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The Evolution of the Four Subunits of Voltage-Gated Calcium Channels: Ancient Roots, Increasing Complexity, and Multiple Losses

Yehu Moran[1,*] and Harold H. Zakon[2,3,4,*]

[1]Department of Ecology, Evolution and Behavior, Alexander Silberman Institute of Life Sciences, Faculty of Science, Hebrew University of Jerusalem, Israel

[2]Department of Integrative Biology and Center for Computational Biology and Bioinformatics, University of Texas, Austin

[3]Department of Neuroscience, University of Texas at Austin

[4]Josephine Bay Paul Center for Molecular Biology and Evolution, Marine Biological Laboratory, Woods Hole, Massachusetts

*Corresponding author: E-mail: yehu.moran@mail.huji.ac.il; h.zakon@austin.utexas.edu.

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Abstract

The alpha subunits of voltage-gated calcium channels (Ca_vs) are large transmembrane proteins responsible for crucial physiological processes in excitable cells. They are assisted by three auxiliary subunits that can modulate their electrical behavior. Little is known about the evolution and roles of the various subunits of Ca_vs in nonbilaterian animals and in nonanimal lineages. For this reason, we mapped the phyletic distribution of the four channel subunits and reconstructed their phylogeny. Although alpha subunits have deep evolutionary roots as ancient as the split between plants and opistokonths, beta subunits appeared in the last common ancestor of animals and their close-relatives choanoflagellates, gamma subunits are a bilaterian novelty and alpha2/delta subunits appeared in the lineage of Placozoa, Cnidaria, and Bilateria. We note that gene losses were extremely common in the evolution of Ca_vs, with noticeable losses in multiple clades of subfamilies and also of whole Ca_v families. As in vertebrates, but not protostomes, Ca_v channel genes duplicated in Cnidaria. We characterized by in situ hybridization the tissue distribution of alpha subunits in the sea anemone *Nematostella vectensis*, a nonbilaterian animal possessing all three Ca_v subfamilies common to Bilateria. We find that some of the alpha subunit subtypes exhibit distinct spatiotemporal expression patterns. Further, all six sea anemone alpha subunit subtypes are conserved in stony corals, which separated from anemones 500 MA. This unexpected conservation together with the expression patterns strongly supports the notion that these subtypes carry unique functional roles.

Key words: voltage-gated calcium channel, ion channel, Cnidaria, *Nematostella vectensis*, evolution of nervous system.

Introduction

Voltage-gated Ca^{2+} channels (Ca_v) play a fundamental role in synaptic transmission and muscle contraction in Bilateria, a group which comprise the vast majority of animal species (Catterall et al. 2005; Tyson and Snutch 2013; Simms and Zamponi 2014). Although much is known about the physiological roles of these channels in Bilateria, little is known about their function or their tissue distribution in nonbilaterian animals. Among these limited data, there are indications for Ca_v playing a role in neuronal and muscular function in cnidarians such as sea anemones and jellyfish (Anderson 1987; Holman and Anderson 1991; Jeziorski et al. 1998). Strong phylogenetic support places the Cnidaria as sister to the Bilateria (Erwin et al. 2011; Ryan et al. 2013), suggesting that they share a common ancestor, which possessed muscles and a nervous system. Nevertheless, recent surprising findings emphasize the independent evolution of striated muscle (Steinmetz et al. 2012) and Na^+-permeable voltage-gated channels (Gur Barzilai et al. 2012) in Cnidaria and Bilateria. Better understanding of these features in Cnidaria may help us grasp the complexity of the nervous and muscular systems of ancient animals that lived more than half a billion years ago.

In bilaterians, the pore-forming α subunits of Ca_vs (α1), which are responsible for conducting the Ca^{2+} ions (fig. 1),

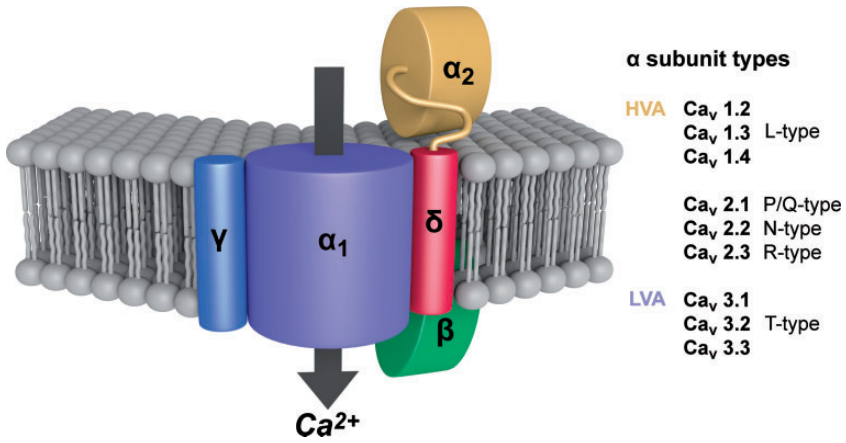

Fig. 1.—Schematic representation of a Ca_V channel (α_1) with its three auxiliary (β, α_2/δ, γ) subunits. The α_1 subunit forms a voltage-gated calcium-permeating channel that functions alone. The trafficking of the α_1 subunit and its biophysical properties are influenced by the other subunits. The α_2 and δ subunits are distinct proteins made from a common precursor protein and linked through a disulfide bond. The β subunit is intracellular whereas the others are transmembrane proteins. On the right is a table of the three α_1 Ca_V channel types. Figure adapted from Khosravani and Zamponi (2006) Voltage-Gated Calcium Channels and Idiopathic Generalized Epilepsies. Physiological Reviews, volume 86, issue 3, p. 945 by permission of the American Physiological Society.

are divided into two broad groups based on physiology: Low-voltage-activated (LVA) channels that open near resting potential and high-voltage-activated (HVA) channels that require a sizable depolarization to open (Catterall et al. 2005). The LVA channels are also termed T type or Ca_V3. The HVA group is further subdivided by their biophysical and pharmacological properties into L type (Ca_V1) and a third category of N, P/Q, and R types (Ca_V2). Two major protostome groups, the Ecdysozoa and Lophotrochozoa, possess three Ca_Vs, one each from the Ca_V1–3 groups (Liebeskind et al. 2011; Cai and Clapham 2012), whereas vertebrates have ten Ca_V channels resulting from the two rounds of genome duplications that expanded the repertoire of many vertebrate genes (Jegla et al. 2009). In contrast to our understanding of Ca_V evolution in Bilateria, we still lack much knowledge regarding the evolution of these channels in nonbilaterian and nonanimal phyla. We know from recent studies that α subunits of Ca_Vs can be found in the representatives of early-branching groups as choanoflagellates and algae and that they are distantly related to calcium channels of fungi (Verret et al. 2010; Liebeskind et al. 2012), yet we do not know much about the evolution of the Ca_V1, 2, and 3 subfamilies. Furthermore, bilaterian Ca_Vs contain auxiliary subunits ($\alpha2\delta$, β, and γ; fig. 1), and the phyletic distribution and evolutionary history of the gene families encoding them are unknown. To address these gaps in knowledge, we analyzed in this work the phyletic distribution of the various Ca_V subunits and reconstructed their phylogenies. Additionally, we used the sea anemone *Nematostella vectensis* to study Ca_V spatiotemporal expression patterns to better understand the functionality of these channels in nonbilaterian organisms.

Materials and Methods

Identification of Ca_V Homologs and Phylogenetic Analysis

Putative Ca_V homologs were detected in GenBank (nr), Broad Institute and Joint Genome Institute databases through BLAST. Transcript clusters were translated to proteins. Accession numbers of proteins used in all phylogenetic analyses can be found in supplementary table S1, Supplementary Material online. Protein models were aligned using MUSCLE and low-quality alignment regions were removed by TrimAl (Edgar 2004; Capella-Gutierrez et al. 2009). ProtTest was used to find the most suitable model for phylogeny reconstruction (Abascal et al. 2005) and this model was used to reconstruct a maximum-likelihood tree with PhyML (Guindon et al. 2010). Support values were calculated using 100 bootstrap replicates. A Bayesian tree was constructed using MrBayes version 3.1.2 with the same model. The run lasted 5,000,000 generations and every 100th generation was sampled. We estimated that the Bayesian analysis reached convergence when the potential scale reduction factor reached 1.0.

RNA Isolation and Polymerase Chain Reaction Amplification of Ca_V Transcript Fragments from *N. vectensis*

Total RNA was isolated from planulae (4 days old) and adult polyps (>5 months old) of *N. vectensis* using Trizol (Life Technologies, USA) according to the manufacturer's instructions. The purified RNA was used as a template for reverse transcription reaction using the SuperScript III reverse transcriptase (Life Technologies) and random primers (New

England Biolabs, USA) according to manufacturer instructions. Advantage2 DNA polymerase mix (Clontech) was used for polymerase chain reaction (PCR) under high-stringency conditions: 94 °C for 2:00 min, 35× (94 °C for 20 s, 65 °C for 20 s, 72 °C for 1 min) and 72 °C for 5 min. The amplified fragments were 1–1.5 kb long (for primer list, see supplementary table S2, Supplementary Material online) and they were purified using Illustra PCR purification kit (GE Healthcare, UK) and ligated into pGEM-T (Promega). The resulting plasmids were sequenced from both ends (performed at MicroSynth, Switzerland) and were used as templates for producing RNA probes for in situ hybridization (ISH).

In Situ Hybridization

For ISH experiments, *N. vectensis* larvae were fixed at 48–168 h postfertilization in ice-cold 3.7% formaldehyde in one-third of seawater with 0.2% glutaraldehyde for 90 s and then in 3.7% formaldehyde in one-third of seawater without glutaraldehyde for additional 60 min. Antisense RNA probes for ISH were generated and labeled by using the T7 or SP6 MEGAscript kits (Life Technologies) and an RNA labeling mix with digoxygenin (Roche, Germany). The ISH procedure was performed as described previously (Genikhovich and Technau 2009). The stained samples were photographed in a Nikon Eclipse 80i microscope with differential interference contrast optics connected to a Nikon Digital Sight DS-U2 camera.

Results

Distribution and Evolution of α_1 Subunits of Ca_v Channels

We collected from publicly available genomic and transcriptomic databases (see Methods and Materials) the putative protein sequences of bilaterian, cnidarians, placozoan (*Trichoplax*), poriferan (sponges), and ctenophore (comb jellies) Ca_v α subunits. In addition, we searched for such homologs in all available data from nonanimal groups. We reconstructed a phylogeny of these proteins, which included only complete models (all four channel domains present) to increase its accuracy (fig. 2). The earliest branching group where we could find homologs of Ca_v α_1 subunits was green algae, such as *Chlamydomonas* and *Micromonas*; however, as their sequences are highly derived and as they also include some characteristics of Na_v channels (Verret et al. 2010; Liebeskind et al. 2012) we did not include them in our phylogenetic analysis. Another organism where we found Ca_v α_1 subunit homologs is *Thecamonas trahens*, a member of the Apusozoa, which according to recent phylogeny is a sister group to all opisthokonts (fungi, animals, and their close protozoan relatives) (Derelle and Lang 2012). However, our phylogeny indicates that both complete *Thecamonas* protein models are clustered with α subunits of voltage-gated sodium channels (Na_v1) and their close homologs (Na_v2) rather than with Ca_vs. The next group we could find Ca_v homologs in was Choanoflagellata, a protist sister group of animals: The genome of the choanoflagellate *Salpingoeca rosetta* (Fairclough et al. 2013) contains two α-subunit homologs, with one of them clustering with HVA channels and the other with LVA channels (fig. 2). In the genome of another choanoflagellate, *Monosiga brevicollis* (King et al. 2008), we could detect only a short gene fragment encoding a partial Ca_v of only 258 amino acids, suggesting either a partial gene deletion or a technical problem in genome assembly at this specific region (data not shown). We found an HVA homolog in the genome of the sponge *Amphimedon queenslandica* (Srivastava et al. 2010) and a homolog of Ca_v2 in the genome of the ctenophore *Mnemiopsis leidyi* (Ryan et al. 2013). In cnidarians and the placozoan *Trichoplax adhaerens* (Srivastava et al. 2008), we found representatives of all three Ca_v α_1 subunit families.

In the genome of *N. vectensis* (Putnam et al. 2007), we identified one Ca_v1 gene, three Ca_v2 genes, and two Ca_v3 genes. Interestingly, in the transcriptomic data available for the stony coral *Acropora millepora* (Meyer et al. 2009; Moya et al. 2012) we identified partial transcript models which demonstrate clear orthologous relationships with each of the Ca_v genes of *N. vectensis* (fig. 3). Taking into account the *Acropora* and *Nematostella* approximate separation time (Shinzato et al. 2011), it is likely that these orthologs are conserved in both lineages for about 500 Myr. Thus, similar to vertebrates, but not protostomes, Ca_v channel genes duplicated in Cnidaria.

Localization of the transcripts of the six Ca_v α_1 subunits of *Nematostella* by ISH demonstrated complex spatiotemporal expression patterns. Ca_v1a expression starts only from the mid-planula stage, where it is diffused in the endoderm. Later in the late-planula and primary polyp stages the expression becomes concentrated in regions along the mesenteries and in the endoderm and ectoderm of the tentacle buds, regions which also include muscles and neurons (Marlow et al. 2009; Renfer et al. 2010; fig. 4). In contrast, Ca_v2a is expressed already from the early planula stage and is specific to nematocytes, the stinging cells which typify Cnidaria (Kass-Simon and Scappaticci 2002; Zenkert et al. 2011). This is clearly evident by the stain-free nematocyst (stinging capsule) structure which is surrounded by stained cytoplasm (fig. 4 and supplementary fig. S1, Supplementary Material online). Ca_v2b, Ca_v2c, and the two Ca_v3 genes of *Nematostella* also exhibit distinct developmental expression patterns (fig. 4), but it is more difficult to postulate on their identity.

Distribution and Evolution of Auxiliary Subunits of Ca_v Channels

As with the study of α_1 subunits, we collected putative protein homologs of the $\alpha_2\delta$, β, and γ subunits of Ca_v channels from

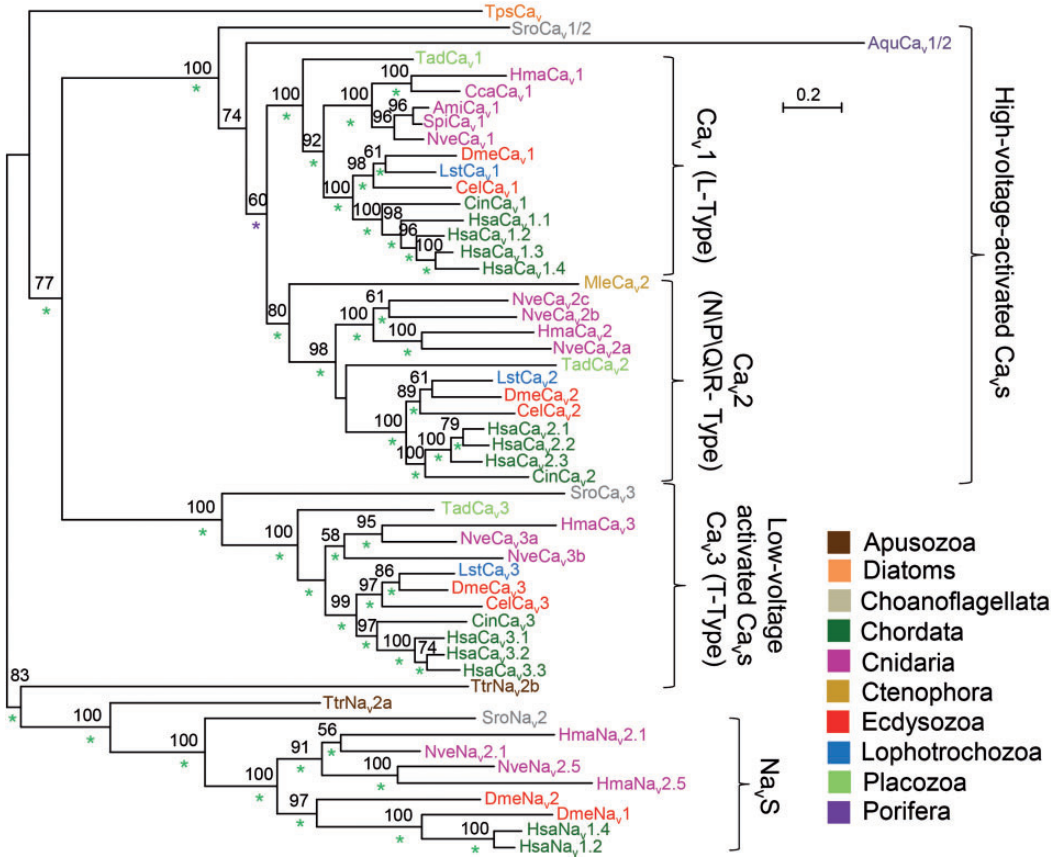

Fig. 2.— Phylogeny of Ca_v α_1 subunits. A maximum-likelihood phylogenetic tree was constructed with the LG model (+G, +F). Bootstrap support values above 50% are indicated above branches. Posterior probability (PP) values of a Bayesian tree constructed with the WAG model are indicated by a green (PP = 1.0), or purple ($0.95 \leq PP < 1.0$) asterisk. Abbreviations of species names are: Aqu, *Amphimedon queenslandica* (sponge); Ami, *Acropora millepora* (stony coral); Cel, *Caenorhabditis elegans* (nematode); Cin, *Ciona intestinalis* (tunicate); Cca, *Cyanea capillata* (jellyfish); Dme, *Drosophila melanogaster* (fruit fly); Hsa, *Homo sapiens* (human); Hma, *Hydra magnipapillata* (hydra); Lst, *Lymnaea stagnalis* (pond snail); Mle, *Mnemiopsis leidyi* (comb jelly); Nve, *Nematostella vectensis* (starlet anemone); Spi, *Stylophora pistillata* (stony coral); Sro, *Salpingoeca rosetta* (choanoflagellate); Tad, *Trichoplax adhaerens* (placozoan); Tps, *Thalassiosira pseudonana* (diatom); Ttr, *Thecamonas trahens*.

transcriptomic and genomic databases and reconstructed their phylogenies. We could not find $\alpha_2\delta$ homologs in sponges and ctenophores or in any nonanimal group. We detected single $\alpha_2\delta$ genes in *Trichoplax*, *Acropora,* and *Nematostella*. In the cnidarian *Hydra magnipapillata* there are three $\alpha_2\delta$ genes, suggesting a lineage-specific expansion (fig. 5). We rooted the phylogenetic tree of the $\alpha_2\delta$ subunits by using the clade of human cache domain containing 1 protein (VWFAC1) and its cnidarian homologs as an outgroup. The VWFAC1 proteins are similar in sequence (~40% similarity) to $\alpha_2\delta$ subunits and seem to be highly conserved in most animals, including cnidarians, arthropods, and vertebrates. However, to the best of our knowledge, the function of these proteins in bilaterians is currently unknown.

Unlike $\alpha_2\delta$ subunits which seem to be metazoan-specific, β subunits can be found in choanoflagellates (fig. 6). We also detected a single β subunit in each cnidarian species and in *Trichoplax*. This suggests that β subunits appeared in the common ancestor of choanoflagellates and animals but were lost independently in sponges and ctenophores.

We could not detect any γ subunits in nonvertebrate species but a single homolog in the hemichordate *Saccoglossus kowalevskii* and a single homolog in each of the two annelids, *Capitella teleta* and *Helobdella robusta* (supplementary table S1, Supplementary Material online). These protein models show only modest similarity to γ subunits of vertebrates (41–46% similarity) but contain a typical claudin-2 domain and have a similar length to their vertebrate homologs (250–300 amino acids). Moreover, when we used them for a reciprocal BLAST query against the human proteome, the best scoring hits were γ subunits of Ca_v, suggesting that these might be true homologs. The extremely patchy phyletic distribution of γ subunits strongly suggests that they were lost independently in multiple lineages. However, there are some indications that other proteins with claudin domains can also influence the expression levels and function of Ca_v channels in invertebrates

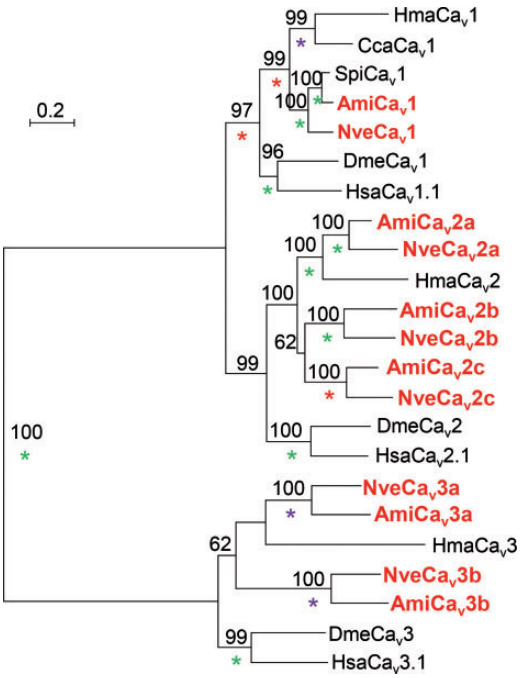

Fig. 3.— Phylogeny of Ca_v α_1 subunit subtypes from *Nematostella vectensis* and *Acropora millepora*. Sequences from these two species appear in red bold. A maximum likelihood phylogenetic tree was constructed with the LG model (+I, +G, +F). Bootstrap support values above 50% are indicated above branches. Posterior probability (PP) values of a Bayesian tree constructed with the WAG model are indicated by a green (PP = 1.0), purple ($0.95 \leq PP < 1.0$), or red ($0.9 \leq PP < 0.95$) asterisk. Abbreviations of species names are: Ami, *Acropora millepora* (stony coral); Cca, *Cyanea capillata* (jellyfish); Dme, *Drosophila melanogaster* (fruit fly); Hsa, *Homo sapiens* (human); Hma, *Hydra magnipapillata* (hydra); Nve, *Nematostella vectensis* (starlet anemone); Spi, *Stylophora pistillata* (stony coral).

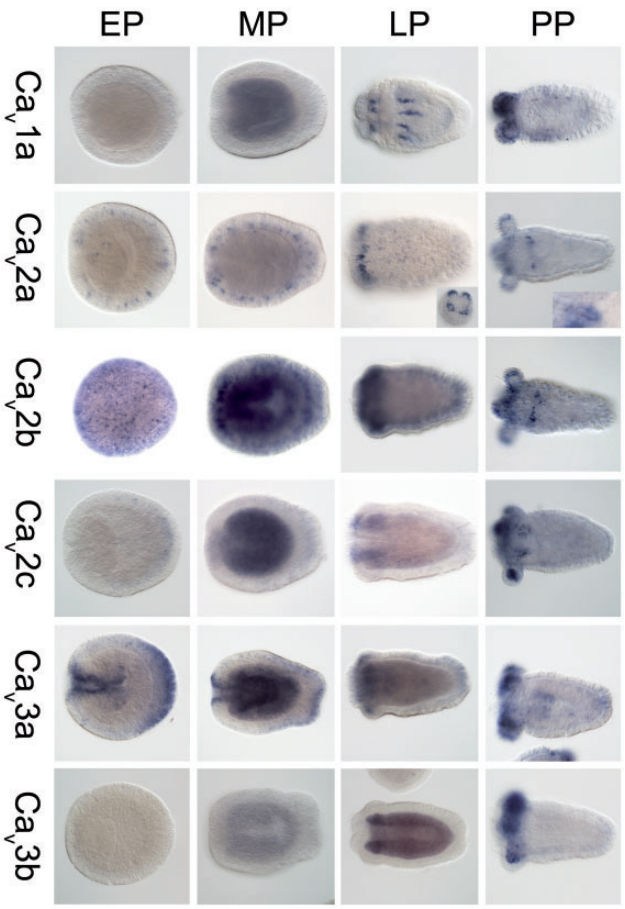

Fig. 4.—The spatiotemporal expression of the Ca_v α subunits subtype from *Nematostella vectensis* as determined by ISH. Gene expression is indicated by blue staining. The insets show the expression of Ca_v2a in nematocytes (stinging cells). In all pictures (not including insets), the oral end of all larvae is to the left. Abbreviations of developmental stages are: EP, early planula; MP, mid-planula; LP, late planula (tentacle buds are noticeable); and PP, primary polyp.

(Simske 2013) and it is possible that high sequence variability of such subunits is masking common ancestral origins.

Discussion

The current work of mapping the phyletic distribution of the four subunits of Ca_v channels and the analysis of their phylogeny together with results of previous research (Verret et al. 2010; Liebeskind et al. 2011; Gur Barzilai et al. 2012; Liebeskind et al. 2012) allows us to reconstruct their evolutionary history. It seems that the first α_1 subunits of Ca_vs appeared very early in eukaryote evolution, already in the common ancestor of Viridiplantae (plants and green algae), Apusozoa, and Opisthokonta. However, it was lost in multiple lineages, such as all extant land plants, Amoebozoa and fungi. The finding of HVA and LVA Ca_v channels in *S. rosetta* indicates that these Ca_v subfamilies already separated in the ancestor of choanoflagellates and animals. Intriguingly, choanoflagellates possess a primordial neurosecretory apparatus (Burkhardt et al. 2011) and we hypothesize that Ca_v channels may play a role in its function. When HVA channels further diverged to Ca_v1 and Ca_v2 is harder to determine, as the order of divergence of sponges and ctenophores is still under debate (Philippe et al. 2011; Ryan et al. 2013). Our phylogeny indicates that the single Ca_v of the sponge *A. queenslandica* is a sister clade to all other animal α_1 subunits of HVA channels, whereas the single α_1 subunit of the ctenophore *M. leidyi* clusters with the Ca_v2 subfamily. This suggests that possibly Ca_v2-like characteristics already appeared in the ancient HVA Ca_vs prior to the divergence of ctenophores. Alternatively, the divergence of Ca_v1 and Ca_v2 might have happened before the divergence of ctenophores from the rest of the animals, and the lineage of *M. leidyi* lost Ca_v1. Gene loss is a general trend in the evolution of Ca_vs, as both sponges and ctenophores seem to have independently lost the Ca_v3 subfamily (fig. 2). Such losses of channel genes in animal lineages may be suspected as artifacts due to technical

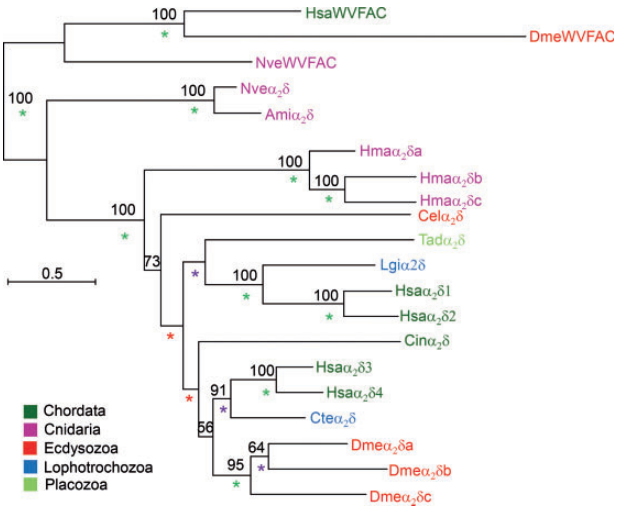

Fig. 5.—Phylogeny of Ca_V $\alpha_2\delta$ subunits. A maximum-likelihood phylogenetic tree was constructed with the LG model (+I, +G, +F). Bootstrap support values above 50% are indicated above branches. Posterior probability (PP) values of a Bayesian tree constructed with the WAG model are indicated by a green ($PP = 1.0$), purple ($0.95 \leq PP < 1.0$), or red ($0.9 \leq PP < 0.95$) asterisk. Abbreviations of species names are: Ami, *Acropora millepora* (stony coral); Cel, *Caenorhabditis elegans* (nematode); Cin, *Ciona intestinalis* (tunicate); Cte, *Capitella teleta* (annelid worm); Dme, *Drosophila melanogaster* (fruit fly); Hsa, *Homo sapiens* (human); Hma, *Hydra magnipapillata* (hydra); Lgi, *Lottia gigantea* (owl limpet); Nve, *Nematostella vectensis* (starlet anemone); Tad, *Trichoplax adhaerens* (placozoan).

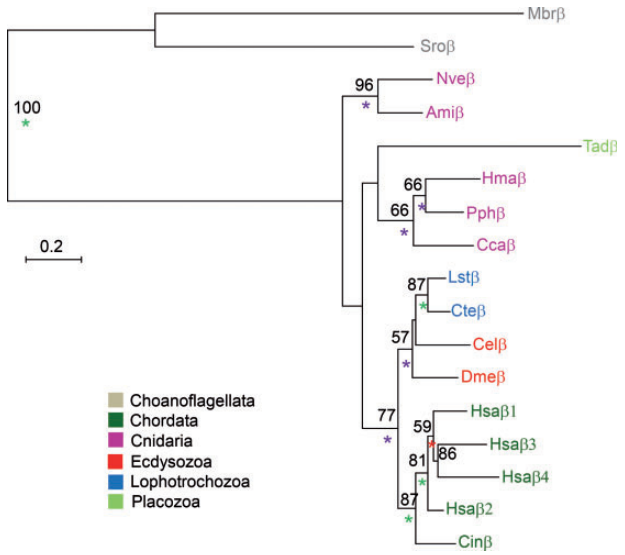

Fig. 6.—Phylogeny of Ca_V β subunits. A maximum-likelihood phylogenetic tree was constructed with the LG model (+G). Bootstrap support values above 50% are indicated above branches. Posterior probability (PP) values of a Bayesian tree constructed with the WAG model are indicated by a green ($PP = 1.0$), purple ($0.95 \leq PP < 1.0$), or red ($0.9 \leq PP < 0.95$) asterisk. Abbreviations of species names are: Ami, *Acropora millepora* (stony coral); Cel, *Caenorhabditis elegans* (nematode); Cin, *Ciona intestinalis* (tunicate); Cca, *Cyanea capillata* (jellyfish); Cte, *Capitella teleta* (annelid worm); Dme, *Drosophila melanogaster* (fruit fly); Hsa, *Homo sapiens* (human); Hma, *Hydra magnipapillata* (hydra); Lst, *Lymnaea stagnalis* (pond snail); Mbr, *Monosiga brevicollis* (choanoflagellate); Nve, *Nematostella vectensis* (starlet anemone); Pph, *Physalia physalis* (hydrozoan cnidarian); Sro, *Salpingoeca rosetta* (choanoflagellate); Tad, *Trichoplax adhaerens* (placozoan).

reasons, such as errors in gene annotation, genome assembly, and/or insufficient sequencing depth. However, it is noteworthy that the sequencing depth of the genomes of *Amphimedon* (9-fold by Sanger sequencing; Srivastava et al. 2010) and *Mnemiopsis* (12-fold coverage by 454 sequencing; Ryan et al. 2013) is more than adequate. Moreover, our searches in the recently sequenced genomes of the ctenophore *Pleurobrachia bachei* (Moroz et al. 2014) and the sponge *Oscarella carmela* (available through the Compagen website; Hemmrich and Bosch 2008) supported the above scenario in which Ca_V3 subfamily and β subunits were lost in sponges and ctenophores.

An intriguing question is what might be the functional value of Ca_V channels in light of the fact that Na_V2 channels are also voltage-gated channels conducting mostly calcium ions (Zhou et al. 2004; Gur Barzilai et al. 2012). The answer to this question might lie in the vastly different voltage-sensitivities of Na_V2 and HVA Ca_V channels, as the latter opens only in relatively high voltages and therefore are not well-suited for conducting neuronal action potentials (Tyson and Snutch 2013; Simms and Zamponi 2014).

Our analyses suggest that auxiliary subunits were gradually added during evolution to the Ca_V complex: β subunits appeared already in the ancestor of choanoflagellates and animals, $\alpha_2\delta$ appeared only later in the common ancestor of placozoans, cnidarians and bilaterians, whereas γ subunits might have appeared only in the bilaterian lineage. As auxiliary subunits can increase the complexity of electrical signaling (Lacerda et al. 1991; Obermair et al. 2005; Dolphin 2012), this trend at the genetic level could support increasing complexity at the neuronal level. However, we also notice that all subunits other than $\alpha_2\delta$ were lost in some lineages, demonstrating a highly plastic evolution of the Ca_V complex.

Our finding by using ISH techniques that each Ca_Vs α_1 subunit of *Nematostella* occupies a distinct spatiotemporal expression domain (fig. 4) raises the possibility that each of these subunits has acquired a specialized role. The expression of Ca_V1a in muscles and/or motor neurons is in accordance with previous works that recorded L-type calcium currents in the muscles of sea anemones and isolated transcripts encoding Ca_V1 from motor neuron-rich regions of a jellyfish bell (Holman and Anderson 1991; Jeziorski et al. 1998). The expression of *Nematostella* Ca_V2a in nematocytes fits a previous report on the isolation of transcripts encoding Ca_V channels from the stinging cells of the cnidarian *Physalia physalis*

(Bouchard et al. 2006) and reports of Ca_v-dependence of nematocyst action (Gitter et al. 1994; Watson and Hessinger 1994). The notion of specialization of cnidarian Ca_vs α_1 subunits is also strongly supported by our finding that the six Ca_v α subunit subtypes are highly conserved for about 500 Myr in the lineage of sea anemone and reef-building corals (fig. 3). The expansion and specialization of Ca_v α subunits is part of a wider trend that seems to be true also for other ion channel families in Cnidaria, such as voltage-gated potassium channels (Jegla et al. 2012; Martinson et al. 2014) and Na_v2 channels (Gur Barzilai et al. 2012).

Supplementary Material

Supplementary figure S1 and tables S1 and S2 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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