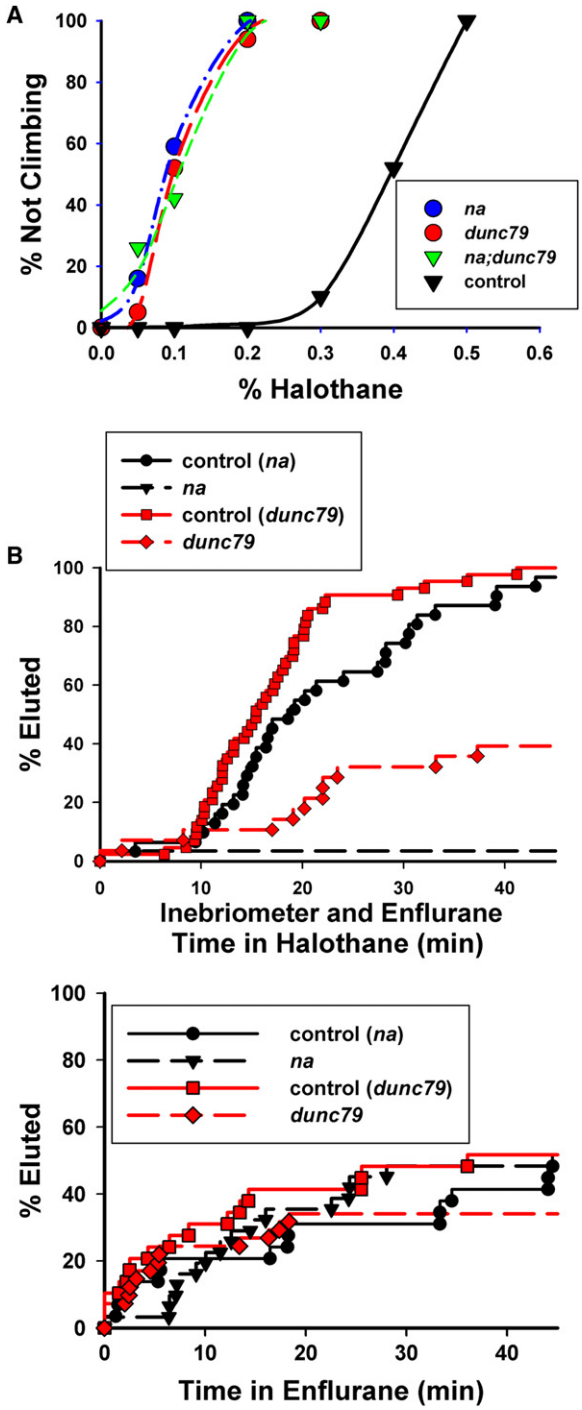

Figure 3. The Effect of a *dunc79* Mutation on Anesthetic Sensitivity in *D. melanogaster*

(A) Distribution tests of reactive climbing. Halothane concentration-response curves are fitted as above with a slope constant of 8. As reported before for different alleles in a different strain background [13, 21], the curve for an *na* mutant (e04385) is greatly left-shifted compared to an isogenic control strain. In the same genetic background [19], a *dunc79* mutation (f03453) confers similar hypersensitivity to halothane, and the *na;dunc79* double mutant is no more sensitive than either single mutant.

(B) Inebriometer tests of postural control. Parallel tests were run simultaneously for the control strain and a single mutant; each such pair is presented as solid and dashed lines of the same color (black for *na*, red for *dunc79*). The step plots show the time-dependent increase in the cumulative fraction of flies of each genotype that tumble out of the inebriometer column. Note that the mutants elute much more slowly than the wild-type when exposed to 0.5% halothane (top) but elute very similarly to the wild-type control when exposed to 0.7% enflurane (bottom).

Table 1. Effects of *unc79*/*dunc79* and *nca*/*na* Mutations

C. elegans		
Assay/condition	*unc-79*	*nca-2;nca-1*
Locomotion/air	fainter	fainter
Immobility/halothane	very hypersensitive	very hypersensitive
Immobility/enflurane	near wild-type	near wild-type
D. melanogaster		
Assay/condition	*dunc79*	*na*
Locomotion/air	hesitant	hesitant
Inebriometer/halothane	slow	very slow
Inebriometer/enflurane	near wild-type	near wild-type

the circuits that subserve endpoints with agent-specific dependence on NCA or NA function, our observations suggest that neurons within them directly depend on these gene products for resisting the effects of halothane. In this population, the putative channel could be a molecular target of halothane (but not enflurane) or could serve to stabilize neuronal performance against the deleterious effects of halothane on some other component in that cell (one that is insensitive to enflurane). In considering these models, one must keep in mind that volatiles are likely to have more than one molecular target, each of which may be necessary but not sufficient to produce the desired endpoint [22]. Thus, although the putative channel may play a critical role in setting anesthesia sensitivity, it is unlikely to be the only factor. Nevertheless, our observations provide a strong clue that, at least for some endpoints, the relationship between the α1U family and halothane action is intimate.

In summary, this work has established that there is a remarkable parallelism between two sets of genes in two distantly related organisms. One set of genes, *unc-79*/*dunc79*, acts as a posttranscriptional regulator of the other set, *nca*/*na*, which encodes a putative ion channel. Moreover, there is a strong parallelism in the phenotypes of animals carrying mutations in these genes, with subtle effects on locomotion and strong effects on sensitivity to certain volatile anesthetics found in both organisms. The conserved nature of the agent-specific effects implies that the channel is present in anesthetic-sensitive neurons and has important effects on the degree to which these neurons resist the effects of volatile agents. Because both sets of genes are found in vertebrates and have been shown to be expressed neuronally, there is every reason to believe that they will strongly influence the clinical effects of volatile anesthetics.

Supplemental Data

Supplemental Data include Experimental Procedures, three figures, and two movies and are available with this article online at: http://www.current-biology.com/cgi/content/full/17/7/624/DC1/.

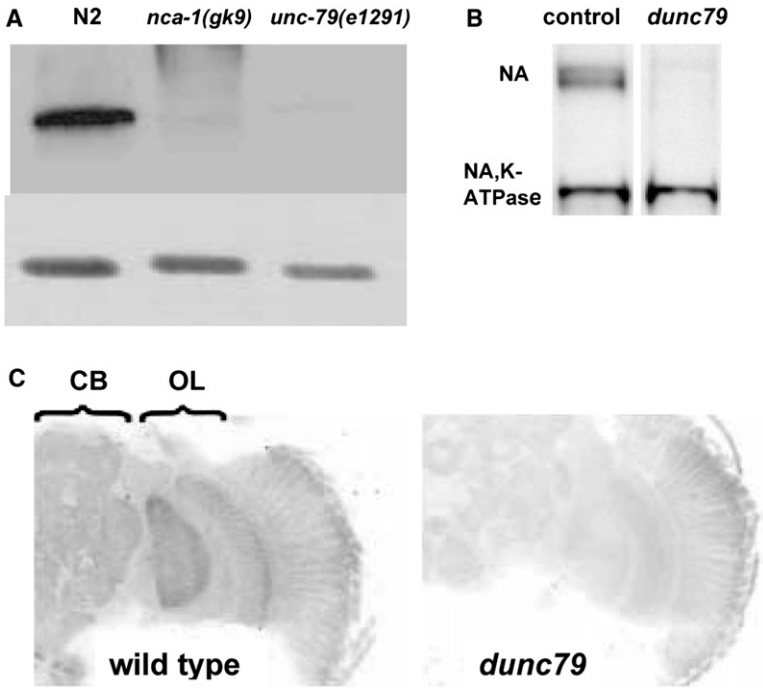

Figure 4. Dependence of *nca/na* Channel Expression on *unc-79/dunc79*

(A) Western blots (top) with anti-NCA-1 of extracts from mixed-age cultures of N2, an *nca-1* deletion mutant (*gk9*), and an *unc-79* mutant (*e1291*). Compared to the N2 wild-type extract, only nonspecific background staining is seen in the mutants. The same outcome is obtained with the comparable experiment (not shown) using anti-NCA-2. The same samples are probed with anti-UNC-1 (bottom) as a control for loading.

(B) Western blot of extracts from fly heads of a *dunc79* mutant and an isogenic control strain probed with anti-NA and anti-Na, K-ATPase. A doublet of Mr ~180 kDa that is specific for NA protein [14] is undetectable in the *dunc79* extract. The rough equivalence of loading in these extracts and the specificity of the *dunc79* effect for NA is demonstrated by the comparable signal from Na,K-ATPase (Mr ~100 kDa).

(C) Staining of cryosections of adult *Drosophila* heads with an antibody specific for the *na*-encoded ion channel. As reported before [14], in wild-type flies (left panel) the channel is expressed widely in the synaptic regions (neuropil) of the brain, particularly in the central brain (CB) and in parts of the optic lobe (OL). In *dunc79* mutant flies (right panel), no staining is detected above background.

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Accession Numbers

The following sequences have been deposited in GenBank with the corresponding accession number: the *C. elegans unc-79* cDNA sequence, DQ858354; the *D. melanogaster dunc79* cDNA sequence, DQ923614; the alternatively spliced *C. elegans nca-1* cDNA sequences, DQ917240 and AY555271; and the alternatively spliced *C. elegans nca-21* cDNA sequences, DQ917241 and AY555272.