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## Review

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# Evolution of voltage-gated Na<sup>+</sup> channels

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### Summary

Voltage-gated Na<sup>+</sup> channels play important functional roles in the generation of electrical excitability in most vertebrate and invertebrate species. These channels are members of a superfamily that includes voltage-gated K<sup>+</sup>, voltage-gated Ca<sup>2+</sup> and cyclic-nucleotide-gated channels. There are nine genes encoding voltage-gated Na<sup>+</sup> channels in mammals, with a tenth homologous gene that has not been shown to encode a functional channel. Other vertebrate and invertebrate species have a smaller number of Na<sup>+</sup> channel genes. The mammalian genes can be classified into five branches in a phylogenetic tree, and they are localized on four chromosomes. Four of the branches representing the four chromosomal locations probably resulted from the chromosomal duplications that

led to the four *Hox* gene clusters. These duplications occurred close to the emergence of the first vertebrates. The fifth branch probably evolved from a separate ancestral Na<sup>+</sup> channel gene. There are two branches in the invertebrate tree, although members of only one of those branches have been demonstrated to encode functional voltage-gated Na<sup>+</sup> channels. It is possible that the other branch may have diverged, so that its members do not represent true voltage-gated Na<sup>+</sup> channels. Vertebrate and invertebrate Na<sup>+</sup> channels appear to be derived from a single primordial channel that subsequently evolved independently in the two lineages.

Key words: Na<sup>+</sup> channel, cloning, phylogeny, evolution, diversity.

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### Introduction

Voltage-gated Na<sup>+</sup> channels play a critical role in controlling the electrical excitability of animal cells, being primarily responsible for the rising phase of the action potential. The electrophysiological properties of all voltage-gated Na<sup>+</sup> channels are not identical, however, and small differences in these properties can have significant effects on the electrical excitability of the cell (for a review, see Hille, 2001). Na<sup>+</sup> channels with different functional or pharmacological properties have been observed in different tissues of the same species and in different species by electrophysiological recording (Mandel, 1992). In addition, a variety of different Na<sup>+</sup> channel isoforms have been cloned, functionally expressed and characterized (Goldin, 1999).

The Na<sup>+</sup> channel consists of a highly processed  $\alpha$  subunit of approximately 260 kDa and contains four homologous domains termed I–IV. Within each domain, there are six transmembrane segments called S1–S6, and a hairpin-like P loop between S5 and S6 that comprises part of the channel pore (Fig. 1). The  $\alpha$  subunit is associated with accessory subunits in the tissues of certain species, such as the  $\beta$  subunits in mammals (Catterall, 1993) and the tipE subunits in flies (Feng et al., 1995). The purpose of this review is to examine the phylogenetic relationships among the different voltage-gated Na<sup>+</sup> channels that have been identified thus far. The structure

and functional characteristics of the isoforms will not be discussed in detail as these topics have recently been reviewed (Catterall, 2000; Goldin, 2001).

For the purpose of this review, the voltage-gated Na<sup>+</sup> channels can be considered in three distinct categories. Mammalian Na<sup>+</sup> channels represent the largest group of genes that have been identified and studied (Table 1). These isoforms have all been classified as members of a single gene family with nine members. These will be referred to using the nomenclature that was proposed recently (Goldin et al., 2000), which consists of the prefix Na<sub>v</sub> to indicate the principal permeating ion and the principal physiological regulator, followed by a number that indicates the gene subfamily (currently Na<sub>v</sub>1 is the only subfamily). The number following the decimal point identifies the specific channel isoform (e.g. Na<sub>v</sub>1.1). There is a tenth isoform termed Na<sub>x</sub> that has approximately 50% sequence identity to the other mammalian Na<sup>+</sup> channels. Because none of the Na<sub>x</sub> channels has been functionally expressed, it is possible that this gene does not encode a voltage-gated Na<sup>+</sup> channel, which is why it has not been assigned a number. Representatives of all nine isoforms and Na<sub>x</sub> have been identified and characterized from humans and rats, with representatives of some of the isoforms from other species. Only full-length sequences of the mammalian Na<sup>+</sup> channels have been used in this analysis.

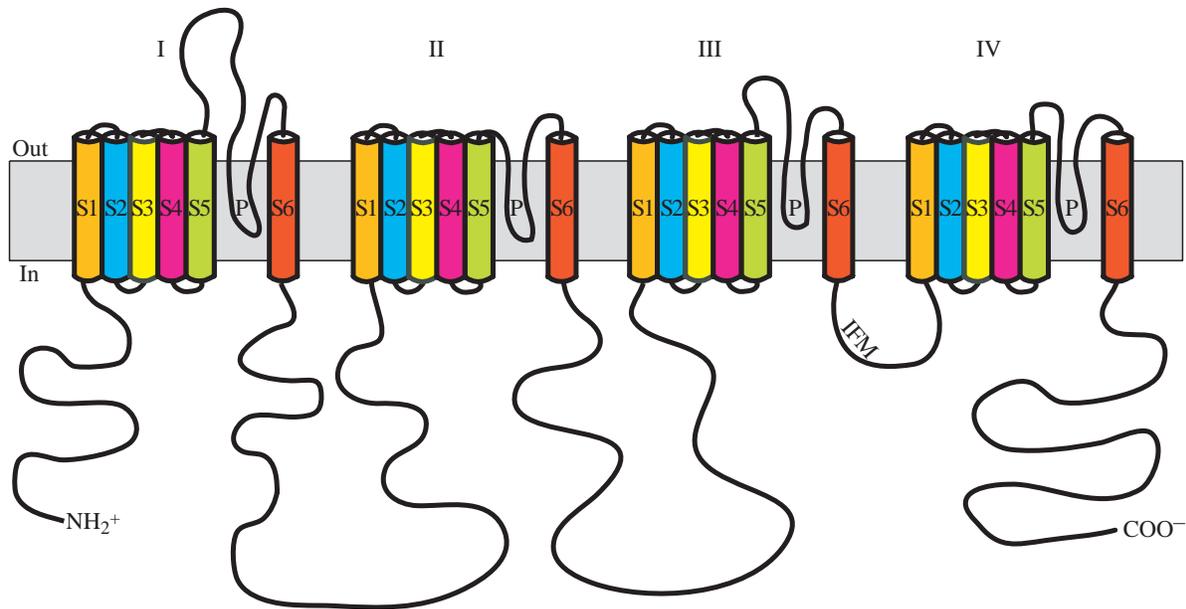


Fig. 1. A schematic diagram of the voltage-gated  $\text{Na}^+$  channel  $\alpha$  subunit. The four homologous domains are labeled I–IV, and the six transmembrane segments in each domain are labeled S1–S6. The critical motif of the fast inactivation particle (IFM) is shown on the cytoplasmic linker connecting domains III and IV. The channel is aligned with the extracellular side of the membrane on the top. The lengths that are shown for the N and C termini and the interdomain cytoplasmic loops are consistent with the sequence of the  $\text{Na}_v1.2$  mammalian  $\text{Na}^+$  channel, but these linkers (except for that between domains III and IV) vary greatly in length and sequence among the different  $\text{Na}^+$  channel isoforms. P, P loop.

The other two groups of  $\text{Na}^+$  channel that will be discussed include the non-mammalian vertebrate  $\text{Na}^+$  channels and the invertebrate  $\text{Na}^+$  channels (Table 2). There is no systematic nomenclature for these channels, and they have been named in a variety of ways. A consistent nomenclature has been adopted for the purposes of this review. The name consists of the first letters of the genus and species, followed by  $\text{Na}_v$  to indicate the principal permeating ion and the principal physiological regulator, and a number that was either part of the original name or is arbitrary.

There are many fewer representatives of non-mammalian  $\text{Na}^+$  channel sequences. Only five full-length sequences have been determined for non-mammalian vertebrate  $\text{Na}^+$  channels, and each of these is from a separate species. Lopreato et al. (2001) have determined partial sequences for six genes in one species, *Sternopygus macrurus*. This is the first attempt to identify all the  $\text{Na}^+$  channel genes in a single non-mammalian vertebrate species, so these data are particularly informative with respect to phylogeny. Therefore, these sequences have been included in the current analysis.

More invertebrate  $\text{Na}^+$  channel genes have been identified, with 13 full-length sequences representing 11 species. However, more than one full-length sequence has been determined for only two invertebrate species, *Blattella germanica* and *Halocynthia roretzi*. Two sequences have been determined from *Drosophila melanogaster*, with one full-length ( $\text{DmNa}_v1$ ) and one that is almost complete ( $\text{DmNa}_v2$ ). Blackshaw et al. (1999) have attempted to identify all the  $\text{Na}^+$  channel genes in an invertebrate species, *Hirudo medicinalis*.

They determined partial sequences for four distinct genes, and these sequences have been included in the analysis.

### Phylogeny of $\text{Na}^+$ channels

Voltage-gated  $\text{Na}^+$  channels are members of a superfamily that includes voltage-gated  $\text{K}^+$  channels, voltage-gated  $\text{Ca}^{2+}$  channels and cyclic-nucleotide-gated channels (Jan and Jan, 1992; Hille, 2001). The  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channel members all contain four homologous domains, whereas the  $\text{K}^+$  and cyclic-nucleotide-gated channels in the superfamily consist of tetramers of single-domain subunits. On the basis of structure alone, it might be expected that  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels would have evolved from the single-domain channels. This is in fact the most widely accepted scheme, for the reasons reviewed by Hille (1987, 1988). Bacteria, like eukaryotic cells, typically have high intracellular  $\text{K}^+$  and low intracellular  $\text{Ca}^{2+}$  concentrations, but many have no requirement for  $\text{Na}^+$  or  $\text{Cl}^-$ . Consistent with this lack of necessity for  $\text{Na}^+$  or  $\text{Cl}^-$ , bacteria do not generally express either  $\text{Na}^+$  or  $\text{Cl}^-$  channels, whereas they clearly express  $\text{K}^+$  channels (Milkman, 1994). Therefore, it is likely that an ancestral primordial prokaryotic channel was more similar to the voltage-gated  $\text{K}^+$  channels (Ranganathan, 1994). In fact, it has been suggested that channels with a cytoplasmic gate, such as the cyclic-nucleotide-gated channels and mechanosensitive channels, may represent the primordial channel from which the voltage-gated channels evolved (Jan and Jan, 1994; Anderson and Greenberg, 2001).

It is likely that the  $\text{Ca}^{2+}$  channels evolved from the  $\text{K}^+$

Table 1. Mammalian Na<sup>+</sup> channel  $\alpha$  subunits

Name	Original name	Species	Common name	Tissue	Size	Gene symbol	Gene location <sup>a</sup>	GenBank accession number	References
rNa <sub>v</sub> 1.1	rat I	<i>Rattus norvegicus</i>	Rat	CNS, PNS	2009	SCN1A	2 [36]	X03638	Noda et al. (1986); Malo et al. (1991)
hNa <sub>v</sub> 1.1	SCN1A HBSCI	<i>Homo sapiens</i>	Human	CNS	2009 Partial		2q24	X65362	Lu et al. (1992); Malo et al. (1994a); Escayg et al. (2000)
rNa <sub>v</sub> 1.2	rat II rat IIA	<i>Rattus norvegicus</i>	Rat	CNS	2005	SCN2A	2 [36]	X03639 X61149	Noda et al. (1986); Auld et al. (1988); Malo et al. (1991); Lu et al. (1992)
hNa <sub>v</sub> 1.2	HBA HBSCII	<i>Homo sapiens</i>	Human	CNS	2005 Partial		2q23–24	M94055 X65361	Litt et al. (1989); Lu et al. (1992); Ahmed et al. (1992)
rNa <sub>v</sub> 1.3	rat III	<i>Rattus norvegicus</i>	Rat	CNS	1951	SCN3A	2 [36]	Y00766	Kayano et al. (1988); Joho et al. (1990); Malo et al. (1991)
hNa <sub>v</sub> 1.3	type III	<i>Homo sapiens</i>	Human	CNS	1951		2q24	AJ251507	Malo et al. (1994b); Chen et al., 2000
rNa <sub>v</sub> 1.4	SkM1, $\mu$ 1	<i>Rattus norvegicus</i>	Rat	Skeletal muscle	1840	SCN4A	11 [64]	M26643	Trimmer et al. (1989); Wang et al. (1992); Ambrose et al. (1992)
hNa <sub>v</sub> 1.4	SkM1	<i>Homo sapiens</i>	Human	Skeletal muscle	1836		17q23–25	M81758	George et al. (1991, 1992b); Wang et al. (1992)
rNa <sub>v</sub> 1.5	SkM2 rH1	<i>Rattus norvegicus</i>	Rat	Denervated skeletal muscle, heart	2018	SCN5A	9 [70]	M27902	Rogart et al. (1989); Kallen et al. (1990); George et al. (1995)
hNa <sub>v</sub> 1.5	H1	<i>Homo sapiens</i>	Human	heart	2016		3p21	M77235	Gellens et al. (1992); George et al. (1995)
rNa <sub>v</sub> 1.6	NaCh6 PN4	<i>Rattus norvegicus</i>	Rat	CNS, PNS	1976	SCN8A	15 [64]	L39018 AF049239 AF049240	Schaller et al. (1995); Burgess et al. (1995); Dietrich et al. (1998); Plummer et al. (1998)
hNa <sub>v</sub> 1.6	Scn8a	<i>Homo sapiens</i>	Human	CNS	1980		12q13	AF050736 AF225988	Burgess et al. (1995); Plummer et al. (1998)
mNa <sub>v</sub> 1.6	Scn8a	<i>Mus musculus</i>	Mouse	CNS	1976			U26707 AF049617	Burgess et al. (1995); Smith et al. (1998)
rNa <sub>v</sub> 1.7	PN1	<i>Rattus norvegicus</i>	Rat	PNS	1984	SCN9A	2 [36] 2	AF000368 U79568	Beckers et al. (1996); Kozak and Sangameswaran (1996); Toledo-Aral et al. (1997); Sangameswaran et al. (1997)
hNa <sub>v</sub> 1.7	hNE-Na	<i>Homo sapiens</i>	Human	Medullary thyroid Ca	1977		2q24 <sup>a</sup>	X82835	Klugbauer et al. (1995)
oNa <sub>v</sub> 1.7	Nas	<i>Oryctolagus cuniculus</i>	Rabbit	Schwann cells	1984			U35238	Belcher et al. (1995)
rNa <sub>v</sub> 1.8	SNS PN3	<i>Rattus norvegicus</i>	Rat	PNS (DRG)	1957 1956	SCN10A	9 [67]	X92184 U53833	Akopian et al. (1996); Sangameswaran et al. (1996); Kozak and Sangameswaran (1996)
hNa <sub>v</sub> 1.8	PN3	<i>Homo sapiens</i>	Human	PNS (DRG)	1956		3p22–24	AF117907	Rabert et al. (1998)
mNa <sub>v</sub> 1.8	SNS	<i>Mus musculus</i>	Mouse	PNS	1958			Y09108	Souslova et al. (1997)
cNa <sub>v</sub> 1.8	NaNG	<i>Canis familiaris</i>	Dog		1962			U60590	Chen et al. (1997)
rNa <sub>v</sub> 1.9	SNS2	<i>Rattus norvegicus</i>	Rat	PNS	1765	SCN11A	9 [71]	AJ237852 AF059030	Dib-Hajj et al. (1998); Tate et al. (1998); Dib-Hajj et al. (1999b); Jeong et al. (2000); Ogata et al. (2000)
hNa <sub>v</sub> 1.9	NaN PN5 SCN12A	<i>Homo sapiens</i>	Human		1791		3p21–24	AF188679 AF109737 AF150882	Dib-Hajj et al. (1999a,b); Jeong et al. (2000)
mNa <sub>v</sub> 1.9	NaN NaT	<i>Mus musculus</i>	Mouse		1765			AF118044 AB031389	Dib-Hajj et al. (1998); Ogata et al. (2000)
rNa <sub>x</sub>	Na-G SCL11	<i>Rattus norvegicus</i>	Rat	Astrocytes PNS (DRG)	Partial 1702	SCN7A <sup>b</sup>	2 [41]	M96578 Y09164	Gautron et al. (1992); Potts et al. (1993); Akopian et al. (1997)
hNa <sub>x</sub>	Na <sub>v</sub> 2.1	<i>Homo sapiens</i>	Human	Heart, uterus muscle	1682	SCN6A <sup>b</sup>	2q21–23	M91556	George et al. (1992a, 1994)
mNa <sub>x</sub>	Na <sub>v</sub> 2.3	<i>Mus musculus</i>	Mouse	Heart, uterus muscle	1681			L36179	Felipe et al. (1994)

<sup>a</sup>Human map location for SCN9A is inferred from the mouse mapping data.

<sup>b</sup>SCN6A and SCN7A probably represent the same gene because they were mapped in human and mouse, respectively (Plummer and Meisler, 1999).

CNS, central nervous system; PNS, peripheral nervous system; DRG, dorsal root ganglion.

Table 2. Non-mammalian Na<sup>+</sup> channel  $\alpha$  subunits

Name	Original name	Species	Common name	Size	GenBank accession number	References
Vertebrates						
CpNa <sub>v</sub> 1		<i>Cynops pyrrhogaster</i>	Japanese common newt	2007	AF123593	
DrNa <sub>v</sub> 1	zfNa <sub>v</sub> 1.6	<i>Danio rerio</i>	Zebrafish	1949	AF297658	Tsai et al. (2001)
EeNa <sub>v</sub> 1	eel Na channel	<i>Electrophorus electricus</i>	Electric eel	1820	X01119	Noda et al. (1984)
FpNa <sub>v</sub> 1	fMNa1	<i>Fugu pardalis</i>	Puffer fish	1880	AB030482	Yotsu-Yamashita et al. (2000)
FrNa <sub>v</sub> 1	fBNA2	<i>Fugu rubripes</i>	Puffer fish	1717	D37977	
SmNa <sub>v</sub> 1	SterNa1	<i>Sternopygus macrurus</i>	Teleost fish	Partial	AF378139	Lopreato et al. (2001)
SmNa <sub>v</sub> 2	SterNa2	<i>Sternopygus macrurus</i>	Teleost fish	Partial	AF378140	Lopreato et al. (2001)
SmNa <sub>v</sub> 3	SterNa3	<i>Sternopygus macrurus</i>	Teleost fish	Partial	AF378141	Lopreato et al. (2001)
SmNa <sub>v</sub> 4	SterNa4	<i>Sternopygus macrurus</i>	Teleost fish	Partial	AF378142	Lopreato et al. (2001)
SmNa <sub>v</sub> 5	SterNa5	<i>Sternopygus macrurus</i>	Teleost fish	Partial	AF378143	Lopreato et al. (2001)
SmNa <sub>v</sub> 6	SterNa6	<i>Sternopygus macrurus</i>	Teleost fish	Partial	AF378144	Lopreato et al. (2001)
Invertebrates						
AcNa <sub>v</sub> 1	SCAP1	<i>Aplysia californica</i>	California sea hare	1993	U66915	Dyer et al. (1997)
ApNa <sub>v</sub> 1	AnemNa1	<i>Aiptasia pallida</i>	Sea anemone	1810	AF041851	see Spafford et al. (1998)
BcNa <sub>v</sub> 1	BdNa1	<i>Bdelloura candida</i>	Turbellarian flatworm	1699	U93074	Jeziorski et al. (1997)
BgNa <sub>v</sub> 1	CSMA para	<i>Blattella germanica</i>	German cockroach	2031 Partial	U73583 U71083	Dong (1997); Miyazaki et al. (1996)
BgNa <sub>v</sub> 2	Bsc1	<i>Blattella germanica</i>	German cockroach	2304	AF312365	Liu et al. (2001a)
CcNa <sub>v</sub> 1	CYNA1	<i>Cyanea capillata</i>	Scyphozoan jellyfish	1739	L15445	Anderson et al. (1993)
DmNa <sub>v</sub> 1	Para	<i>Drosophila melanogaster</i>	Fruit fly	1820	M32078-80	Ramaswami and Tanouye (1989); Loughney et al. (1989)
DmNa <sub>v</sub> 2	DSC1	<i>Drosophila melanogaster</i>	Fruit fly	Partial	X14394-8	Salkoff et al. (1987)
HmNa <sub>v</sub> 1	LeNa1	<i>Hirudo medicinalis</i>	Leech	Partial		Blackshaw et al. (1999)
HmNa <sub>v</sub> 2	LeNa2	<i>Hirudo medicinalis</i>	Leech	Partial		Blackshaw et al. (1999)
HmNa <sub>v</sub> 3	LeNa3	<i>Hirudo medicinalis</i>	Leech	Partial		Blackshaw et al. (1999)
HmNa <sub>v</sub> 4	LeNa4	<i>Hirudo medicinalis</i>	Leech	Partial		Blackshaw et al. (1999)
HrNa <sub>v</sub> 1	TuNa1	<i>Halocynthia roretzi</i>	Ascidian tunicate	2049	D17311	Okamura et al. (1994)
HrNa <sub>v</sub> 2	TuNa2	<i>Halocynthia roretzi</i>	Ascidian tunicate	2221	AB042806	Nagahora et al. (2000)
LbNa <sub>v</sub> 1	squid Na channel	<i>Loligo bleekeri</i>	Squid	1522	D14525	Sato and Matsumoto (1992)
LoNa <sub>v</sub> 1	GFLN1	<i>Loligo opalescens</i>	Squid	1784	L19979	Rosenthal and Gilly (1993)
MdNa <sub>v</sub> 1	Msc Vssc1	<i>Musca domestica</i>	House fly	2108	X96668 U38814	Williamson et al. (1996); Ingles et al. (1996)
PpNa <sub>v</sub> 1	PpSCN1	<i>Polyorchis penicillatus</i>	Hydrozoan jellyfish	1695	AF047380	Spafford et al. (1998)

channels during the evolution of the stem eukaryotes (Hille, 1989). Protozoans generally use Ca<sup>2+</sup> as the inward charge carrier, and purely Na<sup>+</sup>-dependent action potentials are not common until the cnidarians (Hille, 1989; Anderson and Greenberg, 2001). Na<sup>+</sup> channels have not been detected in protozoa, algae or higher plants (Hille, 1989). According to this scenario, the Na<sup>+</sup> channels evolved early in the metazoan era from an ancestral channel resembling the T-type (Ca<sub>v</sub>3 family) Ca<sup>2+</sup> channels, before the separation of diploblasts and triploblasts (Spafford et al., 1999).

The four domains of the Na<sup>+</sup> channel have more similarity to the four domains of the Ca<sup>2+</sup> channels than to each other, further supporting the argument that Na<sup>+</sup> channels evolved after the subunit duplications leading to the Ca<sup>2+</sup> channels (Hille, 1989; Strong et al., 1993). On the basis of sequence similarities, Strong et al. (1993) suggested that the original duplication event resulted in a two-domain channel consisting of domains I/III and II/IV, each of which then duplicated to result in the first four-domain Ca<sup>2+</sup> channel. In this context, it is interesting to note that no channels consisting of two homologous domains have been observed. In addition, no K<sup>+</sup> channels with multiple homologous domains have been observed, suggesting either that there was strong selective pressure against this form of channel or that the selectivity

change from K<sup>+</sup> to Ca<sup>2+</sup> occurred before the gene duplication event (Anderson and Greenberg, 2001). The primordial Na<sup>+</sup> channel then evolved from the Ca<sup>2+</sup> channels, and this primordial Na<sup>+</sup> channel subsequently evolved independently in vertebrates and invertebrates (Fig. 2) (Strong et al., 1993).

### Evolution of vertebrate Na<sup>+</sup> channels

The mammalian vertebrate voltage-gated Na<sup>+</sup> channels represent the best-characterized family in that all the different isoforms have been identified and cloned as full-length channels from two species, human and rat. The isoforms are in five distinct branches on the phylogenetic tree (Fig. 2). This division correlates well with the chromosomal localization of the genes encoding the channels, as described by Plummer et al. (1999). The genes encoding Na<sub>v</sub>1.1, Na<sub>v</sub>1.2, Na<sub>v</sub>1.3, Na<sub>v</sub>1.7 and Na<sub>x</sub> are clustered together on chromosome 2 in humans and mice (Table 1), and these channels form the largest branch of the tree. Na<sub>v</sub>1.1, Na<sub>v</sub>1.2, Na<sub>v</sub>1.3 and Na<sub>v</sub>1.7 share a number of common characteristics, including expression in the nervous system and block by nanomolar concentrations of tetrodotoxin (TTX). Na<sub>x</sub> is unusual in that this channel has sequence differences in functionally important regions, including fewer charges in the S4 voltage sensors and the absence of the IFM

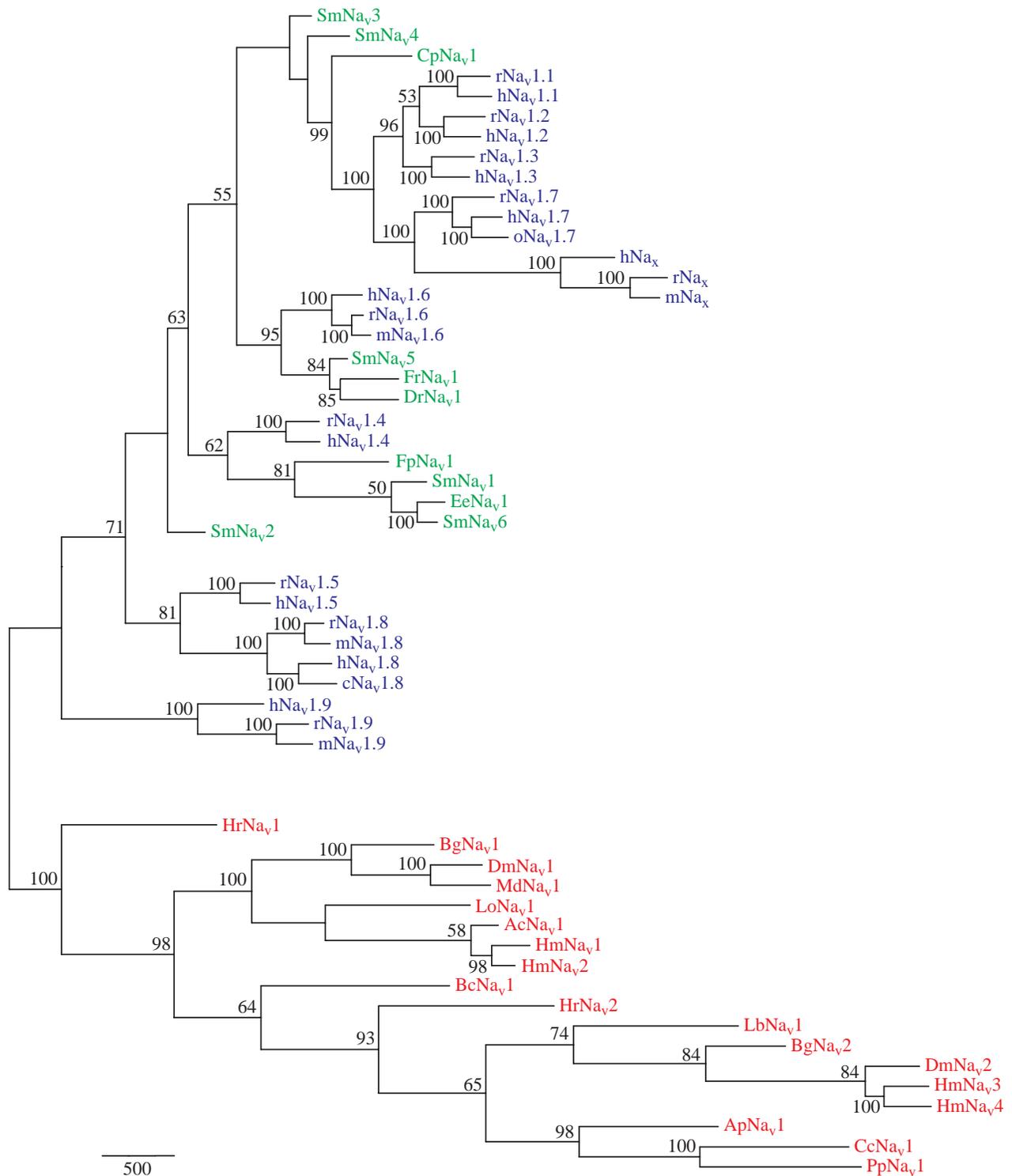


Fig. 2. This proposed phylogenetic tree for the voltage-gated Na<sup>+</sup> channel α subunits was generated using sequences of the channels listed in Tables 1 and 2. The mammalian channels are in blue, the non-mammalian vertebrate channels are in green and the invertebrate channels are in red. This unrooted tree represents the optimal tree based on parsimony analysis of nucleotide sequences. To perform the analysis, the amino acid sequences for all the isoforms were aligned using Clustal W (Thompson et al., 1994). The amino acid sequences in the alignments were then replaced with the published nucleotide sequences, and the nucleotide sequence alignments were subjected to analysis using the program PAUP\* (Swofford, 1998). Divergent portions, including most of the terminal regions and the cytoplasmic loops between domains I and II and domains II and III, were excluded from the PAUP\* analysis. The numbers at the nodes indicate the bootstrap values for 100 replications. When a number is not indicated, the bootstrap value was less than 50. The scale bar represents 500 substitutions. Channels and species are identified in Tables 1 and 2.

motif that is critical for fast inactivation (Fig. 1) (George et al., 1992a; Goldin, 2001). In addition, this is the only mammalian isoform that has not been functionally expressed in an exogenous system. Consistent with these differences, the gene for  $\text{Na}_x$  has diverged more than any of the other channels in this branch of the tree. This divergence suggests that  $\text{Na}_x$  may have evolved a unique functional role. In support of this hypothesis, mice in which the gene encoding  $\text{Na}_x$  has been knocked out demonstrate functional deficits in salt intake behavior, suggesting that this channel has a physiological function other than as a voltage-gated  $\text{Na}^+$  channel (Watanabe et al., 2000).

The other mammalian  $\text{Na}^+$  channel genes that are clustered together are those encoding the  $\text{Na}_v1.5$ ,  $\text{Na}_v1.8$  and  $\text{Na}_v1.9$  isoforms. These genes are clustered on chromosome 9 in humans and the orthologous region of chromosome 3 in mice (Table 1). These isoforms also share functional characteristics, including the fact that they are all considered 'TTX-resistant'  $\text{Na}^+$  channels, requiring micromolar concentrations of TTX before they are blocked. Although the gene for  $\text{Na}_v1.9$  is clustered on the chromosome with the other two genes, this channel appears to represent a distinct branch of the tree, and it may have evolved from a distinct ancestral  $\text{Na}^+$  channel gene (Plummer and Meisler, 1999).

The final two isoforms,  $\text{Na}_v1.4$  and  $\text{Na}_v1.6$ , each represent a separate branch of the tree, and the genes encoding these channels are located on separate chromosomes. The gene for  $\text{Na}_v1.4$  is located on chromosome 11 in humans and chromosome 17 in mice, and the gene for  $\text{Na}_v1.6$  is located on chromosome 15 in humans and chromosome 12 in mice (Table 1). The properties of these two channels are generally similar to those of the channels expressed in the nervous system, including block by nanomolar concentrations of TTX.  $\text{Na}_v1.6$  is the most closely related channel to the branch containing the nervous system  $\text{Na}^+$  channels, and this isoform is also expressed in the nervous system.  $\text{Na}_v1.4$  is the primary channel expressed in adult skeletal muscle, and is unique in this regard.

Plummer et al. (1999) observed that each of the chromosome segments containing the  $\text{Na}^+$  channel genes represents one of the *Hox* gene clusters. They suggested that the initial expansion of the  $\text{Na}^+$  channel genes was associated with the genomic duplications that occurred after the divergence of prevertebrates from invertebrate chordates and resulted in the four mammalian *Hox* gene clusters. Later tandem duplications of the  $\text{Na}^+$  channel genes on chromosomes 2 and 9 in humans (chromosomes 2 and 3 in mice) resulted in the two clusters of  $\text{Na}^+$  channel genes.  $\text{Na}_v1.9$  was more distantly related to  $\text{Na}_v1.5$  and  $\text{Na}_v1.8$  in the analysis of Plummer et al. (1999), and it appears on a separate branch in the current analysis (Fig. 2). Plummer et al. (1999) hypothesized that the ancestral chromosome might have had two  $\text{Na}^+$  channel genes, one that represented the ancestor of  $\text{Na}_v1.9$  and a second that represented the ancestor of the other mammalian  $\text{Na}^+$  channels. This model assumes that the ancestor of  $\text{Na}_v1.9$  was subsequently lost from three of the four chromosome segments.

The analysis of the non-mammalian vertebrate  $\text{Na}^+$  channels that have been characterized thus far is consistent with that of the mammalian channels and provides further information about the timing of the duplication events. The most informative data were obtained by Lopreato et al. (2001), who cloned partial sequences for six separate isoforms from the teleost fish *Sternopygus macrurus*. These sequences probably represent all the  $\text{Na}^+$  channels in that species. As pointed out by Lopreato et al. (2001), these isoforms align well with the branches of the mammalian channels (Fig. 2).  $\text{SmNa}_v3$  and  $\text{SmNa}_v4$  are in the branch with  $\text{Na}_v1.1$ ,  $\text{SmNa}_v5$  aligns with  $\text{Na}_v1.6$ , and  $\text{SmNa}_v1$  and  $\text{SmNa}_v6$  are in the branch with  $\text{Na}_v1.4$ .  $\text{SmNa}_v2$  forms a distinct branch of its own, although it is more closely related to  $\text{Na}_v1.5$  and  $\text{Na}_v1.8$  than to the other  $\text{Na}^+$  channel isoforms.  $\text{SmNa}_v2$  was located on a branch with  $\text{Na}_v1.5$ ,  $\text{Na}_v1.8$  and  $\text{Na}_v1.9$  in the analysis of Lopreato et al. (2001). This branching pattern is not as robust as that for the other vertebrate channels though, because it is based on partial sequence data. Lopreato et al. (2001) suggested that the initial  $\text{Na}^+$  channel duplication into four genes occurred early in vertebrate history close to the emergence of the first vertebrates, so that a common ancestor of mammals and teleost fish already had four distinct genes. This hypothesis is consistent with the conclusions of Plummer et al. (1999). The duplications occurred as an initial event leading to two pairs of *Hox* genes, each of which then duplicated to result in the four clusters that are now present. These initial chromosomal duplications were then followed by tandem duplications, as suggested by Plummer et al. (1999), and these duplications were independent in teleosts and mammals. The relationships between the mammalian and teleost genes shown in Fig. 2 are supported by the corresponding tissue expression patterns of the mammalian and teleost channels encoded within each cluster (Lopreato et al., 2001).

An additional means of generating diversity in  $\text{Na}^+$  channels is through post-transcriptional processing, including alternative splicing and RNA editing. Alternative splicing has been demonstrated for five isoforms present in the mammalian nervous system,  $\text{Na}_v1.1$ ,  $\text{Na}_v1.2$ ,  $\text{Na}_v1.3$ ,  $\text{Na}_v1.6$  and  $\text{Na}_v1.7$  (Ahmed et al., 1990; Sarao et al., 1991; Schaller et al., 1992; Gustafson et al., 1993; Belcher et al., 1995; Plummer et al., 1997, 1998; Dietrich et al., 1998; Oh and Waxman, 1998). The proportion of differentially spliced transcripts depends on various factors, including age of development, the tissue of origin and the presence of modulatory agents such as dibutyryl cyclic AMP (Gustafson et al., 1993; Plummer et al., 1997; Dietrich et al., 1998; Oh and Waxman, 1998). However, electrophysiological differences resulting from alternative splicing have only been demonstrated for two alternatively spliced forms of  $\text{Na}_v1.6$  (Dietrich et al., 1998), so it is not clear how much functional diversity in mammalian  $\text{Na}^+$  channels results from alternative splicing. No alternative splicing has been demonstrated for non-mammalian vertebrate  $\text{Na}^+$  channels, and no RNA editing has been demonstrated for any vertebrate  $\text{Na}^+$  channels. This is in contrast to the situation with the invertebrate  $\text{Na}^+$  channels, which will be discussed below.

### Evolution of invertebrate Na<sup>+</sup> channels

The data concerning the invertebrate Na<sup>+</sup> channels are much less complete than those for mammalian Na<sup>+</sup> channels in that more than one full-length sequence has been determined from only two species, *Blattella germanica* and *Halocynthia roretzi*. A full-length sequence and a collection of sequences comprising most of the coding region have been cloned from *Drosophila melanogaster*, and it is likely that these represent the only Na<sup>+</sup> channel genes in that species (Littleton and Ganetzky, 2000). Blackshaw et al. (1999) have made the most extensive effort to identify all the Na<sup>+</sup> channel genes in an invertebrate species, having isolated four partial clones from *Hirudo medicinalis*. The data for these four species suggest that there are two branches of the Na<sup>+</sup> channel tree for invertebrates (Fig. 2), although HrNa<sub>v</sub>1 is somewhat divergent from the first branch. The existence of two invertebrate Na<sup>+</sup> channel branches was previously suggested by Spafford et al. (1999). The two invertebrate branches are independent of the branching that occurred during vertebrate evolution (Fig. 2). This result is consistent with the conclusion of Plummer et al. (1999) that the vertebrate gene duplications occurred after the divergence of prevertebrates from invertebrate chordates. The four genes from *Hirudo medicinalis* align with two in each of the branches, suggesting that two additional gene duplication events occurred later during the evolution of this species.

It is difficult to evaluate the functional significance of the two branches of invertebrate Na<sup>+</sup> channels. Functional expression has been reported for only three channels, DmNa<sub>v</sub>1 (Feng et al., 1995), MdNa<sub>v</sub>1 (Smith et al., 1997) and BgNa<sub>v</sub>1 (Tan et al., 2001; Liu et al., 2001b), and all these channels are in the same branch of the phylogenetic tree. However, K. Dong and colleagues have preliminary evidence for functional expression of the BgNa<sub>v</sub>2 channel (K. Dong, personal communication). They suggest that the properties of this channel are so unique that it is questionable whether it should be considered a voltage-gated Na<sup>+</sup> channel. If BgNa<sub>v</sub>2 does not represent a true voltage-gated Na<sup>+</sup> channel, it would suggest that there is only one branch of voltage-gated Na<sup>+</sup> channels in invertebrates and that the second branch has diverged to the point that these channels carry out unique functions. If this is the case, then the functional diversity of Na<sup>+</sup> channels in many, if not most, invertebrate species would have to result from post-transcriptional regulatory events, as suggested by Liu et al. (2001a), unless additional Na<sup>+</sup> channel genes remain to be discovered in these species.

The two major post-transcriptional processes that would increase Na<sup>+</sup> channel diversity are alternative splicing and RNA editing. Alternative splicing of Na<sup>+</sup> channels has been shown to occur in two invertebrate species, *Drosophila melanogaster* (Loughney et al., 1989; Thackeray and Ganetzky, 1995) and *Blattella germanica* (Liu et al., 2001a). Spafford et al. (1999) pointed out that 85 % of the intron splice junctions in a jellyfish Na<sup>+</sup> channel (PpNa<sub>v</sub>1) are also found in mammalian Na<sup>+</sup> channels with similar locations, suggesting that the positions of the introns within the coding regions have been retained from a common ancestor. However, no

alternatively spliced variants were detected in PpNa<sub>v</sub>1, suggesting that alternative splicing was not a primordial means of generating diversity in Na<sup>+</sup> channels. RNA editing has been demonstrated for some invertebrate Na<sup>+</sup> channels, including DmNa<sub>v</sub>1 from *Drosophila melanogaster* (Hanrahan et al., 2000; Reenan, 2001). Hanrahan et al. (2000) observed that two of the three characterized RNA editing sites in *Drosophila melanogaster* are also conserved in *Drosophila virilis*, suggesting that this process has been maintained throughout the 61–65 million years of divergence between these two species.

Considering the fact that invertebrate species appear to have only 2–4 Na<sup>+</sup> channel genes, in contrast to the nine mammalian genes, it is likely that invertebrates and vertebrates use fundamentally different means of generating diversity in Na<sup>+</sup> channel function. The major means of generating diversity in invertebrate Na<sup>+</sup> channels may involve alternative splicing and RNA editing, whereas vertebrate Na<sup>+</sup> channel diversity may result primarily from the presence of multiple genes.

### Concluding remarks

Voltage-gated Na<sup>+</sup> channels are encoded by a family of genes that have been highly conserved throughout evolution, which undoubtedly reflects the critical functional role of these proteins in regulating electrical excitability. Because the majority of Na<sup>+</sup> channels have been cloned from mammalian species, there is most information about the diversity of mammalian Na<sup>+</sup> channels. The recent identification of additional Na<sup>+</sup> channels in non-mammalian vertebrates suggests that many of the duplication events leading to the different isoforms occurred early in vertebrate evolution. There is much less definitive information about the invertebrate Na<sup>+</sup> channels. Although a significant number of genes have been identified and sequenced, the limited amount of functional information makes it difficult to determine whether those genes encode true voltage-gated Na<sup>+</sup> channels. Future efforts to express the invertebrate channels should help to more clearly define the roles of Na<sup>+</sup> channel genes in these species.

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