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REVIEW

Molecular evolution of Na^+ channels in teleost fishes

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Abstract

Voltage-dependent sodium channels are critical for electrical excitability. Invertebrates possess a single sodium channel gene; two rounds of genome duplication early in vertebrates increased the number to four. Since the teleost-tetrapod split, independent gene duplications in each lineage have further increased the number of sodium channel genes to 10 in tetrapods and 8 in teleosts. Here we review how the occurrence of multiple sodium channel paralogs has influenced the evolutionary history of three groups of fishes: pufferfish, gymnotiform and mormyriform electric fish. Pufferfish (tetraodontidae) produce a neurotoxin, tetrodotoxin, that binds to and blocks the pore of sodium channels. Pufferfish evolved resistance to their own toxins by amino acid substitutions in the pore of their sodium channels. These substitutions had to occur in parallel across multiple paralogs for organismal resistance to evolve. Gymnotiform and mormyriform fishes independently evolved electric organs to generate electricity for communication and object localization. Two sodium channel genes are expressed in muscle in most fishes. In both groups of weakly electric fishes, one gene lost its expression in muscle and became compartmentalized in the evolutionary novel electric organ, which is a muscle derivative. This gene then evolved at elevated rates, whereas the gene that is still expressed in muscle does not show elevated rates of evolution. In the electric organ-expressing gene, amino acid substitutions occur in parts of the channel involved in determining how long the channel will be open or closed. The enhanced rate of sequence evolution of this gene likely underlies the species-level variations in the electric signal.

Keywords: electric fish, molecular evolution, pufferfish, sodium channels, tetrodotoxin.

INTRODUCTION

In 1837, Charles Darwin sketched in his notes his concept of a phylogenetic tree, with branches dividing into smaller branches, and these splitting into twigs. Some branches were long, others ended suddenly. This simple drawing summarized key aspects of his thinking about evolution: the common origin of life and the radiation, adaptation and extinction of species. Slightly less than 150 years later, Susumu Ohno suggested in a pithy book entitled *Evolution by Gene Duplication* (1970) that genes analogously speciate (gene duplication), adapt (neofunctionalization), or go extinct (pseudogenization, or loss from the genome). Ohno also suggested that an important determinant in the evolution of new species and the dynamics of gene evolution is the duplication of the genome itself; that is, polyploidy. With the advent of rapid and powerful sequencing techniques and new al-

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gorithms for sequence analysis, we can now discern in detail the processes about which Ohno could only speculate.

Here we review the evolutionary history of the voltage-dependent Na^+ channel (Nav) gene family, and provide two examples of how the evolutionary history of this gene family in teleosts had profound implications for the evolution of specific organismal features of three groups of fishes: the tetraodontidae, which have evolved the use of a Na^+ channel-blocking neurotoxin for defense, and the gymnotiforms and mormyriforms, which have independently evolved electric organs for communication and object location functions, and whose electric discharges are shaped by Na^+ channels.

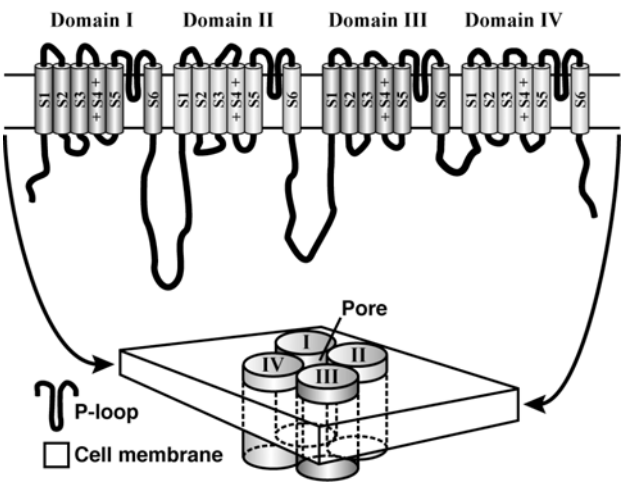

Figure 1 Schematic illustration of a sodium channel. Sodium channels are composed of four domains (I–IV). Each domain is composed of six membrane spanning segments (S1–S6). The four domains contact each other and form a channel through the membrane (bottom part of figure). The S5 and S6 segments of the four domains line the walls of the channel, and the p-loops, which are extracellular loops that dip back into the membrane, form the outer mouth of the channel. Specific amino acids in the p-loops of each domain determine the channel's ion selectivity (i.e. preference for Na^+ ions). The S4 segment is positively charged and is the channel's voltage sensor. Activation of the S4 by membrane voltage causes conformational changes in the S5 and S6, which opens the channel and, with a slight delay, the small loop between domain III and IV to occlude the inner mouth of the channel inactivating it (i.e. stopping the flow of sodium ions through it). Fig. 1 is modified from Jost *et al.* (2008).

Voltage-dependent Na^+ channels are critical for electrical excitability of the nervous system, muscle, heart and neuroendocrine tissues. Like other members of the large voltage-dependent ion channel superfamily, Na^+ channels have a highly conserved structure: six membrane spanning helices labeled S1-S6 (Fig. 1) (Noda *et al.* 1984). This basic structure is repeated four times in Ca^{2+} and Na^+ channels so that they are composed of four repeating domains (DI–IV) (Stuhmer *et al.* 1989). The four domains face each other (Fig. 1, bottom), forming a pore. The S4s with their repeating positively charged amino acids are the channel's voltage sensors; these initiate the conformational changes that cause the channel to open (i.e. activation) upon a depolarizing stimulus (Chanda & Bezanilla 2002). The S5 and S6 segments form the lining of the pore through the membrane. It is believed that movements of S5 and S6 initiate the open or closed conformations of the channel (Zhao *et al.* 2004). The extracellular loops between S5 and S6 (the p-loop) from each of the four domains dip down into the membrane and, facing each other, form the mouth of the pore. The p-loops are responsible for ion selectivity of the channel, its selectivity to Na^+ over other ions (Lipkind & Fozzard 2000). A characteristic feature of Na^+ channels is that they inactivate. That is, after being opened by depolarizing voltages, they then undergo a second structural change that causes the channel to cease conducting. Technically, inactivation is distinct from closing as a channel might still be in the open conformation (determined by S5 and S6) but be inactivated. Inactivation occurs when the small cytoplasmic loop between DIII and DIV twists and occludes the inner mouth of the channel pore (Kellenberger *et al.* 1977, 1997).

THE EVOLUTIONARY TRAJECTORY OF THE VOLTAGE-DEPENDENT NA^+ CHANNEL GENE FAMILY DIFFERS IN TELEOSTS AND TETRAPODS

In a prescient prediction, Ohno suggested in 1970 that the vertebrate genome evolved via two rounds of genome duplications (the so-called 2R hypothesis) from the genome of a chordate ancestor. He then suggested that the teleost genome had undergone an additional duplication. In other words, from the viewpoint of a tunicate, the tetrapod genome is octaploid and the teleost genome is hexadecaploid (sixteenploid)!! Extensive phylogenetic analyses of a number of genes and the identification of

homologous segments of chromosomes within and across species by the order of genes along the chromosomes (i.e. synteny) (Amores *et al.* 1998; Jaillon *et al.* 2004; Christoffels *et al.* 2004; Crow *et al.* 2006) have largely supported Ohno's hypotheses, although this view has been controversial (Gu *et al.* 2002; Hughes & Friedman 2003). While additional losses or gains might obscure this pattern for particular gene families, this scenario is strongly supported by the evolution of the Na^+ channel gene family (Lopreato *et al.* 2001; Novak *et al.* 2006: but see Piontkivska & Hughes 2003).

Tunicates possess a single Na^+ channel gene. Phylogenetic reconstructions of the vertebrate Na^+ channel gene family are consistent with two rounds of genome duplication in that the ancestor of teleosts and tetrapods had four genes (Lopreato *et al.* 2001; Novak *et al.* 2006). At this point, the trajectories of the teleost and tetrapod families diverged. Mammals have 10 Na^+ channel genes distributed on four chromosomes; two chromosome have a single Na^+ channel gene each, one chromosome has three Na^+ channel genes, and a fourth chromosome has five Na^+ channel genes. Therefore, tandem duplications of a founder gene occurred on two of the four chromosomes. The majority of these tandem duplications occurred before the origin of amniotes (approximately 350 Ma) as lizards, chickens and mammals have orthologous Na^+ channel genes clustered in a similar pattern on their chromosomes (Jost *et al.*, unpublished manuscript). Teleosts, in contrast, have eight Na^+ channel genes. A phylogeny of these genes indicates that they occur in pairs. Furthermore, each is on a different chromosome, and the relationship of the Na^+ channel genes and their surrounding genes on these chromosomes indicates synteny (Novak *et al.* 2006). This pattern is consistent with a single genome duplication at the origin of teleosts (also approximately 350 Ma) with no further losses or duplications.

EVOLUTION OF TETRODOTOXIN RESISTANCE IN PUFFERFISH

Pufferfish (family tetraodontidae) comprise approximately 120 species, many of which produce the potent neurotoxin tetrodotoxin (TTX) (most famously *Fugu*, the Japanese delicacy). Or, more appropriately, they harbor bacteria that produce TTX (Lee *et al.* 2000). TTX is a predator deterrent (Gladstone 1987; Caley & Schluter 2003; Frisch 2006) as it lodges in and blocks the pores of most Na^+ channels, presumably leading to numbness in the mouth of predators that bite, and partial or total paralysis of predators that ingest pufferfish. TTX has become such an important part of the biology of pufferfish that it has evolved to serve as a female pheromone in some species (which implies a TTX-specific chemoreceptor) (Matsumura 1995).

Tetrodotoxin is not sequestered within a gland but is accumulated in various body tissues (Mahmud *et al.* 2003). Therefore, a pufferfish's own neurons, heart and muscles are exposed and must be resistant to TTX (Kidokoro *et al.* 1974). Substitutions of key amino acids in the pores of two pufferfish Na^+ channels (Nav1.4a, Nav1.4b) were found to confer TTX resistance (Yotsu-Yamashita *et al.* 2000; Venkatesh *et al.* 2005). However, our finding that teleosts have eight Na^+ channel genes (Lopreato *et al.* 2001; Novak *et al.* 2006), raised the question of how resistance evolved across an entire gene family. In other words, for a fish to utilize TTX it must have evolved TTX-resistance in all of its eight Na^+ channel genes or have evolved resistance in one or a few of those genes accompanied by changes in the expression of those genes so that the TTX-resistant genes become widely expressed.

We studied this by obtaining sequences for Na^+ channel genes from four species of pufferfish either from genome databases (*Takifugu rubripes* Temminnck & Schlegel, 1850 and *Tetraodon nigroviridis* Marion de Procé, 1822) or by cloning (*Arothron nigropunctatus* Bloch & Schneider, 1801 and *Canthigaster solandri* Richardson, 1845) (Jost *et al.* 2008). We recovered a full complement of eight Na^+ channel genes from *Takifugu*, seven from *Tetraodon*, and five or six in the other two species. At this point, we cannot determine whether there has been some loss of genes or, rather, an inability to locate all eight genes by the polymerase chain reaction (PCR). Additionally, pufferfish are noted for having small genomes (Neafsey & Palumbi 2003) (the reason their genomes were chosen for sequencing) so even if some pufferfish have a reduced number of Na^+ channel genes, it is hard to say whether this is a result of the loss of the gene due to disuse of the channel (i.e. the gene was lost because it was not TTX resistant) or the pressure of genome "streamlining."

Importantly, however, we noted a number of amino acid substitutions at sites in the pore of the Na^+ channel that are otherwise conserved in Na^+ channels of other vertebrates (Fig. 2). Some of these had been identified as affecting TTX binding in biophysical studies on toxin-channel interactions (Terlau *et al.* 1991; Penzotti *et al.* 2001; Choudhary *et al.* 2003; Venkatesh *et al.* 2005;

Maruta *et al.* 2008). Every Na^+ channel that we studied had one or more amino acid substitution in sites critical to TTX binding (Jost *et al.* 2008).

We wondered if the evolution of TTX resistance occurred in an ancestral tetraodontid and was inherited in all species in this radiation, or whether evolution of TTX resistance might have occurred independently in various radiations of pufferfish. We observed that approximately half of the amino acid substitutions were present in all four species (which were chosen to provide broad coverage of the family tetraodontidae) (Holcroft 2005), implying an ancestral origin of these substitutions, with others occurring in only some lineages. Therefore, it seems likely that some degree of TTX resistance was already established in an ancestral tetraodontid and that further refinements of the resistance occurred during their radiation. Tetraodontid fish are one family in the order tetraodontiformes. Other families, such as the diodontidae, include fish that have a moderate insensitivity to TTX (Kidokoro *et al.* 1974). We predict that some of the amino acid substitutions that are common to the tetraodontid fish are also found in these closely related families.

EVOLUTION OF NA^+ CHANNEL GENES IN ELECTRIC FISH

Electric fish generate electrical discharges from a muscle-derived electric organ (EO) in the tail. Electric organs have evolved at least half a dozen times in elasmobranch and teleost fishes. In some groups, the EO is discharged infrequently and only to immobilize prey or deter predators (i.e. electric rays, Torpenidae). The most well-developed electric organs are found in those fish that use electric organs and electrosensory systems for communication and the detection and identification of nearby objects. This very sophisticated use of an EO has evolved at least twice in teleosts: the Gymnotiformes of South America and the Mormyriformes of Africa (Bullock *et al.* 2005). Electric organ discharge (EOD) waveforms are species-specific, often sexually dimorphic and/or individually distinct (Stoddard *et al.* 2006). Weakly electric fish are preyed on by electroreceptive predators (electric eels in South America [electric eels are gymnotiforms] and electroreceptor-bearing catfish on both continents) and electric signals have evolved to

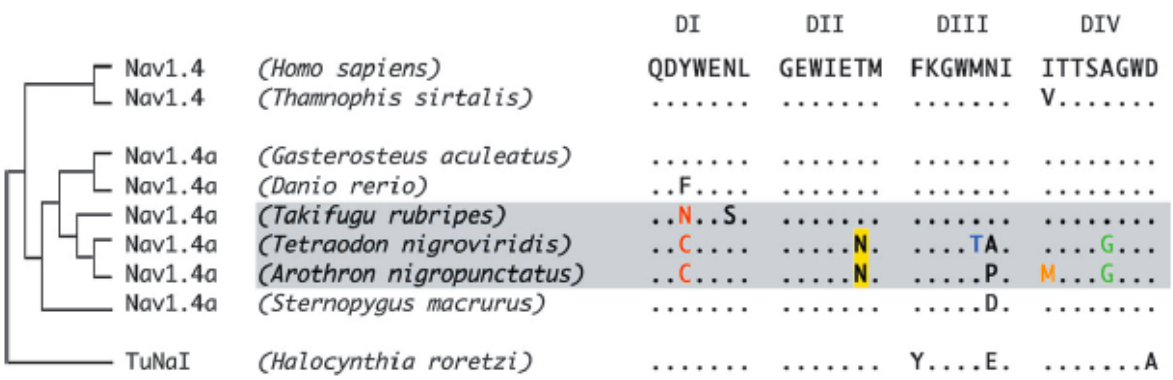

Figure 2 The ion-conducting pore of a Na^+ channel (Nav1.4a) in tetrodotoxin (TTX)-bearing tetraodontid fish shows some amino acid substitutions linked with TTX resistance. Sequences from the I–IV domains of the Na^+ channel pore (right) from a number of species (left). From top to bottom these are the human (*Homo sapiens*) and garter snake (*Thamnophis sirtalis* Linneus, 1758) muscle-expressing Na^+ channel orthologs (Nav1.4), Nav1.4a in non-toxic stickleback (*Gasterosteus aculeatus* L., 1758), zebrafish (*Danio rerio* Hamilton, 1822), the tetraodontids (grey bar) the Fugu pufferfish (*Takifugu rubripes*), green-spotted pufferfish (*Tetraodon nigroviridis*), Black-spotted puffer (*Arothron nigropunctatus*), the weakly electric gold-lined black knifefish (*Sternopygus macrurus* Bloch & Schneider, 1801), and the single Na^+ channel (TuNaI) of the tunicate (*Halocynthia roretzi* von Drasche, 1884), which serves as an outgroup for comparison. The human sequence is used as a reference; amino acids in other sequences that are identical to those in the human channel are indicated by dots. Note the high degree of conservation in the pore region between vertebrates and tunicates (>500 Myr). Fig. 2 is from Jost *et al.* (2008).

minimize detection by these predators (Hanika & Kramer 1999; Stoddard 1999). Therefore, EOD waveforms have been shaped for electrolocation, communication and electrical camouflage.

Two distinct EOD patterns, pulse-type and wave-type, are independently evolved in both groups. Pulse-type fish emit EOD pulses, often with a complex multiphasic structure, at irregular intervals. They discharge at high rates when active, and low rates when resting. Wave-type species discharge in a specific frequency band, and within each species, males, females and juveniles may each discharge within a specific portion of the species bandwidth; each fish may further have its own "personal" frequency within its sex's or age group's band. Wave-type EOD waveforms are usually monophasic pulses whose durations roughly equal the interpulse duration, thereby forming a sine wave-like pattern. Wave-type fish discharge constantly at the same frequency, whether active or resting. A wave-type fish is essentially a pulse-type fish with a very regular discharge.

Electric fish have a diversity of EOD waveforms. Some species produce long duration pulses (20 ms), whereas others generate extremely brief discharges (approximately 200 microseconds) (Fig. 3). Indeed, ultra-brief discharges have evolved in both groups of electric fish (Bennnet 1971). Some wave-type gymnotiforms discharge at low frequencies (50 Hz) and others at high frequencies (>1kHz). Sampling at high frequencies presumably gives fish better temporal resolution of their environment. Generating extremely brief pulses or operating at such high frequencies is difficult given the normal properties of Na^+ channels. Therefore, we can envision that there have been strong selection pressures for amino acid substitutions influencing rates of channel opening, closing, inactivation or recovery from inactivation.

The EOD pulse is mainly shaped by Na^+ currents (Shenkel & Sigworth 1991; Ferrari *et al.* 1995) so electric fish are good model animals to examine whether evolutionary changes have occurred in Na^+ channel genes. Furthermore, because electrogenesis has evolved independently in the unrelated gymnotiforms and mormyriforms we can also examine whether parallel evolution has occurred on the molecular level. Because electric organs derive from muscle, we focused on the teleostean orthologs of the mammalian muscle Na^+ channel gene, Nav1.4; namely, Nav1.4a and Nav1.4b.

A PCR analysis showed that Nav1.4a and Nav1.4b are expressed in the muscles of non-electric fishes

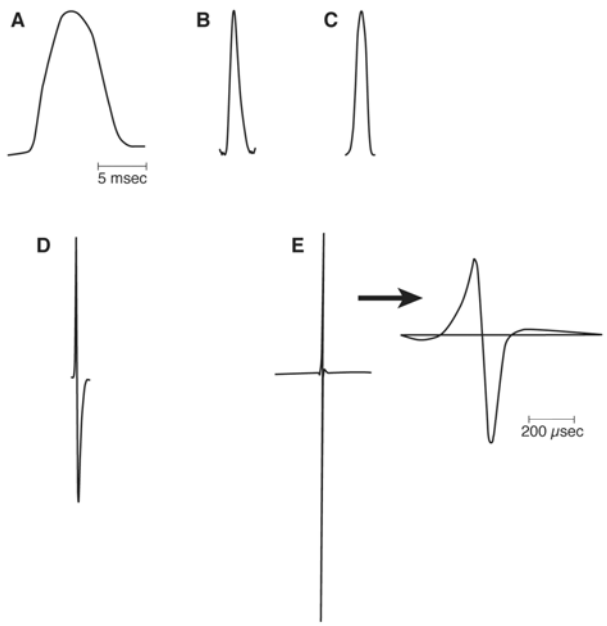

Figure 3 Electric organ discharge (EOD) pulses from the five species of electric fish used in the molecular analysis in Fig. 4. (A) A single pulse of the wave-like EOD of *Sternopygus macrurus*, the gold-lined black knifefish; (B) a single pulse of the wave-like EOD of *Apteronotus leptorhynchus*, the brown ghost; (C) *Electrophorus electricus*, the electric eel; (D) *Brachyhypopomus pinnicaudatus*, Hopkins, 1991 pintail knifefish; (E) *Gnathonemus petersii Günther, 1862*, elephant nose mormyrid. *S. macrurus* and *A. leptorhynchus* are wave-type fish, whereas the other three species are pulse fish. All are gymnotiforms, except for *G. petersii*. All pulses are on the same time scale; the EOD pulse of *G. petersii* is expanded to the right (indicated by the arrow). Note the different time scale. Fig. 3 is modified from Zakon *et al.* (2006).

(Venkatesh *et al.* 2005; Novak *et al.* 2006; Zakon *et al.* 2006). We do not know whether both genes are expressed in every muscle fiber or whether each gene is separately expressed in subsets of muscle fibers, such as slow versus fast muscle. This is currently being tested by *in situ* hybridization. In electric fishes, however, Nav1.4b is still expressed in muscle and might also be expressed in the electric organ in some species. However, Nav1.4a expression has been lost from muscle and gained in the evolutionarily novel electric organ in both groups of electric fish (Zakon *et al.* 2006; Arnegard *et al.*, 2008

unpublished data).

An exception to this pattern is the weakly electric gymnotiform the brown ghost, *Apteronotus leptorhynchus* Ellis, 1912. The large radiation of apteronotid species has a muscle-derived electric organ that is retained only for the first few weeks of larval life. During the first weeks of life, the EOD frequency of *A. leptorhynchus* is a few hundred Hz (Kirschbaum 1977). As they mature, their EOD frequencies increase to 750–1000 Hz. In some apteronotid species, adult EOD frequencies may be as high as 1600 Hz (Kramer *et al.* 1980). During this time, the myogenic larval electric organ degenerates and the axons of the motor neurons that innervate it become the new electric organ (Pappas *et al.* 1975). The speed and synchronization needed to operate at such high frequencies is efficiently maintained by electrotonic coupling from the brain down to the motor neuron with the single chemical synapse (neuro-electrocyte junction) in the pathway eliminated. Because the EO of mature apteronotids is not derived from muscle we believe that neither Nav1.4a nor Nav1.4b, but some other neurally-expressed Na^+ channel gene(s), is responsible for generating the rapid EOD frequencies of apteronotids. PCR or *in situ* hybridization analyses of the motor neurons will identify the Na^+ channels in the adult EO.

Na^+ channel genes are under strong negative selection, as evidenced by the large number of diseases attributed to mutations in Na^+ channel genes (Wei *et al.* 1999; Bendahhou *et al.* 2002; Splawski *et al.* 2002; Tan *et al.* 2003; Berkovic *et al.* 2004; Tian *et al.* 2004; Wang *et al.* 2004). However, Nav1.4a in both lineages of electric fish should be freed from many selective constraints associated with muscle expression because electrocytes are noncontractile and mutations in Nav1.4a would not affect a fish's motility. Furthermore, Nav1.4a is evolving under a different set of selection pressures than when it was expressed in muscle: optimization of the EOD in different sensory environments or for the generation of

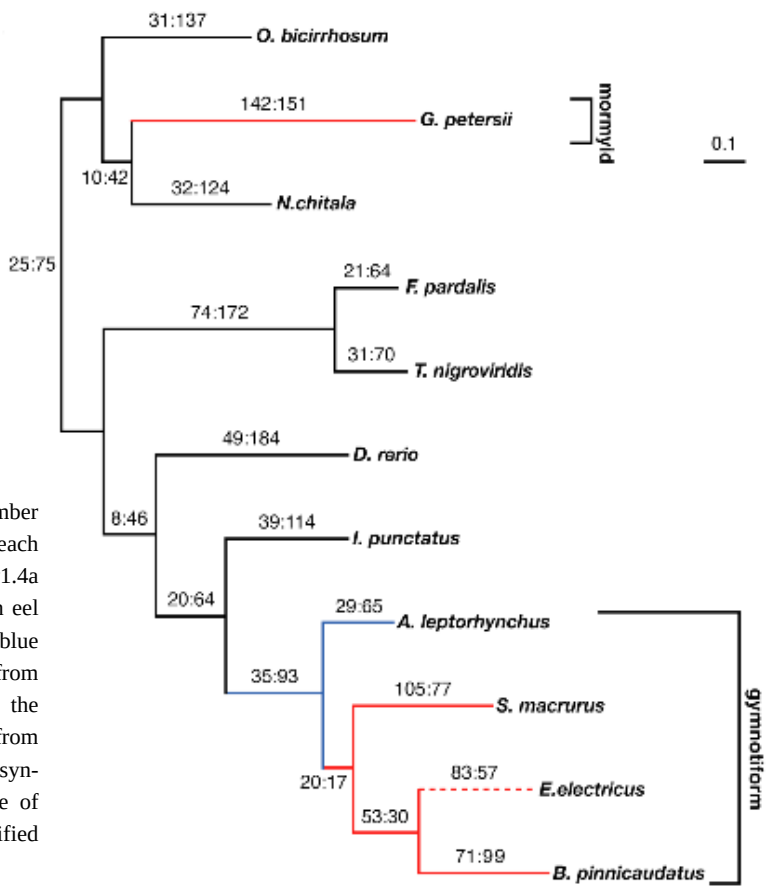

Figure 4 Gene tree of Nav1.4a with the estimated number of non-synonymous/synonymous substitutions for each branch. Red branches indicate lineages in which Nav1.4a is known to be lost from muscle (the dotted line in eel indicates that its loss is likely but not yet tested), and blue branch indicates that Nav1.4a expression is not lost from muscle. Note the much longer branch lengths of the electric fish lineages from which Nav1.4a was lost from muscle and the high ratio of non-synonymous to synonymous substitutions in these branches indicative of elevated rates of evolutionary change. Fig. 4 is modified from Zakon *et al.* (2006).

species -specific communication signals. The release from negative selection in muscle and putative increase in positive selection in the EO should be evident by an increase in the ratio of non-synonymous (nucleotide substitutions that change the amino acid) over synonymous (or "silent" substitutions: nucleotide substitutions that do not change the amino acid) substitutions per codon in the Nav1.4a genes of electric fish. Specifically, we proposed that: (i) Nav1.4a has evolved at a higher rate in electric than non-electric fishes: (ii) changes in the rate of evolution of Nav1.4a in electric fish occur following or concurrent with its loss of expression from muscle and gain of expression in the EO; and (iii) amino acid changes in the channel will be evident in regions of the channel involved in voltage-dependent gating and, specifically, inactivation because the distinguishing feature of EOD is that they may vary in duration.

We obtained sequences for Nav1.4a from six non-electric, one mormyriform, and four gymnotiform species, constructed a phylogenetic tree and estimated the numbers of nonsynonymous versus synonymous changes per codon in each lineage (Zakon *et al.* 2006) (Fig. 4). We found that Nav1.4a of all electric fish except for *Apteronotus* show elevated ratios of nonysnonymous/synonymous substitutions compared to Nav1.4a of non-electric teleosts. This is consistent with the idea that loss of Nav1.4a expression from muscle was permissive for elevated rates of amino acid substitutions. We have since confirmed these results with a larger dataset from both electric lineages (Arnegard *et al.*, 2008 unpublished data). To control for the possibility that the elevation in non-synonomous/synonymous ratio in Nav1.4a is the result of an increase in this ratio in all genes of these species, we performed the same analysis on Nav1.4b (which is still expressed in the muscle in both groups of weakly electric fish) and find that there is no difference in the rates of evolution in this gene between electric and non-electric fish (Arnegard *et al.*, unpublished data). Likelihood-based analyses (Phylogenetic analysis by maximum likelihood, PAML) support the contention that these changes are the result of positive selection.

Electric organ discharge pulses of various species often vary in duration, and this could be related to the rate of inactivation of the Na^+ channel. We examined the amino acid sequences in our dataset of the key parts of the Na^+ channel involved in the final step of inactivation and found a number of amino acid substitutions at sites critical for inactivation (Fig. 5). The final step in inactivation occurs when the highly conserved intracellular loop connecting domains III and IV swings into position in the cytoplasmic side of the channel, binds to the S4-S5 linkers from domains II–IV, and occludes the conduction of Na^+ ions through the channel (Kellenberger *et al.* 1977, 1997; Popa *et al.* 2004). Despite the conservation of amino acids in these regions across approximately 500 Myr of evolution (tunicates–vertebrates), we noted changes in key amino acids in these sites in both groups of weakly electric fish. In the mormyrid, we noted amino acid substitutions at two key residues in the domain III–IV loop "inactivation ball" (Fig. 5D), whereas in the gymnotiforms we noted substitutions in the domain III S4–S5 linker (Fig. 5C), which is one of the binding partners of the inactivation ball. We also noted amino acid substitutions in the S4–S5 linker in domain II (Fig. 5B) in both groups. In agreement with our suggestion that amino acid mutations that occur in these regions will be selected against because they might cause pathology, we indicate mutations that have been noted in the human clinical literature and are associated with muscular, cardiac or neurological disease (Wei *et al.* 1999; Bendahhou *et al.* 2002; Splawski *et al.* 2002; Tan *et al.* 2003; Berkovic *et al.* 2004; Tian *et al.* 2004; Wang *et al.* 2004). These mutations flank (Fig. 5B,C,E) or occur at the same (Fig. 5C) amino acids at which we witness evolutionary changes in electric fish channels.

We conclude that the diversity of EOD waveforms in both groups of electric fish is correlated with accelerations in the rate of evolution of the Nav1.4a Na^+ channel gene (Li *et al.*, 2005). The placement of some of these amino acid substitutions in key regions involved in inactivation further suggests that these substitutions will affect the rates of Na^+ current inactivation (Zakon *et al.* 2006). We are currently testing the effect of these substitutions with site-directed mutagenesis.

CONCLUSIONS

Gene and genome duplications provide a substrate for evolution. We have demonstrated how these processes have contributed to the evolution of the Na^+ channel gene family in vertebrates. We have shown how duplications of Na^+ channel genes have enhanced or constrained organismal evolution in three groups of fishes.

In the case of pufferfish (tetraodontidae), the presence of eight Na^+ channel paralogs has likely complicated the evolution of tetrodotoxin resistance. Different species of insects that have a single Na^+ channel gene have independently evolved resistance to Na^+ channel toxins, such as pyrethroid insecticides in the 50 years that they have been exposed to them (Dong 2007). The evolution of

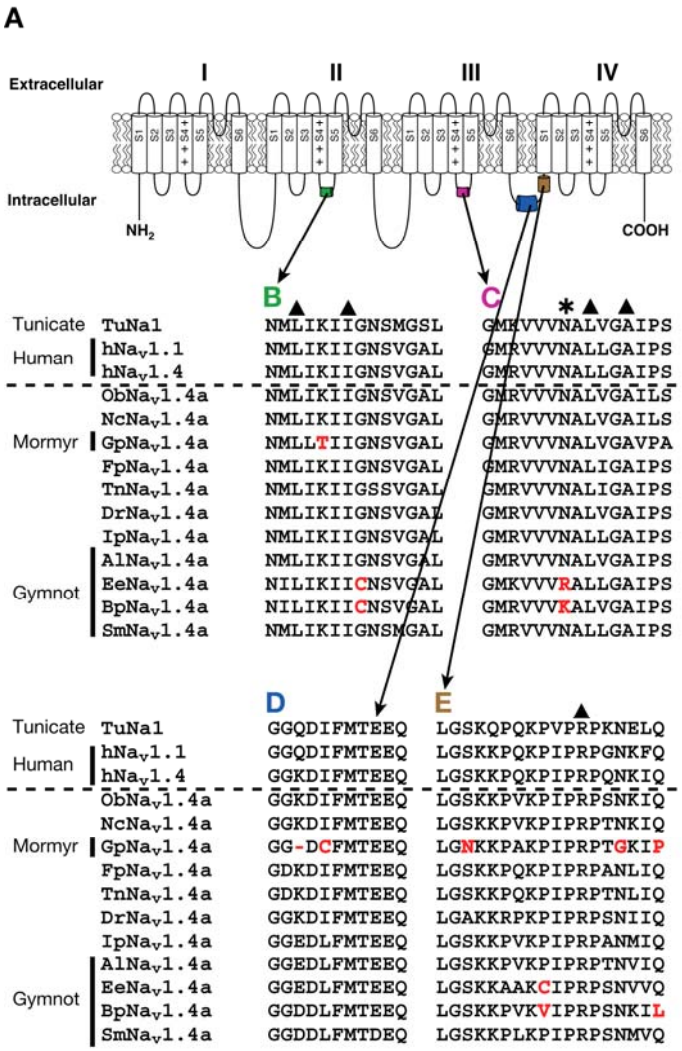

Figure 5 Non-conserved amino acid substitutions occur in a number or regions of the Na+ channel involved in inactivation of the Na^+ current. (A) Schematic illustration of the Na^+ channel; (B) S4-S5 linker in domain II; (C) S4-S5 linker in domain III; (D) and (E) different parts of the inactivation "ball" or hinged lid in the loop between domains III and IV. Amino acid sequences below the dotted line were used in the (phylogenetic analysis by Maximum likelihood, PAML) analysis. Those above were not used in the analysis but are shown for reference. Red letters are non-conservative amino acid substitutions. Triangles represent amino acid sites at which mutations cause diseases in human Na^+ channels. The asterisk represents a site at which there are amino acid changes in gymnotiform fish and at which a mutation in humans is related to a disease. Fig. 5 is modified from Zakon *et al.* (2006).

TTX resistance in a single Na^+ channel would not have conferred complete TTX resistance in pufferfish; the Na^+ channels encoded by seven other genes would be vulnerable. Therefore, solely based on the number of genes involved, it must have been more difficult for pufferfish to evolve resistance to TTX than insects to pyrethroids. However, TTX resistance has also evolved in newts (salamandridae) that, like pufferfish, produce TTX, as well as in some populations of garter snakes that prey on newts. In both groups, there is evidence that their resistance is due to amino acid substitutions in Na^+ channels (Kaneko *et al.* 1997; Geffeney *et al.* 2005). Therefore, while evolutionary change across a gene family must be more difficult than with a single gene, it has likely happened multiple times in the case of TTX resistance.

The story is quite different for the gymnotiform and mormyriform fishes. In this case, if a single Na^+ channel were shared between the muscle and the EO, the strong negative selection on the Na^+ channel in muscle would have severely constrained its evolution in the EO. The duplication of a gene that is expressed in muscle in other vertebrates (Nav1.4) allowed one gene to remain in muscle, whereas the second became selectively expressed in the novel electric organ. This is a classic case of evolutionary changes in expression leading to subsequent sequence evolution and neofunctionalization (Li *et al.* 2005).

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