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REVIEW

Evolution of voltage-gated ion channels at the emergence of Metazoa

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ABSTRACT

Voltage-gated ion channels are large transmembrane proteins that enable the passage of ions through their pore across the cell membrane. These channels belong to one superfamily and carry pivotal roles such as the propagation of neuronal and muscular action potentials and the promotion of neurotransmitter secretion in synapses. In this review, we describe in detail the current state of knowledge regarding the evolution of these channels with a special emphasis on the metazoan lineage. We highlight the contribution of the genomic revolution to the understanding of ion channel evolution and for revealing that these channels appeared long before the appearance of the first animal. We also explain how the elucidation of channel selectivity properties and function in non-bilaterian animals such as cnidarians (sea anemones, corals, jellyfish and hydroids) can contribute to the study of channel evolution. Finally, we point to open questions and future directions in this field of research.

KEY WORDS: Voltage-gated ion channels, Animal evolution, Ion selectivity

Introduction: the superfamily of voltage-gated ion channels

This review aims to cover and clarify the evolution and diversification of metazoan voltage-gated ion channels, particularly at the beginning of multicellularity in the lineage leading to Metazoa and at the emergence of nervous systems. Voltage-gated ion channels are imperative for neuronal signaling, muscle contraction and secretion, and are thought to play a critical role in the evolution of animals (Hille, 2001). Nonetheless, these channels are also found in prokaryotes and viruses, although their roles in these organisms are largely unknown (Martinac et al., 2008; Plugge et al., 2000). The superfamily of voltage-gated ion channels is characterized by the ability to rapidly respond to changes in membrane potential (hence 'voltage-gated'), which results in selective ion conductance. This ion channel superfamily includes voltage-gated potassium channels (K_Vs), voltage-gated calcium channels (Ca_Vs) and voltage-gated sodium channels (Na_Vs). Their α-subunits are composed of four domains (DI–IV), with each domain containing six transmembrane segments (S1–S6; Fig. 1) (Noda et al., 1984; Noda et al., 1986; Guy and Seetharamulu, 1986; Tanabe et al., 1987). Voltage-dependent activation is enabled by conserved positively charged residues at every third position in S4 (voltage sensor) of the four domains, which move outwards upon changes in membrane potential (Noda et al., 1984; Catterall, 1986; Guy and Seetharamulu, 1986), inducing a conformational change that results in opening of the channel pore (Armstrong and Bezanilla, 1974; Stühmer et al., 1989; Papazian et al., 1991; Yang et al., 1996). The pore is formed by the segments S5 and S6, and the selectivity to specific ions is enabled by the selectivity filter, which is composed of conserved residues, specific for the ion conducted by the channel, and these residues are situated at the pore-lining loops (p-loops) connecting S5 to S6 in the four domains (Fig. 1) (Yool and Schwarz, 1991; Heinemann et al., 1992; Tang et al., 1993; Schlief et al., 1996; Doyle et al., 1998; Jiang et al., 2003; Payandeh et al., 2011). Further, many voltage-gated ion channels include in addition to the α-subunit one or more auxiliary subunits that modify their expression levels, folding efficiency, functional properties and subcellular localization (Yu and Catterall, 2004).

In K_V channels, each domain is a separate protein consisting of six transmembrane segments (6-TM; Fig. 1B) (Papazian et al., 1987; Pongs et al., 1988) and the channel assembles into a tetramer built of four protein units (Jiang et al., 2003; Long et al., 2005). In contrast, the pore-forming units of Na_Vs and Ca_Vs consist of a protein encompassing all four domains (Fig. 1C) (Noda et al., 1984; Noda et al., 1986; Guy and Seetharamulu, 1986; Tanabe et al., 1987; Mikami et al., 1989). K_Vs are the most diverse of all voltage-gated channels as they are found in a wide array of eukaryotes and prokaryotes. They are thought to have evolved by the addition of the voltage-sensing module (S1–S4) to the basic structural motif of ion channels consisting of two transmembrane segments (2-TM) connected by a p-loop (pore-module; Fig. 1A), which are homologous to the pore module of K_Vs (Doyle et al., 1998; Lu et al., 2001; Long et al., 2005). Ligand-gated channels such as calcium-activated potassium channels (K_{Ca}), cyclic nucleotide-gated (CNG) channels and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are also composed of a 6-TM domain protein that assembles into a tetrameric channel, and are members of the K_V channel family. However, these channels are only weakly voltage gated; rather, they open in response to the binding of calcium (Moczydlowski and Latorre, 1983; Köhler et al., 1996) or cAMP or cGMP (Kaupp et al., 1989; Altenhofen et al., 1991; DiFrancesco and Tortora, 1991; Ludwig et al., 1998) which bind at the intracellular C-terminus of the channel (Xia et al., 1998; Xia et al., 2002; Schumacher et al., 2001; Goulding et al., 1994; Zagotta et al., 2003; Clayton et al., 2004). HCN channels activate at hyperpolarized voltages and the binding of cyclic nucleotides increases their open probability (DiFrancesco and Tortora, 1991; Gauss et al., 1998; Ludwig et al., 1998). Furthermore, the CNG and HCN channels do not exhibit selectivity for a specific ion, but rather are cation-conducting channels with varying preferences for potassium, sodium and calcium ions (Frings et al., 1995; Gauss et al., 1998).

The genes encoding Na_Vs and Ca_Vs might have evolved by two rounds of gene duplication of a single-domain channel gene

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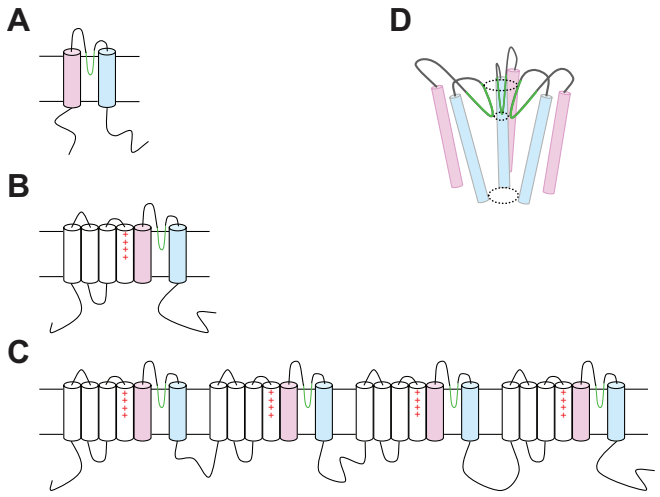

Fig. 1. Schematic illustration of the voltage-gated ion channel α-subunits and the pore region. (A) Schematic view of a 2-transmembrane (TM) domain protein unit that assembles into a tetrameric ion-conducting channel (K_{ir}). (B) Schematic view of a 6-TM domain protein unit that assembles into a tetrameric channel (K_Vs, K_{Ca}, CNG, HCN); and (C) of a channel protein comprising all four domains, DI to DIV (Ca_Vs, Na_Vs, NALCN). Each domain contains six α-helical membrane-spanning segments (represented by cylinders) of which S1–S4 constitute the voltage-sensing module, and S5–S6 with the pore-lining loops form the pore module. The fourth segment (voltage sensor) in each domain bears positively charged residues. (D) Schematic presentation of the pore region. Only three of the four domains are shown for clarity. The pore-lining loops (p-loops) that bear the selectivity filter are in green.

encoding an ancestral K_V that initially converted the single domain into a two-domain protein, which then duplicated again to form the four-domain channel. This hypothesis is supported by the fact that domains I and III of Na_Vs are more similar to one another than to domains II and IV, which themselves are similar to one another (Strong et al., 1993). Na_Vs are thought to have evolved as a result of gene duplication and diversification of an ancestral Ca_V, as the four domains of Na_Vs show higher sequence similarity to the corresponding domains of Ca_Vs than to each other and have in turn the lowest levels of similarity with K_Vs (Hille, 1989). Moreover, the fact that Ca_Vs have a wider phylogenetic distribution than Na_Vs supports this scenario as well (Verret et al., 2010) (see below). The discovery of bacterial sodium-selective channels (NaChBac) (Ren et al., 2001; Yue et al., 2002; Koishi et al., 2004) has somewhat blurred the evolutionary history of Ca_Vs and Na_Vs as NaChBac genes encode a single 6-TM domain that assembles into a tetramer (Durell and Guy, 2001; Ren et al., 2001; Payandeh et al., 2011), and as their selectivity pattern resembles that of animal Na_V channels but their filter sequence resembles that of Ca_Vs, it was suggested that NaChBac channels rather than K_Vs might be the true ancestors of animal Na_Vs and Ca_Vs (Charalambous and Wallace, 2011). However, recent phylogenetic analyses do not support this scenario (Liebeskind et al., 2013).

Sodium leak channels, non-selective (NALCN) are four-domain channels, similar to Ca_Vs and Na_Vs, and were shown to have diverged from voltage-gated channels before the diversification of Ca_V and Na_V channels (Lee et al., 1999; Liebeskind et al., 2012). These channels are non-selective and voltage insensitive, rely on accessory proteins for their function and their open state was shown to be affected by various neurotransmitters and calcium (Lu et al., 2009; Swayne et al., 2009; Lu et al., 2010).

In order to elucidate the evolution of voltage-gated ion channels in animals and their relatives, it is necessary to first look into the evolutionary history of the organisms expressing them.

The evolution of animals and the genomic revolution

Since the introduction of the evolutionary theory and its core idea that all the organisms on Earth have a common ancestor (Darwin, 1859), up to about 20 years ago, the study of the evolutionary relationships between species was based mostly on morphological characters. However, in many cases, morphological characters can converge, meaning the same character might have appeared more than once independently in separate lineages. The introduction of DNA sequencing at the end of the 1970s (Sanger et al., 1977) and especially of high-throughput sequencing at the 2000s revolutionized the study of phylogeny. These new tools allowed researchers to resolve previously obscure evolutionary relationships between phylogenetic groups (e.g. Campbell et al., 2011; Philippe et al., 2011a; Pisani et al., 2012; Smith et al., 2011). Despite its advantages, phylogenomics (phylogeny of species based on dozens or hundreds of genes) suffers from new problems and limitations, and different technical approaches within this field can lead to strikingly different results (Dunn et al., 2008; Hejnol et al., 2009; Philippe et al., 2011b; Ryan et al., 2013; Schierwater et al., 2009).

Regardless of whether morphological or molecular approaches are employed, one can divide life forms into three domains: Eukarya, Bacteria and Archaea (Woese et al., 1990). Eukarya can be further divided into Kingdoms; however, the number of Kingdoms and even the number of Domains is debatable (Cavalier-Smith, 2004). Members of the voltage-gated ion channel superfamily are found in all Domains of life, suggesting that these channels are extremely ancient, possibly as ancient as the last common ancestor of all organisms on Earth. As this review will focus mostly on channels from animals (Kingdom Metazoa, formerly known as Animalia) and their relative Phyla, we will look deeper into the evolution of this Kingdom. Metazoans are typified by being heterotrophic, multicellular, motile (at least through some part of their life cycle) and, unlike many other multicellular eukaryotes, lacking a rigid cell wall. Most extant animals belong to the Bilateria, which exhibit bilateral symmetry and triploblasty (meaning that at the gastrulation of the blastula stage, three distinct germ cell layers form). Bilaterians are further divided into deuterostomes (chordates, hemichordates, echinoderms and possibly *Xenoturbella*) and protostomes (the rest of the bilaterian animals). While in deuterostomes the blastopore, the first opening formed in the embryo, becomes the anus, in the protostomes the blastopore becomes the mouth. The ancestor of all bilaterians is called the ‘urbilaterian’: the nature of this animal as well as the history of body plan development within Bilateria and especially the evolution of the blastopore fate are all currently under debate (De Robertis, 2008; Martindale and Hejnol, 2009).

Non-bilaterian animals form the following four Phyla: Porifera (sponges), Ctenophora (comb jellies), Cnidaria (corals, sea anemones, jellyfish and hydroids, among others) and Placozoa (*Trichoplax*). Traditionally, the fact that both Cnidaria and Ctenophora are diploblastic (having two germ-layers) and have neurons and muscles, which are missing from sponges and *Trichoplax*, led zoologists to cluster them as one group called Coelenterata, a sister group to bilateria. This view is also supported by at least one phylogenomic study (Philippe et al., 2009) (Fig. 2). The existence of neurons and muscles in these two non-bilaterian Phyla puts them in a pivotal position for studies of the evolution of voltage-gated channels. Moreover, the ease of culture of some cnidarian species and the development of new tools for molecular

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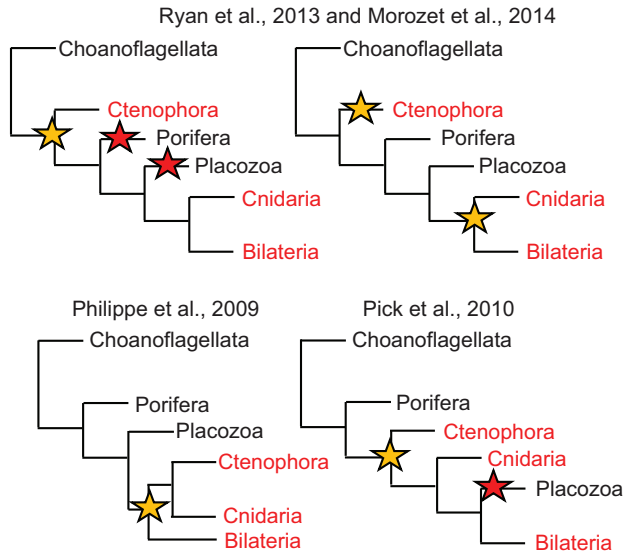

Fig. 2. Animal phylogenomics. Simplified phylogenomic trees showing the relationship between several animal groups and their relatives. Animal groups with muscles and neurons appear in red font. Gold stars represent the likely appearance of neurons and muscles and red stars represent their loss. The trees are based on those presented elsewhere (Moroz et al., 2014; Philippe et al., 2009; Pick et al., 2010; Ryan et al., 2013).

manipulations make them an even stronger comparative model (Technau and Steele, 2011). However, most recent molecular phylogenies suggest that Ctenophora and Cnidaria are not sister groups. Some of them propose ctenophores as the earliest branching animal group that still exists (Dunn et al., 2008; Hejnol et al., 2009; Ryan et al., 2013), whereas other recent phylogenies suggest that Porifera is the earliest branching animal group (Philippe et al., 2009; Pick et al., 2010; Srivastava et al., 2010), followed by Placozoa (Fig. 2). The first scenario, ctenophores being the most basal group, has far-reaching consequences for animal evolution as it means that nervous systems and muscles might have either evolved twice independently or, alternatively, were lost from both sponges and placozoans. The independent evolution of a nervous system in Ctenophora and the cnidarian–bilaterian clades has recently gained further support from the finding that many neuronal markers and neurotransmitters are either missing from ctenophores or expressed in a non-neuronal context (Moroz et al., 2014; but see Marlow and Arendt, 2014). An alternative scenario suggested by yet another phylogenomic study positioned Placozoa as the most basally branching animal group, fitting nicely with the fact that *Trichoplax adhaerens* has the most simple body plan of all extant animals, containing only four cell types (Schierwater et al., 2009). However, no other study supports this basal position of *Trichoplax* and a recent study suggests that this enigmatic animal might have a more complex collection of cell types than initially appreciated (Smith et al., 2014).

Metazoa are considered to be part of the supergroup Opisthokonta, which also includes fungi and several protists (Baldauf and Steenkamp, 2004). The members of one of the opistokont groups, the choanoflagellates, are considered to be the closest unicellular relatives of Metazoa and show striking morphological similarity to specialized cells in sponges called choanocytes (King, 2005). The study of choanoflagellates, as well as other unicellular opisthokonts, at the genomic level has revealed that various gene families, previously thought to be animal specific, actually appeared in early opisthokonts before multicellular organism had even evolved in this lineage (e.g. Sebé-Pedrós et al., 2011; Sebé-Pedrós et al., 2012; Young et al., 2011). Thus, sequencing of genomes of representatives of key lineages may reveal important facts about the evolutionary history of gene families, biological pathways and whole systems. Among other topics, this review will describe how the revolution of genomics also changed our understanding of the evolution of voltage-gated ion channels.

Voltage-gated potassium channels: a diverse and ancient family

Potassium is a common ion in cells and diverse potassium channels are found in most living forms. Because of their wide phyletic distribution, K_Vs are thought to be the very first voltage-gated ion channels to have evolved. In metazoan excitable cells, K_Vs are responsible for the termination of the action potential and for returning the cell to the resting state. These channels are tetramers, with each protein unit encoded by a gene for a 6-TM domain. The tetramerization domain (T1) located at the cytoplasmic N-terminus of each domain enables subfamily-specific assembly into homotetrameric and heterotetrameric channels (Covarrubias et al., 1991; Li et al., 1992; Shen and Pfaffinger, 1995; Xu et al., 1995). The selectivity filter that enables potassium-selective ion conductance constitutes the amino acid residues TVGYG located at the p-loop of all four domains and is highly conserved in all potassium channels from prokaryotes to mammals (Heginbotham et al., 1994). Metazoan K_Vs exhibit two means of channel inactivation: (i) the fast N-type, in which the channel cytoplasmic N-terminal inactivation domain occludes the pore (Hoshi et al., 1990; Zagotta et al., 1990), and (ii) the slow C-type that involves a conformational change at the pore region (Liu et al., 1996; Cordero-Morales et al., 2007; Cuello et al., 2010) and the channel cytoplasmic C-terminus, which influences the inactivation rate (Hoshi et al., 1991; Ogielska et al., 1995).

These voltage-gated channels have likely evolved by the fusion of a voltage-gating module with a 2-TM pore module as both modules were shown to also exist as independent functional units, such as voltage-gated phosphatases (Murata et al., 2005) and voltage-gated proton channels (Ramsey et al., 2006; Sasaki et al., 2006), both lacking a pore-module, and KcsA from the bacterium *Streptomyces lividans* (Schrempf et al., 1995; Doyle et al., 1998) and the eukaryotic K_{ir} family (Ho et al., 1993; Kubo et al., 1993; Whorton and MacKinnon, 2011), which are 2-TM potassium ion channels missing the voltage-gating module. Moreover, there are combinations of voltage-gating modules and pore modules, such as channels made of two 2-TM pore modules (K_{2P}) (Goldstein et al., 1996; Lesage et al., 1996; Brohawn et al., 2012), a 6-TM domain and two 2-TM pore module found in fungi (TOK1) (Ketchum et al., 1995; Zhou et al., 1995), as well as two 6-TM domains (TPC) (Ishibashi et al., 2000).

K_V channels are found in some prokaryotes, such as KvAP from the archaea *Aeropyrum perni* (Jiang et al., 2003) and KvLm from the bacteria *Listeria monocytogenes* (Santos et al., 2006). Putative K_V channels are found in the choanoflagellate *Monosiga brevicollis* (Fig. 3), as well as in the basal metazoans *T. adhaerens* and the ctenophore *M. leidyi* (Fig. 3) (Dubas et al., 1988), whereas in the sponge *Amphimedon queenslandica* only 2-TM K_{ir} channels were shown to be present (Tompkins-MacDonald et al., 2009), but no K_V channels, probably as a result of gene loss. Cnidarians are considered among the earliest animals to have evolved a nervous system, and indeed multiple K_V channels of various biophysical

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Fig. 3. Sequence alignment of Shaker-like K_V channels. Sequences of putative *Trichoplax adhaerens* and *Monosiga brevicollis* voltage-gated potassium channels were obtained by homology search to the *D. melanogaster* Shaker protein sequence (NP_728123.1) using a BLAST search (http://blast.ncbi.nlm.nih.gov/), and of putative *Mnemiopsis leidyi* channels using the *Mnemiopsis* Genome Project Portal (http://research.nhgri.nih.gov/mnemiopsis/). When more than three results were obtained, only the first three were used in the alignment. $hK_V1.2$ corresponds to the human channel (NCBI ref. NP_004965.1). Only partial alignment is shown, with the residues constituting the selectivity filter highlighted by a red box; the fourth segment is indicated by a blue box and carries the conserved positively charged residues that enable voltage-dependent gating. The alignment was generated using CLUSTAL 2.1 (BLOSUM).

properties from the hydrozoan jellyfish *Polyorchis penicillatus* were cloned and expressed (Jegla et al., 1995; Grigoriev et al., 1997; Sand et al., 2011). Recently, 44 genes encoding K_Vs were found to be present in the sea anemone *Nematostella vectensis* (Jegla et al., 2012), and 35 K_V genes in *Hydra magnipapillata* (Chapman et al., 2010; Jegla et al., 2012). As voltage-gated ion channel diversity is believed to be involved in the evolution of animal neuronal complexity and because cnidarians are considered to have a 'simple' decentralized nervous system, the finding of such a variety of channels in cnidarians is surprising. In comparison, in humans 27 genes encoding K_Vs are present (Jegla et al., 2009), while in many protostome invertebrates only a few K_V genes are present (Bargmann, 1998; Holt et al., 2002; McCormack, 2003; Jegla et al., 2009; Jegla et al., 2012), like for example in *Drosophila melanogaster*, which has only five genes encoding K_Vs (Littleton and Ganetzky, 2000), yet functional diversity is enabled by extensive alternative splicing and RNA editing (Kamb et al., 1988; Timpe et al., 1988; Schwarz et al., 1988; Hoopengardner et al., 2003; Ryan et al., 2008; Ingleby et al., 2009). Jegla and co-workers showed that the 44 K_V genes found in *N. vectensis* correspond to the four channel subfamilies K_V1–4 (Shaker, Shab, Shaw and Shal, respectively) that are conserved in Bilateria (Butler et al., 1989; Wei et al., 1990; Strong et al., 1993), but many of them are the products of cnidarian-specific gene duplication events followed by diversification. The Shaker channel family is a distinct K_V family in Metazoa (Jegla et al., 2009, 2012) and arguably the most diverse family in Bilateria as mammals carry eight *shaker* genes. Heterologous expression of 18 of the 20 K_V1 (Shaker) channels of the sea anemone demonstrated that the numerous channel genes indeed encode potassium channel subunits that assemble into homotetramers and heterotetramers with diverse functional properties (Jegla et al., 2012). Further, 11 of these Shaker genes seem to encode regulatory subunits that by themselves do not assemble into functional channels, but in combination with other subunits they produce channels with distinct biophysical properties, thus further increasing channel variability (Jegla et al., 2012). The existence of multiple K_V1 genes plus the many regulatory subunits in the *Nematostella* genome may increase the complexity of shaker-based electrical signaling in sea anemones to levels even higher than those of mammalian K_V1. It is noteworthy that many of the human K_V1 channel family exhibit fast inactivation only when in association with certain beta auxiliary subunits (Rettig et al., 1994; Heinemann et al., 1996), which also modify the channel activation and inactivation kinetics, thus further increasing channel diversity (Pongs et al., 1999). However, whereas the auxiliary beta subunit is conserved in Bilateria (Yu and Catterall, 2004), no such homologs were found in the *Nematostella* genome (Jegla et al., 2012), suggesting that these auxiliary subunits probably evolved in the urbilaterian. Of the 18 *Nematostella* genes, 14 are intron-less (Jegla et al., 2012) and, similarly, of the eight mammalian Shaker genes that are a result of vertebrate-specific gene duplication, seven are also intron-less (Bardien-Kruger et al., 2002; Chandy et al., 1990). Moreover, the Shaker gene in the genome of a deuterostome – the sea urchin *Strongylocentrotus purpuratus* – is intron-less as well (Sodergren et al., 2006), suggesting that chordate and most cnidarian Shaker genes may have evolved from a common intron-less ancestor (Jegla et al., 2012).

A recent study of the functional properties of another family of cnidarian K_V channels uncovered the properties of ancestral channels: in the study, Martinson et al. (Martinson et al., 2014) functionally expressed the *Nematostella* homologs of the bilaterian Ether-a-go-go related channel (Erg, also known as $K_V11.1$). One of the *Nematostella* homologs (NvErg1) exhibits similar electrical characteristics to those of the human channels, whereas another (NvErg4) behaves similarly to the *Drosophila* channel. Phylogeny reveals that NvErg1 and the mammalian channels represent the ancestral electrical behavior, whereas NvErg4 evolved in a convergent manner to the *Drosophila* channel. Surprisingly, it seems that nematode Erg channels also acquired this feature independently, suggesting that this electrical behavior evolved at least three times during animal evolution (Martinson et al., 2014).

Voltage-gated calcium channels: a widely, yet sparsely, distributed channel family

Ca^{2+} currents are associated with locomotion via flagellar movements in paramecium (Plattner, 2014) and algae (*Chlamydomonas*) (Wakabayashi et al., 2009; Fujiu et al., 2009), stress responses in diatoms (Vardi et al., 2006), protein secretion, motility differentiation and infection in parasitic protozoa (Moreno and Docampo, 2003; Nagamune et al., 2007) and light emission in dinoflagellates (von Dassow and Latz, 2002). Hence, it would seem that four-domain Ca_Vs would be widespread in protozoa. Surprisingly, they are absent in many protist lineages (Verret et al., 2010), although well represented in a few lineages such as ciliates.

Most of the increases in cytosolic Ca^{2+} influx in protists appear to be through ligand-gated channels, transient receptor potential (TRP) channels or internal stores. Ca_Vs are also absent in many fungi. However, Ca^{2+} is important in fungal biology – the loss of Ca^{2+} influx that typically occurs in the presence of a mating pheromone leads to a phenotype called mating-induced death – but Ca^{2+} influx occurs via a family of non-voltage-gated channels most closely related to the NALCN channels of metazoans (Paidhungat and Garrett, 1997; Fischer et al., 1997; Liebeskind et al., 2012). Because of the phylogenetic distance to the metazoans, the loss of Ca_Vs genes in many lineages (especially in fungi) and the high likelihood of horizontal gene transfers, a well-resolved phylogeny of protist Ca_Vs, especially their relationship to metazoan Ca_Vs, has been elusive. Moreover, it is clear that multiple losses of Ca_Vs have occurred in other lineages such as land plants (Verret et al., 2010). However, the presence of Ca_Vs in green algae by itself is sufficient to strongly suggest that these channels were already present in the common ancestor of plants and animals (Fujiu et al., 2009; Verret et al., 2010). Alternatively, one can hypothesize that this extremely patchy distribution is the result of an ancient horizontal gene transfer, but we could not find supporting evidence for such a scenario.

Metazoan Ca_Vs open in response to depolarization and allow calcium influx that increases the local calcium concentration. This increase mediates many crucial functions. The most famous function is arguably the fusion of neurotransmitter vesicles to the plasma membrane, which results in neurotransmitter release. Other functions include activation of gene transcription and of various calcium-dependent proteins (reviewed by Clapham, 2007). Ca_Vs of animals can be divided into two broad groups: low-voltage activated (LVA) channels that open near resting potential and high-voltage activated (HVA) channels that require a sizable depolarization to open (Armstrong and Matteson, 1985; Bean, 1985; reviewed in Simms and Zamponi, 2014). LVA channels are typically referred to as T-type or Ca_V3, whereas the HVA group are further sub-divided by their biophysical and pharmacological properties to give mainly N-, P/Q-, R- and L-types. L-Type calcium channels, also known as Ca_V1 channels, form a clear monophyletic group and are characterized pharmacologically by their sensitivity to dihydropyridines, phenylalkylamines and benzothiazepines (reviewed by Catterall et al., 2005). N-, P/Q- and R-type channels are collectively called Ca_V2 and are characterized by their sensitivity to various peptide neurotoxins (Catterall et al., 2005). Choanoflagellates have a single LVA T-type channel and a single HVA type. This finding, together with the fact that up till now LVA channels could not be detected in sponges or ctenophores, suggests that the Ca_V3 channel family was independently lost in several early branching metazoan lineages (Moran and Zakon, 2014). The choanoflagellate and sponge HVA channels occupy a phylogenetic position as a sister group to all metazoan Ca_V1 and Ca_V2 channels, suggesting that they may represent a basal channel HVA group (Moran and Zakon, 2014). In the metazoan lineage leading to placozoans, cnidarians and bilaterians, the ancestral HVA channel has duplicated into an L-type channel and an ancestor to the N/P/Q lineage. This pattern of three Ca_Vs is still the case in most invertebrate bilaterians (Jegla et al., 2009). However, Ca_V channel genes independently duplicated in Cnidaria (six Ca_V channels in sea anemones and reef-building corals) and vertebrates. Moreover, the cnidarian-specific Ca_V genes have been conserved for at least 500 million years, suggesting each of them carries some unique functions (Moran and Zakon, 2014). Unlike their cnidarian counterparts, vertebrates increased their number of Ca_Vs to 10 by two rounds of genome duplications that expanded the vertebrate repertoire of many genes, including those encoding ion channels (Dehal and Boore, 2005; Jegla et al., 2009; Lagman et al., 2013).

Heterologous expression of an L-type Ca_V from the scyphozoan jellyfish *Cyanea capillata* revealed a unique selectivity pattern: unlike most bilaterian L-type Ca_Vs, the jellyfish channel is more permeable to Sr^{2+} than to Ca^{2+}. Moreover, Ca^{2+} was conducted better than Ba^{2+}, again a rare feature in bilaterian channels (Jeziorski et al., 1998). Electrical recordings from muscle cells of the sea anemone *Calliactis tricolor* revealed calcium-based currents. However, unlike most bilaterian Ca_Vs, the channels in this preparation also had a preference for calcium over barium ions (Holman and Anderson, 1991). These results demonstrate how the electrophysiological study of non-bilaterian ion channels has the potential to reveal novel biochemical characteristics and that some significant changes in pore-organization of Ca_Vs probably occurred during animal evolution.

The multiple evolutionary routes to voltage-gated sodium channels

Na_Vs are responsible for the initiation and propagation of electrical signaling in excitable cells and are characterized by voltage-dependent activation, fast inactivation and sodium-selective ion conductance. The fast-inactivation process was shown to be coupled to the outward movement of the voltage sensor in DIV (Chen et al., 1996; Cha et al., 1999; Kühn and Greeff, 1999; Sheets et al., 1999), which leads to occlusion of the pore by the inactivation particle (West et al., 1992; Rohl et al., 1999) from the intracellular side in a hinged-lid mechanism a few milliseconds after channel activation (Vassilev et al., 1988; Stühmer et al., 1989; Patton et al., 1992).

In the Na_V1 family that is found in most bilaterians, the selectivity to sodium ions is enabled by the highly conserved selectivity filter consisting of the four residues Asp-Glu-Lys-Ala (DEKA), with Asp situated at the p-loop of DI, Glu in DII, Lys in DIII and Ala in DIV (Heinemann et al., 1992). The Lys at DIII was shown to be crucial for sodium selectivity as replacing this residue increased potassium and calcium conductance (Schlief et al., 1996; Favre et al., 1996). Moreover, the position of the residues is critical for sodium selectivity and interchanging their position in the domains was shown to result in the loss of sodium selectivity (Schlief et al., 1996).

The genomic revolution of the passing decade revealed that homologs of Na_Vs are present in the unicellular eukaryotes *Thecamonas trahens* of the Apusozoa (Cai, 2012) as well as in *M. brevicollis* (Liebeskind et al., 2011). These findings imply that Na_V-like channels emerged before nervous systems or even multicellularity evolved, more than a billion years ago. Examination of the selectivity filters of the *Monosiga* and *Thecamonas* channels showed they are constituted of the residues DEEA and DEES, respectively (Liebeskind et al., 2011; Cai, 2012). In *Trichoplax* and *Mnemiopsis*, all Na_V channels were found to have the selectivity filer DEEA (Liebeskind et al., 2011; Gur Barzilai et al., 2012). However, in sponges, no Na_V channel homologs are present. Na_Vs with the selectivity filter DEEX (Na_V2 channels) are found in most animal Phyla, with the exception of vertebrates, which have only Na_V1 channels (Fig. 4) (Liebeskind et al., 2011; Gur Barzilai et al., 2012). Heterologous expression of insect and sea anemone Na_V2 channels in *Xenopus* oocytes showed that despite their sequence similarity to Na_V1, these channels are relatively non-selective, conducting sodium and potassium and calcium ions, with a clear preference for the calcium ions (Zhou et al., 2004; Zhang et al., 2011; Gur Barzilai et al., 2012).

In contrast to the calcium-conducting Na_V2 channels, which appeared before the fungal–metazoan split, as we know from their

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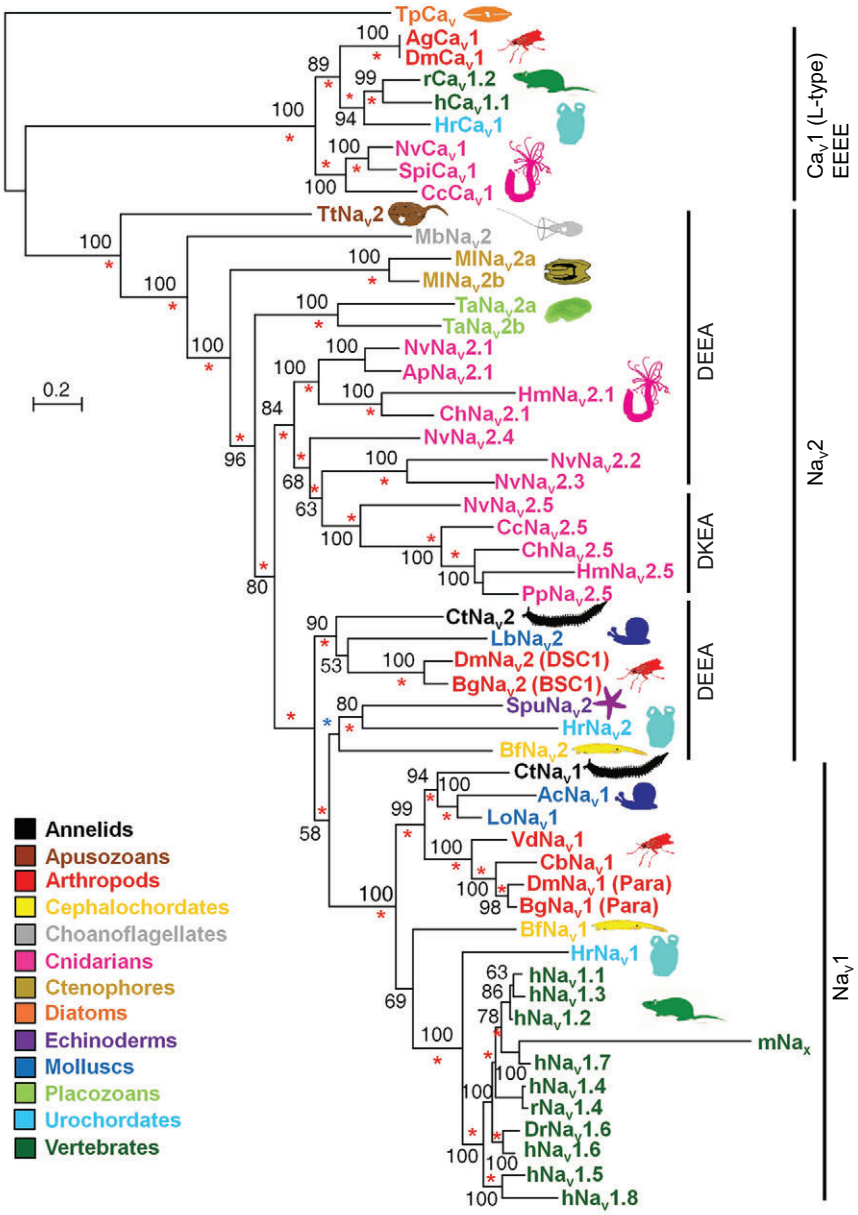

Fig. 4. Phylogeny of voltage-gated sodium channels. A maximum likelihood tree was constructed using the LG (+F +G +I) model (modified from Gur Barzilai et al., 2012). The bootstrap support out of 100 is indicated at the branches. A Bayesian analysis using the WAG model resulted in identical topology. Posterior probabilities of 1.0 are indicated by a red asterisk and those of 0.95<*X*<1.0 are indicated by a blue asterisk. All sequences are from cloned cDNA unless otherwise mentioned. Protein models from genomic sequences were only used after bioinformatic verification that the prediction is sufficiently accurate. Animal clades are indicated by colors. Ac, *Aplysia californica* (sea slug; Mollusca); Ag, *Anopheles gambiae* (mosquito; Arthropoda); Ap, *Aiptasia pallida* (sea anemone; Cnidaria); Bf, *Branchiostoma floridae* (lancelet; Cephalochordata); Bg, *Blatella germanica* (cockroach; Arthropoda); Cb, *Cancer borealis* (crab; Arthropoda); Cc, *Cyanea capillata* (jellyfish; Cnidaria); Ch, *Clytia hemisphaerica* (hydrozoan jellyfish; Cnidaria); Ct, *Capitella teleta* [segmented worm; Annelida (predicted proteins)]; Dm, *Drosophila melanogaster* (fruit fly; Arthropoda); Dr, *Danio rerio* (zebrafish, Vertebrata); h, human (Vertebrata); Hm, *Hydra magnipapillata* (hydra; Cnidaria); Hr, *Halocynthia roretzi* (sea squirt; Urochordata); Lb, *Loligo bleekeri* (squid; Mollusca); Lo, *Loligo opleans* (squid; Mollusca); m, mouse (vertebrata); Mb, *Monosiga brevicollis* (choanoflagellate; Choanoflagellida); Ml, *Mnemiopsis leidyi* [comb jellies; Ctenophora (predicted protein)]; Nv, *Nematostella vectensis* (sea anemone; Cnidaria); Pp, *Polyorchis penicillatus* (jellyfish; Cnidaria); r, rat (Vertebrata); Spi, *Stylophora pistillate* (coral; Cnidaria); Spu, *Strongylocentrotus purpuratus* [sea urchin; Echinodermata (predicted protein)]; Ta, *Trichoplax adhaerens* (Placozoa); Tp, *Thalassiosira pseudonana* [diatom; Heterokontophyta (predicted protein)]; Tt, *Thecamonas trahens* [Apusozoa (predicted protein)]; Vd, *Varroa destructor* (mite; Arthropoda).

presence in *Thecamonas*, the sodium-selective Na_V1 channels are found exclusively in Bilateria. Interestingly, in *Nematostella*, five genes encoding Na_V2 homologs are present (Putnam et al., 2007; Gur Barzilai et al., 2012), of which three have the selectivity filter DEEA, one has DEET and one has DKEA (the $NvNa_V2.5$ channel). In comparison, in the genome of the fruitfly *D. melanogaster*, only one Na_V1 and one Na_V2 encoding gene is present (Littleton and Ganetzky, 2000) and each of these genes gives rise to tens of isoforms with different functional properties by extensive alternative splicing and RNA editing (Thackeray and Ganetzky, 1994; Tan et al., 2002; Song et al., 2004; Olson et al., 2008; Zhang et al., 2011). In the human genome there are 10 genes encoding Na_Vs ($Na_V1.1$–$Na_V1.9$ and Na_X). It is thought that in the common ancestor of vertebrates only one Na_V1 gene was present, which underwent two rounds of duplication in the basal vertebrate and further duplication and diversification in teleosts and tetrapods (Widmark et al., 2011; Zakon et al., 2011). As in the case of K_Vs discussed before, the finding of such a diversity of sodium channels in a sea anemone was highly unexpected (Gur Barzilai et al., 2012). Heterologous expression of $NvNa_V2.5$ showed this channel to be sodium selective, similar to Na_V1 channels (Gur Barzilai et al., 2012). Moreover, replacing the selectivity filter of $NvNa_V2.5$ (DKEA) with that from Na_V1 (DEKA) resulted in the loss of sodium selectivity (Gur Barzilai et al., 2012), while replacing the selectivity filter of Na_V1 with that from $NvNa_V2.5$ also resulted in the loss of sodium selectivity (Schlief et al., 1996). These results suggest that sodium selectivity in $NvNa_V2.5$ is achieved in a different manner from that in Na_V1 channels.

Phylogenetic analysis with a broad dataset of sodium channel sequences showed that while sodium-selective Na_V1 channels appeared first in the urbilaterian, sodium-selective $Na_V2.5$ channels emerged exclusively in cnidarians, as $NvNa_V2.5$ forms a sub-cluster within the DEEX-bearing Na_V2 channels together with several Na_Vs of hydrozoans (Spafford et al., 1998) and scyphozoan jellyfish (Anderson et al., 1993) (Fig. 4). All the channels of this sub-cluster bear the unique DKEA ion selectivity filter (Gur Barzilai et al., 2012), suggesting that the $Na_V2.5$ subtype diverged from a channel bearing a DEEA selectivity filter after a gene duplication event in

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the common ancestor of all extant cnidarians more than 540 million years ago (Park et al., 2012). Remarkably, each of the cnidarians whose genome or transcriptome has been sequenced up till now carries at least one $Na_V2.1$ and one $Na_V2.5$ channel.

NaChBac are a family of bacterial sodium-selective voltage-gated channels (Ren et al., 2001; Yue et al., 2002; Koishi et al., 2004). However, their selectivity filter is composed of the residues EEEE, resembling that of Ca_Vs (Durell and Guy, 2001; Ren et al., 2001; Koishi et al., 2004; Payandeh et al., 2011). Replacing the selectivity filter in the mammalian Na_V1 brain channel with EEEE, the Ca_Vs selectivity filter, was shown to render the sodium channel calcium selective (Heinemann et al., 1992). As both Ca_V and Na_V2 channels seem to be more ancient than the bilaterian-specific Na_V1, we can assume that sodium selectivity in voltage-gated ion channels evolved independently in at least three lineages on the tree of life: in bacterial NaChBac channels, in the cnidarian $Na_V2.5$ sub-family and in the Na_V1 channels. The obvious question is why calcium-conducting Na_V2 channels dominated in Metazoa until the emergence of the sodium-selective Na_V1 channels in bilaterians, and why cnidarians also evolved a sodium-selective channel. The common view is that sodium selectivity is advantageous for the evolution of nervous systems, mainly due to the separation of intracellular calcium signaling from neuronal signaling (Hille, 2001; Petersen et al., 2005; Meech and Mackie, 2007). Moreover, as K_Vs generates the falling phase of the action potential, while Na_Vs is responsible for its rising phase, there is a clear functional advantage in separating the sodium and potassium ion fluxes and increasing the selectivity of Na_V channels to sodium ions. It is therefore likely that the pore regions in both the urbilaterian Na_V1 and the primordial $Na_V2.5$ cnidarian channel evolved under selective pressure to cease potassium and calcium ion conductance, resulting in sodium-selective channels.

Despite the advantages of sodium selectivity, *Nematostella* $Na_V2.5$ was shown to be expressed only in a subset of cells, while many other putative neurons express calcium-conducting Na_V2 channels (Gur Barzilai et al., 2012), indicating that cnidarian signaling is heavily based on calcium. Hence, it is likely that when early neuronal networks emerged, their signaling was calcium based, as exemplified by contemporary ctenophores (Hille, 2001; Meech and Mackie, 2007). Furthermore, several animal lineages with simple nervous systems (e.g. nematodes and echinoderms) appear to have independently lost the Na_V1 channels (Fig. 4) (Littleton and Ganetzky, 2000; Jegla et al., 2009; Widmark et al., 2011), and Na_V2 channels with a calcium preference were retained in parallel with sodium-selective channels in many animal groups such as ascidians, insects and cnidarians (Fig. 4) (Nagahora et al., 2000; Liebeskind et al., 2011; Cui et al., 2012). In flies, the Na_V2 channel DSC1 was found to be expressed in various tissues and is thought to contribute to neuronal excitability regulation and to the stability of the nervous system function in response to environmental stresses (Zhang et al., 2013). Furthermore, flies mutated at the DSC1 gene exhibited olfactory impairment (Kulkarni et al., 2002; Zhang et al., 2013) and were more susceptible to heat shock and starvation compared with wild type (Zhang et al., 2013). Therefore, it seems that calcium-based action potentials are not merely an evolutionary relic but may be advantageous in simple neuronal circuits. Interestingly, all cnidarians can react very quickly to stimuli and some are even capable of sensing and incorporating visual inputs or exhibit behavioral patterns more complex than any ctenophore, echinoderm or nematode (Meech and Mackie, 2007; Garm et al., 2011). It is possible that this higher behavioral complexity may, at least in part, be the result of integration of the sodium-selective $Na_V2.5$ channels in selected neuronal circuits, which require faster signaling and higher precision, such as motor neurons or neurons controlling polyp tentacles involved in capturing moving prey.

Open questions, challenges and future directions

As discussed in the previous sections of this review, the genomic revolution uncovered the channel repertoire in many organisms and provided a much clearer view on channel evolution. This is a major leap forward considering the state of knowledge in this field in the 1990s (Strong et al., 1993) or even the early 2000s (Goldin, 2002). However, the advancement in physiological and biochemical knowledge about channels of non-bilaterian animals during the last decade was modest. This is probably due to the difficulties in achieving expression in heterologous systems of many of these channels (e.g. Anderson et al., 1993; Gur Barzilai et al., 2012). Moreover, even when achieving such expression, it might not fully reflect the native physiological properties of the channel. As rare as electrophysiological studies in heterologous expression systems have been in the past decade, such studies in the native neurons and muscles of non-bilaterians have almost disappeared, probably because of the technical challenges they raise. Notably, during the 1970s, 1980s and 1990s, studies measuring electrical currents in the neurons and muscles of cnidarians, mostly jellyfish, provided us with important knowledge about the electrical properties and function of non-bilaterian ion channels in preparations of dissociated tissue (Anderson and Mackie, 1977; Anderson, 1987; Holman and Anderson, 1991; Mackie and Meech, 1985; Spafford et al., 1996). The disappearance of this ‘art’ can have profound effects on our future understanding of the evolution of voltage-gated ion channels, as no genomic study on its own can reveal function. One of the major obstacles in performing such measurements is the identification of neurons, as in many non-bilaterian animals they are small and lack defining morphological traits if their extensions cannot be seen after dissociation (Holman and Anderson, 1991). A promising solution for this problem might rise from the ability to generate transgenic lines in cnidarians such as *Hydra*, *Hydractinia* and *Nematostella* (Künzel et al., 2010; Renfer et al., 2010; Wittlieb et al., 2006). Indeed, a transgenic *Nematostella* line carrying a fluorescent reporter in their neurons was recently published (Nakanishi et al., 2012). Electrophysiological studies of these neurons will hopefully teach us about the native characteristics of their voltage-gated ion channels and will provide us with a functional perspective about their evolution.

From the study of K_Vs, Na_Vs and Ca_Vs at the genomic level, it is apparent that all voltage-gated ion channel families are present in the closest relatives of animals, the Choanoflagellata (Liebeskind et al., 2011; Liebeskind et al., 2012; Gur Barzilai et al., 2012). Studying channel function in these single-cell organisms by application of blockers and modulators might teach us about channel function and evolution in early animals.

As noted above, most major voltage-gated ion channels (Na_Vs, Ca_Vs and Shaker K_Vs) demonstrate lineage-specific expansion within Cnidaria. It was suggested that expansion of Na_V subtypes in vertebrates correlated with increased neuronal complexity (Zakon et al., 2011) and it is tempting to hypothesize that the same might be true for the expansions in Cnidaria. However, a functional approach is required for testing this hypothesis. A logical future research route is to use the gene knockdown approaches that were established in Cnidaria (Technau and Steele, 2011; Layden et al., 2013) in order to test the role of each channel and the effect of its downregulation on animal physiology and behavior.

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The authors declare no competing or financial interests.

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