Evolution of sodium channels predates the origin of nervous systems in animals

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Voltage-dependent sodium channels are believed to have evolved from calcium channels at the origin of the nervous system. A search of the genome of a single-celled choanoflagellate (the sister group of animals) identified a gene that is homologous to animal sodium channels and has a putative ion selectivity filter intermediate between calcium and sodium channels. Searches of a wide variety of animal genomes, including representatives of each basal lineage, revealed that similar homologs were retained in most lineages. One of these, the Placozoa, does not possess a nervous system. We cloned and sequenced the full choanoflagellate channel and parts of two placozoan channels from mRNA, showing that they are expressed. Phylogenetic analysis clusters the genes for these channels with other known sodium channels. From this phylogeny we infer ancestral states of the ion selectivity filter and show that this state has been retained in the choanoflagellate and placozoan channels. We also identify key gene duplications and losses and show convergent amino acid replacements at important points along the animal lineage.

eumetazoan | inactivation gate | pore motif

Early animals radiated explosively in the Precambrian (1). This radiation was facilitated by the previous evolution of genes for cell adhesion that presaged the evolution of multicellularity (2). Another key animal innovation was the nervous system, which is present in all but a few animals (i.e., sponges and placozoans). Rapid, specific, long-distance communication among excitable cells is achieved in bilaterian animals and a few jellyfish (cnidarians) through the use of action potentials (APs) in neurons generated by voltage-dependent sodium (Na_v) channels. Voltage-dependent calcium (Ca_v) channels evolved in single-celled eukaryotes and were used for intracellular signaling. It has been hypothesized that Na_v channels were derived from Ca_v channels at the origin of the nervous system (3), thereby conferring the ability to conduct action potentials without interfering with intracellular calcium. This view was reinforced by the apparent lack of sodium currents in sponges (4).

To test this hypothesis, we searched newly available genome databases from two animals with simple nerve nets (the sea anemone *Nematostella vectensis* and the ctenophore *Mnemiopsis leidyi*), a placozoan with no nervous system (*Trichoplax adhaerens*), a sponge (*Amphimedon queenslandica*), a single-celled eukaryote (the choanoflagellate, *Monosiga brevicollis*), as well as fungi and additional single-celled eukaryotes for homologs of Ca_v and Na_v channels. We then verified the expression of these genes in *M. brevicollis* and *T. adhaerens* and examined amino acid changes in these genes throughout the history of animal evolution.

Choanoflagellates are widely distributed unicellular protists (5, 6) that form the sister group to the multicellular animals (7). Placozoans are an early-diverging animal lineage that has been proposed to be sister to the eumetazoans, that is, to all animals with nervous systems (8). However, phylogenetic placement of the basal animal lineages is not yet fully resolved (9–12), and many aspects of placozoan life cycles remain unknown (13–15). Choanoflagellates and placozoans have received considerable attention due to their possession of numerous genes once thought to be exclusive to eumetazoans (2, 16–18).

Ca_v and Na_v channels have four domains, each of which has a pore loop (Fig. 1). A single amino acid at the deepest part of each pore loop is responsible for ion selectivity in the pore. Ca_v channels have acidic residues (E and D) in the pore of domains I–IV (usually E/E/E/E or E/E/D/D). Selectivity for sodium, on the other hand, is based on the residues D/E/K/A in the pore. Sodium channels also have a cytoplasmic loop between their third and fourth domains that swings up and occludes the channel pore just milliseconds after activation (Fig. 1). This fast inactivation makes sodium signaling reliable on the millisecond time scale, and mutations at this region in human Na_v channel genes cause many well-known pathologies (19). Calcium channels do not have a similar motif at the homologous region. Because of the differences in the amino acids responsible for ion selectivity, and because proteins are likely to be under strong evolutionary constraints along every point of their evolution (20), it has been suggested that channels with intermediate pore sequences may exist in extant taxa (3, 21), and some invertebrate channels have been proposed as representatives of these intermediate states (21, 22). The phylogenetic relationships of these channels are not clear however (22–24), and no suggestion of an ancestral metazoan pore state has been put forth.

Our objective was to find voltage-gated ion-channel genes in basal animals and their close unicellular relatives, determine whether the genes are expressed in a few key species, and analyze the evolutionary history of the genes for Na_v and Ca_v channels. We examined pore motifs, inactivation gate sequence, and inactivation gate secondary structure and then mapped these states onto our phylogeny. This work provides a unique view of Na_v and Ca_v channel evolution and the evolution of excitable tissues in animals.

Results

Sodium Channel Homologs in Early-Diverging Animals and Choanoflagellates. We found that the genomes of *M. brevicollis*, *T. adhaerens*, *N. vectensis*, and *M. leidyi* contain genes for ion channels that group with the Na_v family (Fig. 2), and we used these genomic sequences as references for further analyses. We found pairs of Na_v paralogs in *Trichoplax*, *Nematostella*, and *Mnemiopsis*, which we name α and β. The genome of the sponge *A. queenslandica* did not contain Na_v homologs but did have one gene for a Ca_v channel. No Na_v homologs were found in the genomes of *Aspergillus niger*, *Saccharomyces cerevisiae*, or any other fungi in the Joint Genome Institute database. We sequenced the entire ORF of an mRNA

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Data deposition: Accession numbers and relevant databases are provided in Table S1. The sequences reported in this paper have been deposited in the GenBank database (accession nos. JF827087, JF905561, JF905562 and JF905563).

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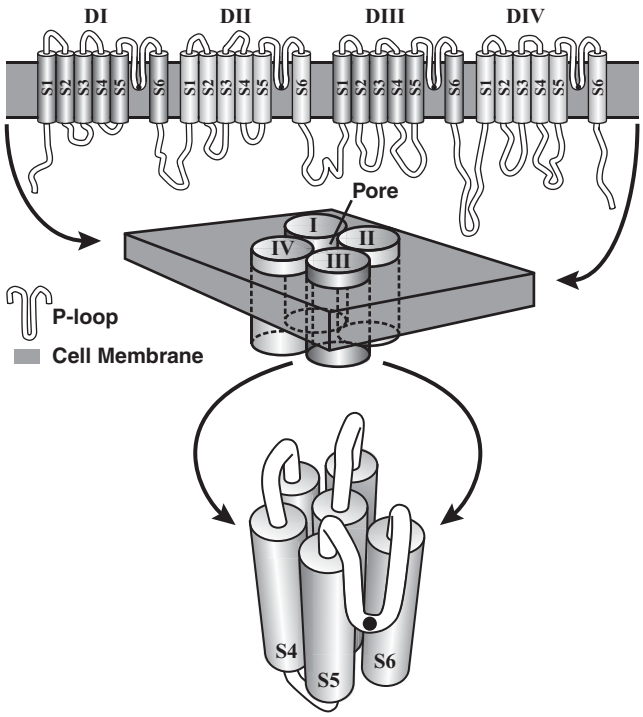

Fig. 1. Hypothetical secondary structure of a sodium-channel protein. (*Top*) Transmembrane domains (DI–DIV), their component segments (S1–S6), and their connecting loops (in white). The pore loops (P loop), which dip down into the membrane, form the ion-selectivity filter. The inactivation gate resides on the long loop between DIII/S6 and DIV/S1. (*Middle*) How the domains cluster to form the protein and its pore. (*Bottom*) Fine structure of one of the domains with the pore loop in the foreground. The black dots on the pore loops in the *Top* and *Bottom* represent the location of the amino acids, which makes up the pore motif.

transcript from *Monosiga* and partial transcripts from the two genes in *Trichoplax*, thereby demonstrating that these genes are expressed. The genes have a pore motif D/E/E/A that is intermediate between Ca_v and Na_v channels and is the same as some previously described invertebrate channels (21, 23).

For one of the paralogs, *Trichoplax* β, only three of the four domains typical to Ca_v and Na_v channels were found in the genome, likely due to a problem with the genome assembly. It is unlikely that a three-domain protein could function as an ion channel alone, but it is not yet known whether the genome sequencing effort simply missed part of the genome, whether our BLAST analysis misidentified the exons for the last domain, or whether it is actually a splice variant or some other regulatory transcript. The ctenophore Na_v homologs and the sponge Ca_v channel are missing amino acids in the putative pore regions, perhaps also due to incomplete assembly.

Four overlapping segments from choanoflagellate mRNA were compiled to yield 4,589 nucleotides, which we believe includes the whole ORF. This sequence was 93.4% identical to the reference sequence obtained with BLAST (*SI Materials and Methods* and Fig. S1).

Sequencing of the *Trichoplax* genes yielded 868 bp from the *Trichoplax* α gene, which aligned to the reference with 92.7% identity, and 1,062 bp from the *Trichoplax* β gene, which aligned with 91.0% identity. Many of the mismatches in the *Trichoplax* β segment are from indeterminate nucleotides and may be due to the fact that this segment was sequenced directly from the PCR products rather than from cloned genes. Although further confirmation of the exact sequences is needed, the presence of these sequences in the mRNA demonstrates that both *Trichoplax* genes are indeed transcribed.

Phylogenetic Analyses. We performed maximum likelihood (ML) analyses on a data set consisting of our sequenced choanoflagellate gene, a putative Ca_v gene from *Monosiga*, and Na_v and Ca_v genes from all major animal lineages and two fungal species, *Aspergillus* and *Saccharomyces* (Fig. 2). The phylogenetic placement of the ion channel genes agrees with the well supported parts of the phylogeny for animals, choanoflagellates, and fungi (8, 9, 12), and the topology was robust to analyses on other platforms and removal of taxa (*SI Materials and Methods*). The placement of the *Amphimedon* Ca_v channel as basal to *Monosiga* Ca_v (Fig. 2) is probably an artifact due to long-branch attraction (LBA). This seems likely because the *Amphimedon* branch is long, and *Monosiga* Ca_v is a partial sequence. The placement of these two sequences within Ca_v channels is not consistent with an LBA artifact, however, and is strongly supported by bootstrap analysis, indicating that these are true Ca_v channels. The fungal Ca_v channels were resolved as the sister group to all animal and choanoflagellate channels. These results support the hypothesis that Na_v genes evolved from Ca_v genes, because the Na_v family emerges from within animal and fungal Ca_v channels.

Our phylogeny supports the view that placozoans, which have the simplest animal body plan, branched off the animal stem after ctenophores and are therefore likely to be secondarily simplified. This scenario was found in both Na_v and N/P/Q type Ca_v genes (*SI Materials and Methods*).

Bootstrapping scores indicate strong support for critical nodes of the Ca_v/Na_v gene phylogeny. The position of the choanoflagellate Na_v channel gene at the base of animal Na_v channel genes was supported in 100% of the bootstrap replicates. The bootstrapping analysis also provides strong support for the monophyly of known groups of Ca_v and Na_v channel genes, including the bilaterian Na_v 1 clade and the three major groups of Ca_v channels. The clades containing channels with pore motifs D/E/E/A in both Cnidaria and Bilateria were less well supported in the bootstrap analysis (50–65% of replicates).

Secondary Structure. Analysis of secondary structure of the inactivation gate revealed conservation of two important α-helices across the Na_v channel family (Fig. S2). This structure was absent in Ca_v channels, including the yeast Ca_v channel.

Discussion

Rooting the Na_v and Ca_v Gene Families. The choanoflagellate *M. brevicollis* and the placozoan *T. adhaerens* express ion-channel genes that group phylogenetically with previously described sodium channels (Fig. 2) and have key molecular signatures of sodium channels (Fig. 3). Others have proposed that Na_v channels evolved from an ancient Ca_v channel resembling the T-type channels (3) and that there may therefore be extant channels that have properties midway between Ca_v and Na_v channels (21). Candidates for such channels have been proposed (21, 22), but the origin and genetic history of Na_v channels have remained obscure. Our phylogenies show that the Na_v ion channel family originated not only before the advent of the nervous system, but probably even before the advent of multicellularity. These results support the idea that Na_v channels arose from Ca_v channels, but push back this divergence date to at least the common ancestor of animals and choanoflagellates. This demonstrates that complex systems like excitable tissues can evolve by coopting existing genes for new functions, rather than by de novo evolution of new genes.

Voltage-Gated Ion Channels and the Animal Phylogeny. The phylogenetic placement of basal animal lineages (sponges, ctenophores, placozoans, and cnidarians) is not yet fully clear, although some placements are less controversial than others. The placement of sponges as sister to all other animals, and of cnidarians as sister to bilaterians, are fairly consistent results (12). The placements of ctenophores and placozoans, however, are less certain

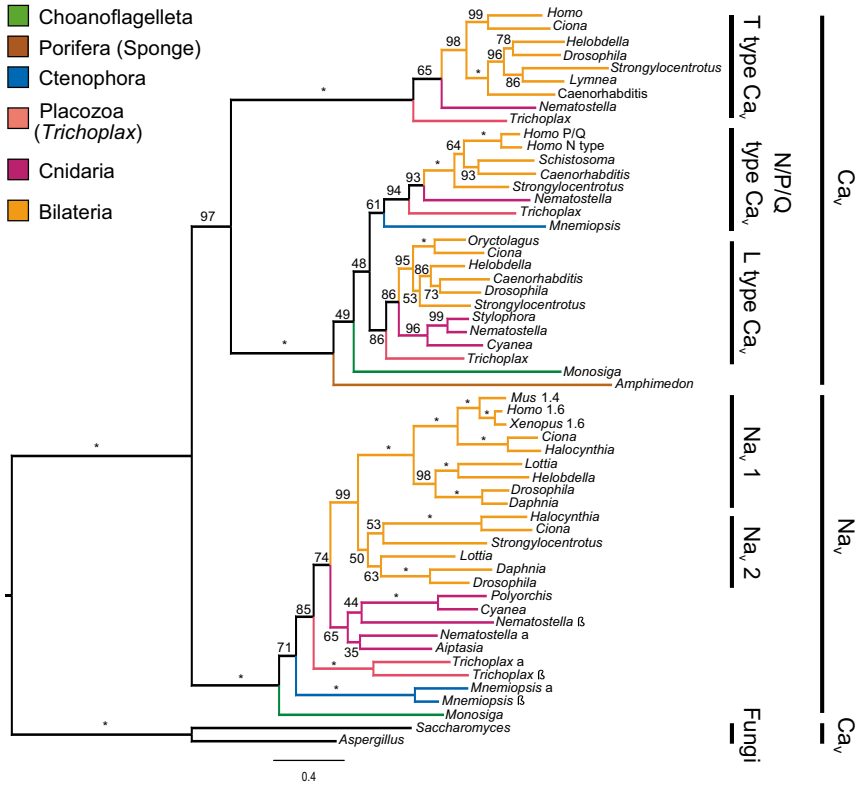

Fig. 2. Maximum likelihood phylogeny of Na_v and Ca_v channels. Bootstrap scores are indicated on branches, with stars indicating scores of 100%. Clades corresponding to major ion channel groups are detailed on the *Right*.

(*SI Materials and Methods*). Our results are consistent with the traditional phylogenetic placement of sponges and cnidarians, but place ctenophores, which have a nervous system, outside of the placozoans, cnidarians, and bilaterians (Figs. 2 and 4). This would suggest that placozoans have lost their nervous system or, much less likely, that the nervous system evolved twice in ctenophores and the cnidarian–bilaterian ancestor. Although our analysis has relatively strong bootstrap support, it has sparse taxon sampling, which has been shown to meaningfully affect phylogenetic inference (11, 25) and cannot therefore be considered a decisive species phylogeny.

Also interesting is the apparent loss of Na_v homologs in the sponge *Amphimedon*, an event that may reflect the sedentary lifestyle of these animals. Electrical impulse conduction has not been shown in demosponges, the group that includes *Amphimedon*, but it has been shown in a hexactinellid sponge (4). Hexactinellids differ drastically from demosponges in terms of morphology; further analysis of hexactinellids will be needed to determine whether Na_v homologs have been retained in this group.

Genetic History—Bilateria. Our results help clarify the diversity of pore states observed in animal Na_v channels. The topology of our tree suggests that D/E/E/A is the ancestral pore sequence of the Na_v gene family and that genes with this motif have been retained in every metazoan lineage that we examined, except for sponges, vertebrates, and the cnidarian subgroup Medusozoa (Figs. 3 and 4). The topology of the Na_v 1 and Na_v 2 clades supports the hypothesis that a gene duplication occurred around the time of the bilaterian radiation and before the split of protostomes and deuterostomes (24). The Na_v 1 duplicate evolved a pore motif D/E/K/A and underwent further duplications in early tetrapods, creating the genes for Na_v 1.1–1.9 in mammals (26). The other duplicate retained the ancestral pore motif and was lost in vertebrates.

Genetic History—Cnidaria. Cnidarians diverged before the bilaterian gene duplication and do not have D/E/K/A channels, but the medusozoans have an amino acid substitution in the second domain pore loop, resulting in a clade of channels with the pore motif D/K/E/A. Although the topology of cnidarian channels with glutamic acid (E) in the second domain was not well supported, the clade of D/K/E/A channels was repeatedly found to represent a derived state and was monophyletic with 100% support. In species-tree analyses, the medusozoans share a common ancestor that is not shared with the anthozoans (8–12). The medusozoan subgroups represented here are Hydrozoa (*Polyorchis*) and Scyphozoa (*Cyanea*), both of which have D/K/E/A in the pore, whereas the anthozoan representatives (*Aiptasia* and *Nematostella*) both have D/E/E/A channels (Fig. 3). Our Na_v tree is therefore consistent with proposed species trees and suggests a lysine (K) substitution in the common ancestor of medusozoans (Fig. 4). There is also a *Nematostella* channel whose pore sequence D/E/E/T is unique among sampled ion channels.

Sodium-based APs have been reported in both *Cyanea* (27) and *Polyorchis* (28), whereas APs in anthozoans and ctenophores seem to be carried mostly by calcium (29, 30). The pore motif D/K/E/A has been shown to be less selective for sodium than the D/E/K/A pore but more so than the D/E/E/A pore (21, 31–33). Channels with D/E/E/A have a higher affinity for calcium than sodium. The convergence to lysine in different domains of medusozoan and bilaterian ion channels may therefore have resulted from similar evolutionary pressure for sodium selectivity, as this would allow for less disruption of calcium homeostasis because Ca^{2+} is used for intracellular signaling in eukaryotes (3). Some medusozoans have concentrated nerve clusters and complex sense organs, which likely emerged convergently with the bilaterian central nervous system, as such nerve concentration is absent in anthozoans (34). It is not known whether the Na_v genes function in these organs.

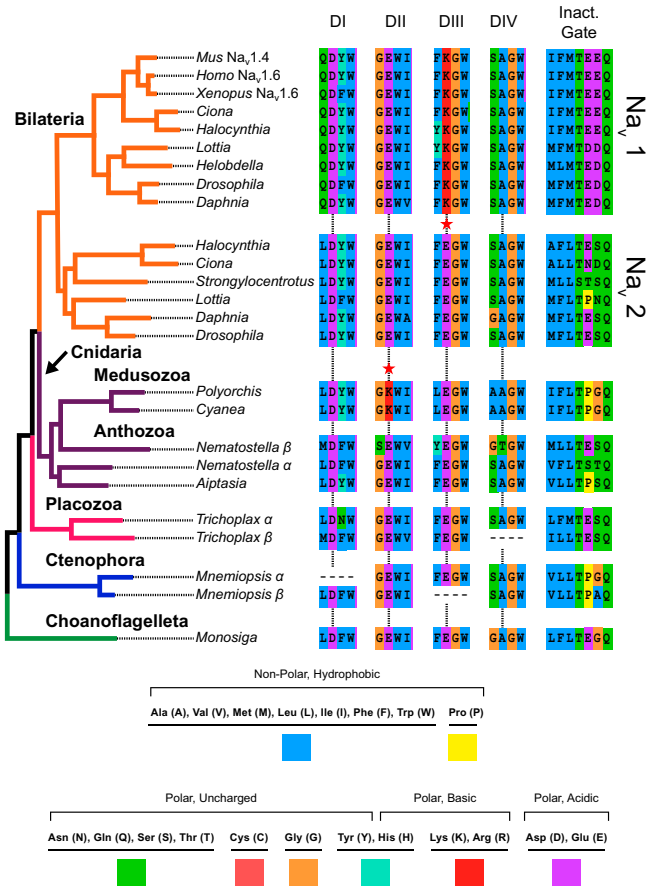

Fig. 3. Phylogeny of Na_v channels with key amino acid sequences mapped to their corresponding taxa. Taxa are color coded the same as in Fig. 2. The amino acids are alignments of the pore loops of all four domains (DI–DIV) and the critical inactivation particle on the inactivation gate. The critical amino acids in the pore are indicated by the vertical lines, and there are red stars next to convergent lysines (red K). Note the functional conservation of the hydrophobic triplet called the "inactivation particle" (first three amino acids on the inactivation gate).

Evolution of Sodium Selectivity and Fast Inactivation. The pore sequence D/E/E/A is intermediate between Ca_v channel and Na_v channel pore motifs. It may also have an intermediate selectivity between calcium and sodium. The function of D/E/E/A channels in such a wide range of organisms and the reason for their apparent loss in medusozoans and vertebrates remains unknown. Mutation studies of the DSC1 channel (called *Drosophila* Na_v 2 here) showed an effect in olfactory behavior in flies (35), but no function for these channels has been suggested in other organisms. The widespread retention of these channels suggests that they probably have important, yet possibly divergent, functions (e.g., not all lineages with D/E/E/A channels have olfaction). The sea urchin *Strongylocentrotus purpuratus* is only known to have an Na_v 2 ortholog (24).

Hydrophobic sites on the domain III/IV linker that are critical for inactivation (19) are functionally conserved in all of the sodium channels that we investigated here, albeit with a wide range of different amino acid combinations at homologous sites (Fig. 3). Secondary structure of the inactivation gate is also relatively conserved. Two helices on either side of the hydrophobic triad that forms the "inactivation particle" have been predicted before and may act to stabilize and direct the inactivation particle as it swings up and binds to the channel (36, 37). These two helices are present across the Na_v family, but not in the Ca_v families (Fig. S2). These findings suggest that all of the Na_v homologs presented here may include an inactivation gate, even in the single-celled choanoflagellate.

Na_v Channels in the Animal Genetic Repertoire. This study adds to the growing evidence that much of the genetic repertoire for animal development, cell signaling, and even the nervous system was already present in the common ancestor of choanoflagellates and animals. Choanoflagellates have genes for cell-adhesion proteins (2, 38), tyrosine kinases and related proteins (2), proteins related to the postsynaptic density of neurons (18), and a remarkable complement of calcium signaling proteins (17). Some choanoflagellate species have a colonial life stage (7), and these genes may function in colony maintenance.

The function of sodium channel homologs in choanoflagellates or placozoans is unknown. They may create calcium-based APs, as suggested by the presence of such APs in ctenophores (30), but there are other possibilities. Both organisms can inhabit coastal marine areas with abundant fresh water runoff (5, 13). *Trichoplax* is restricted to warm coastal waters and is known to be sensitive to lowered salinity (13). It is possible that the channels act as osmosensors or osmoregulators in these organisms. Choanoflagellates have a long flagellum that they use to swim and to capture prey, and *Trichoplax* has a ciliated ventral layer that it uses for gliding across surfaces. It is possible that the channels control flagellar or ciliary beating through the influx of calcium, which triggers actin, or sodium, which is known to mediate flagellar motors in bacteria (39). *Trichoplax* has a layer of contractile fiber cells that form a syncytium and seem to function as muscle and a nervous system simultaneously (40). It is possible that the channels function in this dual purpose tissue.

Functional assays of Na_v-channel homologs will shed light on their biological function and on the evolution of Na_v channels as a whole. Determining the ion selectivity of these channels is critical to understanding how sodium selectivity can evolve from calcium selectivity by sequential mutations. Gaining insight into the function of these channels will not only enlighten the history of this protein's "adaptive walk" (20), it will also help elucidate the evolution of the nervous system.

Materials and Methods

Sources of RNA. *M. brevicollis* and *T. adhaerens* were cultured in the laboratory using previously described and publicly available protocols (41). Placozoans were provided by Andreas Heyland. Choanoflagellate cells were fed on the bacteria present in the inoculum, and the placozoans were fed *Cryptomonas* sp. (LB 2423) from the University of Texas at Austin collection of algae. To extract RNA from *M. brevicollis*, we mixed and centrifuged 2 mL of the culture medium at 4 °C. Whole RNA was extracted using a RNA STAT-60 kit (Tel-Test) and then stored at –20 °C. The same protocol was used to isolate and store RNA from 15 *T. adhaerens* individuals that had been kept in algae-free seawater for 2 d to reduce the chance of contamination with algal RNA.

Gene Amplification and Sequencing. Specific primers were designed from the BLAST sequences for RT and PCR reactions. RT reactions were conducted with a SuperScript II kit (Invitrogen) using both specific and poly-T primers to prevent bacterial RNA contamination. Primers are reported in *SI Materials and Methods*. PCR reactions were carried out with the following cycle for 39 repetitions: Denaturation at 94° (30 s), annealing at a primer-specific temperature (30 s), and elongation at 72° (1 min/kb). This cycle was preceded by an initial denaturation at 94° for 3 min 10 s and followed by a final elongation at 72° for 7 min. PCR products were visualized and purified with gel electrophoresis and then cloned using a TOPO cloning kit (Invitrogen) and One Shot Top 10 (Invitrogen) chemically competent *Escherichia coli*. We sequenced the *M. brevicollis* gene in four overlapping segments using vector-specific primers after cloning.

Sequence Analysis. We performed a maximum likelihood phylogenetic analysis using the translated mRNA sequence from *M. brevicollis* and amino acid sequences from online databases for the other organisms. The latter were obtained either from catalogued, known channels or from BLAST searches of available genomes (for accession numbers, see Table S1). Amino

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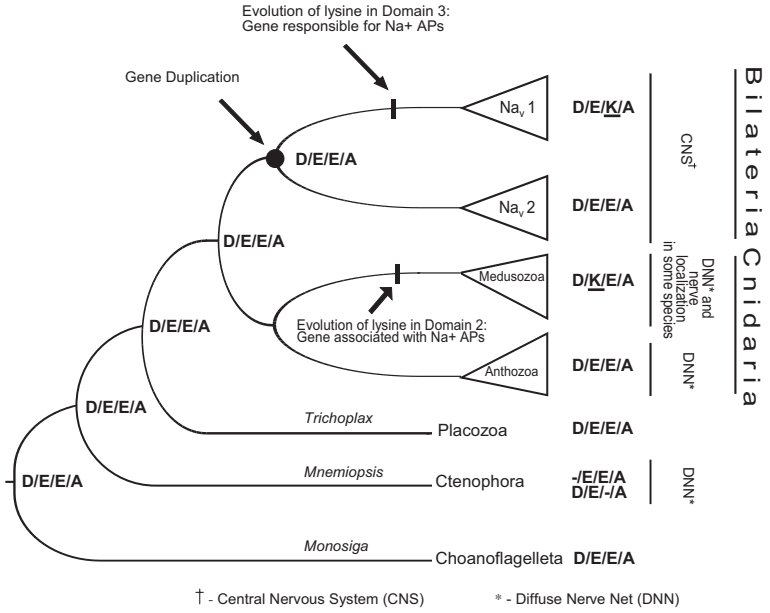

Fig. 4. Simplified gene tree of the Na_v family showing inferred ancestral states of the pore motifs. The gene duplication leading to the bilaterian Na_v 1 and Na_v 2 clades is noted, as are the points where we reconstruct fixation of lysines (K) in pore loops. Taxonomic information and information about the nervous system is also given. The *Nematostella* β and *Trichoplax* β genes have been left out for simplicity, but their addition would not change the proposed ancestral states. Pore states for both *Mnemiopsis* genes are shown because neither has a complete pore motif.

acid sequences were aligned using the E-INS-I strategy in MAFFT (42). We used the Guidance algorithm available on the Guidance server to remove columns that had a score below 0.377 from the alignment (43). Maximum likelihood phylogenetic analysis and bootstrapping were performed in Garli (44), using a model of amino acid replacement selected using the Akaike Information Criterion in ProtTest (45). The model of protein evolution selected in the ProtTest analysis was WAG + I + G + F (Whelan and Goldman model, with invariant sites, parameter for gamma-distributed rate heterogeneity, and amino acid frequencies matched to the observed data). The maximum likelihood tree was obtained using Garli set to use the WAG + I + G + F model. The full amino acid alignment was analyzed for four search repetitions operating across 5 million generations each. A total of 100 bootstrap samples were collected using a halved topological termination condition, as recommended in the Garli manual, and a stop time of 1 million generations. All bootstrap outputs were analyzed in PAUP (46).

Secondary structure of the inactivation gate region was examined using the online server PsiPred (47), the results of which are reported in Fig. S2.

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Supporting Information

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SI Materials and Methods

Gene Sequencing. There were two notable differences between the *Monosiga* reference sequence and our mRNA sequence (Fig. S1). First, the beginning of the ORF did not align with the reference. We took the first methionine to be the beginning of the ORF, but because we did not find any stop codons at the beginning of our sequence, we cannot exclude the possibility that there is an earlier methionine that we did not find, or that a later one begins the actual protein. Second, we found 125 bp of sequence that was not identified in our BLAST analysis. This sequence was in the Joint Genome Institute (JGI)'s *M. brevicollis* genome, but was classified as an intron. However, as we located this sequence in the mRNA, and because the translation remained in frame, we believe it is an exon that was misclassified by the BLAST analysis and is called the missing exon in this alignment. The rest of the alignment had very few differences, none of which threw the sequence out of frame.

Phylogenetic Methods. Besides the tree we report here, we also made maximum likelihood (ML) trees using Garli (1) with datasets that excluded the sponge and ctenophore sequences, which yielded completely consistent results. Analyses that further excluded two sodium channel genes (*Trichoplax* β and *Nematostella* β, which are relatively divergent), and a partially sequenced calcium channel (*Monosiga* Ca_v) using two different programs, Garli and SATe (2), yielded consistent results as well. We have used several different masking schemes throughout these analyses. These include masking with Guidance (3), as in the reported tree; removing the fast evolving cytoplasmic loops; and running the analyses without any masking. These different schemes can affect the exact placement of D/E/E/A channels within Bilateria and Cnidaria, indicating low phylogenetic signal in these channels, but does not affect the main conclusions of the paper. The *Monosiga* Na_v homolog always grouped with the Na_v family and ancestral state reconstruction was not affected.

Metazoan Phylogeny. A careful reanalysis of previous studies that had suggested nontraditional placements for basal groups finds that some of the inconsistencies regarding the basal placement of sponges can be resolved by removing genes of dubious orthology from alignments or increasing the taxon sampling (4). Doing so returns sponges to a basal position in the animal lineage and cnidarians to a sister group relation with bilaterians, a result which also agrees with the phylogenies in the *Trichoplax* and *Amphimedon* genome papers (5, 6). The revised phylogenies are not well supported, however, revealing low phylogenetic signal (4). The precise placement of ctenophores and placozoans is even less certain in these analyses, and one or the other group is left out of several studies (5–7). However, Philippe et al. (4) proposed that ctenophores should provisionally be placed in a group with cnidarians, with placozoans being sister to this group plus bilaterians. They call the group of animals with nervous systems "Eumetazoa," which contrasts with Srivastava et al. (6) who use this word to mean all animals with nervous systems plus *Trichoplax* (i.e., all animals except sponges). Knowing the precise placement of ctenophores and placozoans is critical because ctenophores have a fully developed nervous system, whereas *Trichoplax* has the simplest animal body plan. It is not possible to give a satisfying account of the evolution of the nervous system, or animal complexity in general, without knowing the phylogenetic positions of these groups.

PCR Primers. The following pairs of primers were used to amplify the *Monosiga brevicollis* gene segments during PCRs, with lower primers reported in their antisense form:

Segment 1 RT: 3′-GCGGAACCGGGGTCAAGGGC-5′. Primers: 5′-TTTTGTCGTCTTTATCATTTTTGGC-3′ and 3′-TCACTTTCTAGAAGATTGCACACGT-5′.
Segments 2 and 3 RT: 3′-GCGGAACCGGGGTCAAGGGC-5′.
Segment 2 primers: 5′-GAGTGGATTGAACTTCTATGGGAGA-3′ and 3′-TTGAGTCCGATACACCCTATGATAA-5′.
Segment 3 primers: 5′-ATGCATCCCTGCCCAAGCGCGC-3′, 3′-CTCGTTCAGTACAATGGGCGTAGA-5′.
Segment 4 RT: 3′-GATGATTTCAACGATGGACA-5′. Primers: 5′-TCGGAAGTTTGGTCAGACTGAGCCC-3′ and 3′-TCCACCCAGATGCAAAGTAGGAACG-5′.

The upper primer used in the third segment was designed from the putative beginning of the ORF, but actually bound further toward the 3′ end of the gene. It was later revealed that the part of the gene that this primer was designed from did not match the reference sequence, so the upper primer from the third segment should not be used.

The following primers were used to sequence ~1,000 bp from both *Trichoplax* genes:

Trichoplax α RT: 3′-CAACTAATGCTTCTAAAACG-5′. Primers: 5′-TTGGATCTTTTTTCTCATTAAACCT-3′ and 3′-CATGAAAATGCTGTCGCTGAGTTAT-5′.
Trichoplax β RT: 3′-GAGAGTAAAAAAGGTGCCAA-5′. Primers: 5′-ATCAGTCTTCAAGGCCACGACTTAC-3′ and 3′-TGCCATGTTAAGCCATTATCTAAAC-5′.

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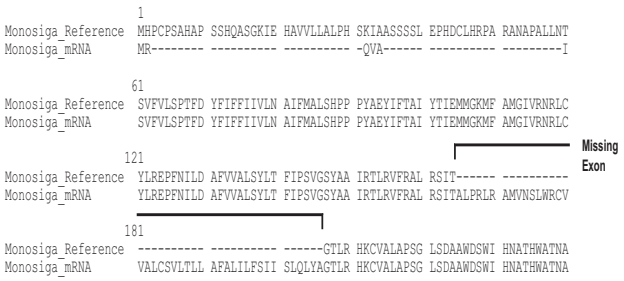

Fig. S1. Alignment of the beginning of the sequenced ORF from *Monosiga* with the reference obtained from JGI.

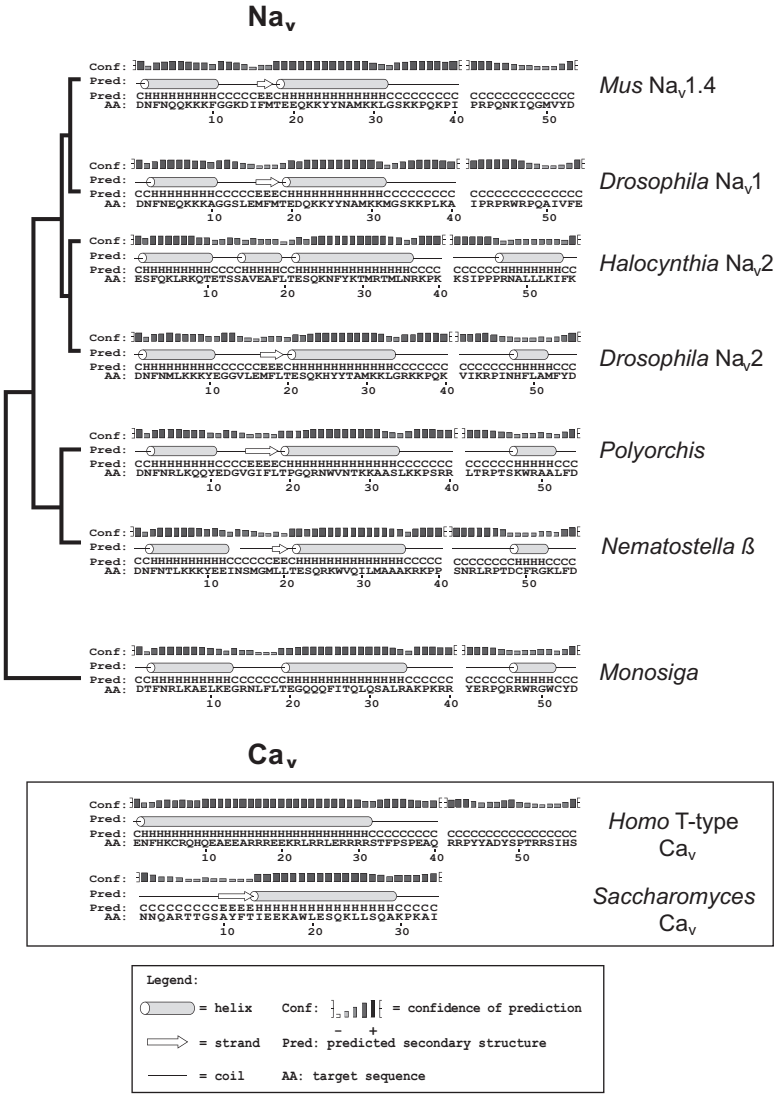

Fig. S2. Representative secondary structure predictions for the Na$_v$ inactivation gate mapped onto a simplified phylogeny. The two major helices are present in Na$_v$ genes but absent in the Ca$_v$ genes.

Table S1. Accession numbers for the genes used in the phylogenetic analysis

	Channel	Accession number/protein ID	Database
T type			
	Homo	O95180.4	Swiss-Prot
	Ciona	269719	JGI-Genome Portal
	Helobdella	66349	JGI-Genome Portal
	Drosophila	NM_001103419.1	RefSeq
	Strongylocentrotus	GLEAN3_25833	SpBase
	Lymnea	AAO83843.1	GenBank
	Caenorhabditis	WP:CE36117	WormBase
	Nematostella	170705	JGI-Genome Portal
	Trichoplax	21513	JGI-Genome Portal
N/P/Q			
	Homo P/Q	O00555.2	Swiss-Prot
	Homo N	O55017.1	Swiss-Prot
	Strongylocentrotus	GLEAN3_11692	SpBase
	Schistosoma	AAK84313.1	GenBank
	Caenorhabditis	WP:CE31225	WormBase
	Nematostella	59997	JGI-Genome Portal
	Trichoplax	53006	JGI-Genome Portal
	Mnemiopsis	JF905561	GenBank
L type			
	Oryctolagus	P15381.1	Swiss-Prot
	Ciona	239620	JGI-Genome Portal
	Helobdella	128998	JGI-Genome Portal
	Caenorhabditis	WP:CE31165	WormBase
	Drosophila	NM_080365.2	RefSeq
	Strongylocentrotus	GLEAN3_07770	SpBase
	Nematostella	88037	JGI-Genome Portal
	Stylophora	AAD11470.1	GenBank
	Cyanea	AAC63050.1	GenBank
	Trichoplax	18642	JGI-Genome Portal
Other			
	Monosiga	23875	JGI-Genome Portal
	Amphimedon	228755	Metazome
Na_v			
	Mus 1.4 Na_v1	NM_133199.2	RefSeq
	Xenopus 1.6 Na_v1	464193	JGI-Genome Portal
	Homo 1.6 Na_v1	NM_014191.2	RefSeq
	Ciona Na_v1	249763	JGI-Genome Portal
	Halocynthia Na_v1	662385	NCBI
	Lottia Na_v1	177540	JGI-Genome Portal
	Helobdella Na_v1	109965	JGI-Genome Portal
	Drosophila Na_v1	150421666	NCBI
	Daphnia Na_v1	50283	JGI-Genome Portal
	Halocynthia Na_v2	8096345	NCBI
	Ciona Na_v2	259743	JGI-Genome Portal
	Strongylocentrotus	GLEAN3_25997	SpBase
	Lottia Na_v2	161240	JGI-Genome Portal
	Daphnia Na_v2	40660	JGI-Genome Portal
	Drosophila Na_v2	166215092	NCBI
	Polyorchus	3005564	NCBI
	Cyanea	994814	NCBI
	Nematostella α	122010	JGI-Genome Portal
	Aiptasia	2791840	NCBI
	Nematostella β	88459	JGI-Genome Portal
	Trichoplax β	54699	JGI-Genome Portal
	Trichoplax α	23340	JGI-Genome Portal
	Mnemiopsis α	JF905562	GenBank
	Mnemiopsis β	JF905563	GenBank
	Monosiga	JF827087	GenBank
Fungi			
	Saccharomyces	1323391	NCBI
	Aspergillus	55835	JGI-Genome Portal