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Review

Molecular evolution of communication signals in electric fish

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Summary

Animal communication systems are subject to natural selection so the imprint of selection must reside in the genome of each species. Electric fish generate electric organ discharges (EODs) from a muscle-derived electric organ (EO) and use these fields for electrolocation and communication. Weakly electric teleosts have evolved at least twice (mormyriforms, gymnotiforms) allowing a comparison of the workings of evolution in two independently evolved sensory/motor systems. We focused on the genes for two Na^+ channels, *Nav1.4a* and *Nav1.4b*, which are orthologs of the mammalian muscle-expressed Na^+ channel gene *Nav1.4*. Both genes are expressed in muscle in non-electric fish. *Nav1.4b* is expressed in muscle in electric fish, but *Nav1.4a* expression has been lost from muscle and gained in the evolutionarily novel EO in both groups. We hypothesized that *Nav1.4a* might be evolving to optimize the EOD for different sensory environments and the generation of species-specific communication signals. We obtained the sequence for *Nav1.4a* from non-electric, mormyriform and gymnotiform species, estimated a phylogenetic tree, and determined rates of evolution. We observed elevated rates of evolution in this gene in both groups coincident with the loss of Nav1.4a from muscle and its compartmentalization in EO. We found amino acid substitutions at sites known to be critical for channel inactivation; analyses suggest that these changes are likely to be the result of positive selection. We suggest 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 due to changes in selection pressure on the gene once it was solely expressed in the EO.

Key words: sodium channels, molecular evolution, communication, electric fish, electric organ, fish.

Introduction

Neuroethology abounds with examples of animals' sensory systems shaped by natural selection for optimal encoding of sensory information from their environments or communication signals from conspecifics. Visual systems may be optimized for night vision, specific colors or color contrast, or detection of movement (Land, 1993; Warrant et al., 2004; Kern et al., 2005). Auditory systems may be specialized for high frequencies, low frequencies, specific frequency bands, or specific combinations of harmonics or temporal patterns (Kössel and Russell, 1995; Feng et al., 2006). Olfactory systems are similarly specialized (Rouquier et al., 2000; Niimura and Nei, 2003; Nozawa and Nei, 2007).

However, the functioning and evolution of sensory systems is also intimately tied to the functioning and evolution of motor systems in two ways. First, when acquiring information from the world around them many animals are moving through their environments. An animal's motor systems must be tuned so that the rate of information flow generated by its movement through the world matches or at least does not exceed the information-encoding rate of its sensors (Snyder et al., 2007). For example, dusk- or night-active insects, whose retinas integrate slowly, fly more slowly than day-active insects (Warrant et al., 2004). Second, animals must detect conspecific communication signals. Thus, each species' sensory systems have evolved to detect the signal generated by their motor systems (Gilbert and Strausfeld, 1991; Land, 1993). In situations where rapid speciation is occurring, there must be rapid co-evolution of the sensory and motor systems for species-specific signals (Mendelson and Shaw, 2005; Arnegard et al., 2006). The most precise matching of motor output and sensory tuning occurs in animals such as dolphins and bats that navigate in the ultrasonic range, and electric fish that emit and detect their own electric fields.

All of these examples raise the question as to how on a molecular level these sensory systems evolved their species-specific properties and how these co-evolved with each species' motor systems. Most sensory/motor systems are under complex multigenic control precluding the easy identification of candidate genes (Hoy et al., 1977). Our deepest understanding of this process comes from cases of molecules specifically and obviously expressed in sensory receptors such as opsins (Terai et al., 2002) or olfactory receptors (Rouquier et al., 2000; Niimura and Nei, 2003; Nozawa and Nei, 2007). Electric fish have a number of attributes that make them good model systems for studying these questions.

Electric fish as models for molecular evolution

Electric organs (EOs) have evolved at least six times in teleost and elasmobranch fish. The EOs of some groups generate strong discharges to stun prey or predators (torpenid rays, uranoscopid teleosts, malapterurid catfish). The EOs of others are seldom discharged and their function is poorly understood (Rajidae, synodontid catfish). However, the use of EOs and electrosensory systems for sophisticated communication (electrocommunication) and for the detection and identification of nearby objects (electrolocation) has evolved independently in teleosts at least twice – the gymnotiforms of South America and the mormyriforms of

Africa. This allows us to compare the workings of evolution in two independently evolved sensory/motor systems (Bullock et al., 2005). Their sensory receptors, EOs, and central sensory processing and motor control areas are remarkably similar in a striking case of parallel evolution. Electric fish are, therefore, good model organisms for investigating whether parallel evolution has also occurred on the molecular level.

Electric fish lend themselves to a molecular evolutionary analysis in that electric signals are already in the currency of the nervous system: electricity. Because of this, electric signals are easily understood and analyzed in terms of the biophysics of ion currents and associated with a manageable number of candidate genes (mainly encoding Na^+ and K^+ channels) whose expression can be localized to sensory and motor structures.

Electric fish are excellent animals for studying the coordinated evolution of sensory and motor processes. Electric fish generate EO discharges (EODs) from a muscle-derived EO and sense these fields with special sensory receptors called electroreceptors, presumably derived from the same embryonic source as lateral line hair cells (Bullock et al., 2005). Electric fish detect nearby objects by sensing how those objects perturb their own EODs in a process called electrolocation. EOD waveforms are species specific, usually sexually dimorphic, and are often individually distinct (Stoddard et al., 2006), and electric fish communicate with conspecifics by detecting each other's EODs. Furthermore, weakly electric fish are preyed on by electric eels in South America (electric eels are gymnotiforms) and electroreceptor-bearing catfish on both continents, and electric signals have evolved to minimize detection by these predators (Hanika and Kramer, 1999; Stoddard, 1999). Thus, EOD waveforms and the sensory capabilities of electroreceptors have been shaped by the needs of electrolocation, communication and predator avoidance.

Electric fish show a number of presumably adaptive specializations. Two distinct discharge patterns occur, independently evolved in both groups: pulse- and wave-type patterns. Pulse fish emit EOD pulses, often with a complex multi-phasic structure, at irregular intervals. They vary the repetition rate of the EOD as needed, discharging at high rates when active and low rates when resting. Wave-type species discharge in a specific frequency band; males, females and juveniles may each discharge within a portion of the species bandwidth, and each fish may furthermore have its own 'personal' frequency (many species of electric fish are capable of shifting their individual frequency to a new value if they are 'jammed' by another fish with a similar frequency). Wave-type EOD waveforms are usually monophasic pulses of a duration roughly equal to the interpulse duration, thereby forming a sine wave-like pattern. Wave-type fish discharge constantly whether they are active or inactive.

Electric fish show a diversity of EOD waveforms. Some species produce long duration pulses (20 ms) whereas others generate extremely brief discharges (~200 μs; Fig. 1). This is a hundredfold difference in EOD pulse duration. Indeed, ultra-brief discharges have evolved in both groups of electric fish (Bennnet, 1971). The briefer the discharge the broader the power spectrum of the signal; broad power spectra are beneficial for detecting a wider range of complex impedences from objects in the environment (von der Emde and Ringer, 1992). Some wave-type gymnotiforms discharge at low frequencies (50 Hz) and others at very high frequencies (>1 kHz). Presumably, sampling at high frequencies gives fish better temporal resolution of their world. Generating such brief pulses or operating at high frequencies may be possible because electric fish are 'ion channel specialists'. That is, they have specialized in the evolution and regulation of ion channels as a means to generate species-specific signals [the anatomical structure of the EO varies greatly among species and also plays a dominant role in shaping the EOD (Hopkins, 1999)].

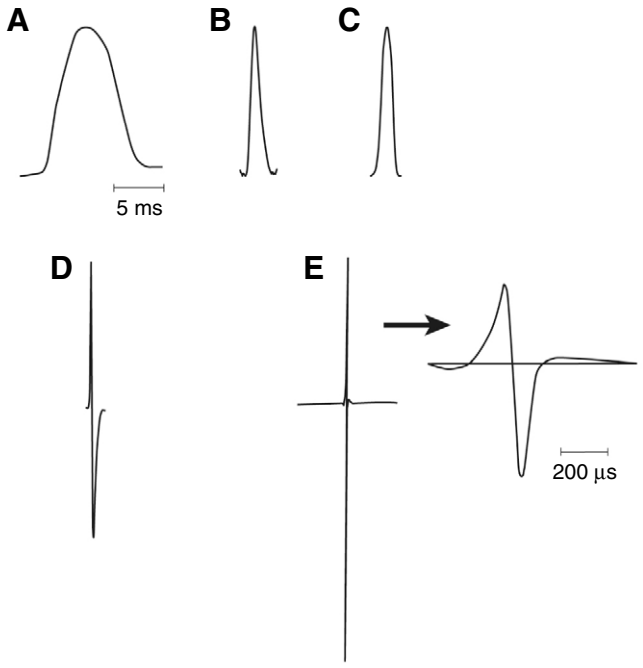

Fig. 1. EOD pulses from the five species of electric fish used in the molecular analysis in Fig. 2. (A) A single pulse of the wave-like EOD of *Sternopygus macrurus*, the gold-lined black knifefish; (B) a single pulse of the wave-like EOD of *Apteronotus leptorhynchus*, the brown ghost; (C) *Electrophorus electricus*, the electric eel; (D) *Brachyhypopomus pinnicaudatus*, the pintail knifefish; and (E) *Gnathonemus petersii*, the 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 also shown on an expanded time scale to the right (indicated by the arrow). Reproduced with permission (Zakon et al., 2006).

Evolution of Na^+ channel genes in electric fish

Voltage clamp analysis of the ion currents in the electrocytes, the cells of the EO, show that the EOD pulse is mainly shaped by Na^+ currents (Shenkel and Sigworth, 1991; Ferrari et al., 1995). We therefore focused our molecular evolutionary analysis on Na^+ channel genes. When we undertook this work it was known that mammals possess 10 Na^+ channel genes but there was no information on the number of Na^+ channel genes in fish, despite the fact that the first Na^+ channel gene cloned was from the EO of the electric eel, a gymnotiform (Noda et al., 1984). Therefore, we cloned Na^+ channel genes from representative species of both groups of weakly electric fish, a catfish and a lamprey (as an outgroup for gnathostomes), and assembled additional Na^+ channel genes by cloning or from genome databases for zebrafish and pufferfish. We found that teleosts have seven to eight Na^+ channel genes. Phylogenetic studies indicate that the common ancestor of teleosts and tetrapods had four Na^+ channel genes and that these duplicated at the origin of teleosts (Lopreato et al., 2001; Novak et al., 2006), presumably as part of a teleost-specific genome-wide duplication (i.e. ploidy) event (Amores et al., 1998; Jaillon et al., 2004; Crow et al., 2006).

Since EOs derive from muscle, we were especially interested in the orthologs of the mammalian muscle Na^+ channel gene *Nav1.4*

(*Nav1.4=scn4a*). We found that fish possess two duplicate copies: *Nav1.4a* and *Nav1.4b*. Not surprisingly, analysis of muscle mRNA by PCR showed that both genes are expressed in the muscles of non-electric fish (Venkatesh et al., 2005; Zakon et al., 2006; Novak et al., 2006). At this level of resolution, however, we do not know whether both genes are expressed redundantly in every muscle fiber or whether each gene is separately expressed in a subset of muscle fibers (slow *vs* fast, hypaxial *vs* epaxial, etc). This can be tested by *in situ* hybridization or single cell PCR.

The situation is different in electric fish. *Nav1.4b* is also expressed in muscle in electric fish and, in addition, may be expressed in the EO in some species. However, *Nav1.4a* expression has been lost in muscle and gained in the evolutionarily novel EO in both groups of electric fish (Zakon et al., 2006) (M. Arnegard, D.Z., Y.L. and H.H.Z., unpublished observations).

A notable exception to this pattern is the weakly electric fish the brown ghost, *Apternotus leptorhynchus*. This species (and the large radiation of other apteronotid species) has a myogenically derived larval EO that is retained only for the first few weeks of larval life during which time its EOD frequency is only a few hundred hertz (Kirschbaum, 1977). As maturation continues its EOD frequency increases to 750–1000 Hz or, in some apteronotid species, up to 1600 Hz (Kramer et al., 1980). As the myogenic larval EO degenerates, the axons of the motorneurons that innervated it are altered and become the new EO (Pappas et al., 1975). In this way, speed and synchronization are most efficiently maintained by electrotonic coupling from the brain down to the motorneuron and the elimination of the single obligate chemical synapse (neuro-electrocyte junction) in the pathway. Because the EO of mature apteronotids is not from muscle we believe that neither *Nav1.4a* nor *Nav1.4b*, but some other neurally expressed Na^+ channel gene(s), must be responsible for generating a rapid EOD frequency in apteronotids. Intracellular recordings from the axons of neurogenic electrocytes show that they are capable of discharging at or over 1000 Hz (Schaefer and Zakon, 1996). PCR or *in situ* hybridization analyses of the motorneurons and of the larval EO will provide the final identity of the Na^+ channels in the adult and larval EOs.

The fact that *Nav1.4a* has twice lost its expression from muscle and gained it in the novel environment of the EO is potentially informative as to possible constraints on the evolution of *Nav1.4a*. Indeed, in zebrafish, a non-electrogenic teleost, *Nav1.4a* is expressed in fewer tissues than *Nav1.4b* (Novak et al., 2006). Thus, it may be that fewer tissues would have been affected by the loss of expression of *Nav1.4a* than *Nav1.4b*. We do not know what molecular events account for altered expression of *Nav1.4a* in electric fish, but analysis of regulatory regions in these genes compared with the regulatory regions in related non-electric fish will probably shed light on this.

Na^+ channel genes are normally under strong negative selection as evidenced by the large catalog of channelopathies (diseases attributed to mutations in ion channels) (Wei et al., 1999; Bendahhou et al., 2002; Splawski et al., 2002; Tan et al., 2003; Tian et al., 2004; Wang et al., 2004; Berkovic et al., 2004). We reasoned that in both lineages of electric fish *Nav1.4a* would be freed from many selective constraints associated with muscle expression as electrocytes are not contractile and mutations in *Nav1.4a* would have no effect on a fish's motility. Furthermore, it seemed likely that *Nav1.4a* is evolving under a new set of selection pressures to optimize the EOD in different sensory environments or for the generation of species-specific communication signals. This would be evident by an increase in the number 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.

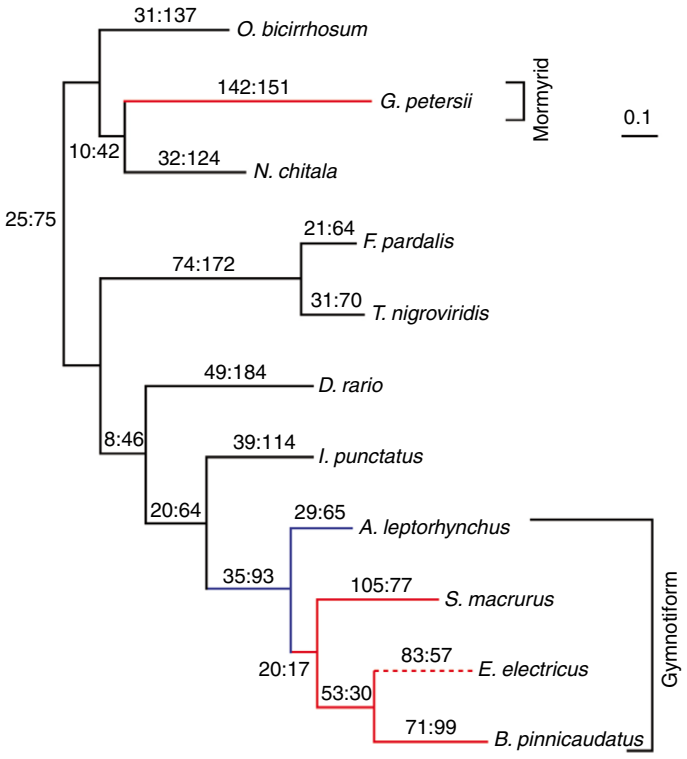

Fig. 2. Gene tree of *Nav1.4a* with the estimated number of non-synonymous/synonymous substitutions for each branch. Red branches indicate lineages in which *Nav1.4a* is known to be lost from muscle (the dotted line in *E. electricus* indicates that its loss is likely but not yet tested), and the blue 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. Reproduced with permission (Zakon et al., 2006).

We tested the hypotheses that: (1) *Nav1.4a* has evolved at a higher rate in electric than non-electric fish; (2) changes in the rate of evolution of *Nav1.4a* in electric fish occur following its loss of expression from muscle and gain of expression in the EO; (3) amino acid changes in the channel will be evident in regions of the channel involved in voltage-dependent gating and, specifically, inactivation (the closure of the channel despite maintained depolarization) since the distinguishing feature of EODs is that they may vary in duration.

We obtained the sequence for *Nav1.4a* from six non-electric, one mormyriform and four gymnotiform species, constructed a phylogenetic tree and estimated the number of non-synonymous *vs* synonymous changes per codon in each lineage (Zakon et al., 2006) (Fig. 2). We found that the single mormyrid and all the gymnotiform electric fish except for *Apteronotus* showed elevated ratios of non-synonymous/synonymous substitutions in *Nav1.4a* compared with the same gene in non-electric teleosts. This is consistent with our hypothesis 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 data set from both electric lineages (M. Arnegard, D.J.Z., Y.L. and H.H.Z., unpublished observations). Furthermore, in order to confirm that the elevation in the non-synonomous/synonymous ratio was not due to an increase in this ratio in all genes of these species, we performed the same

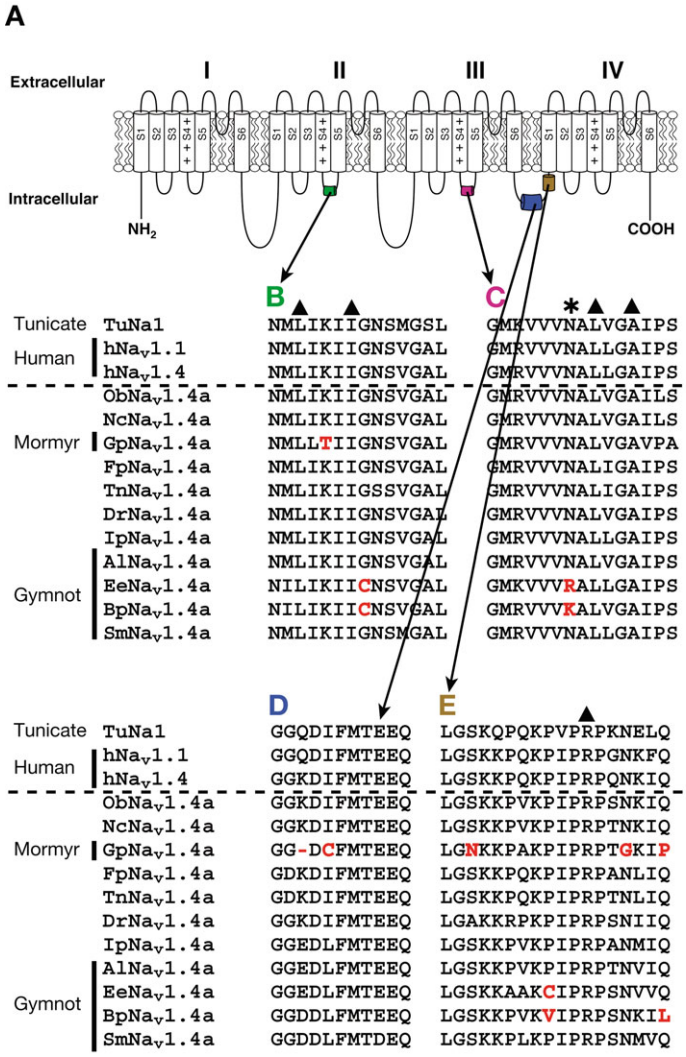

Fig. 3. Non-conserved amino acid substitutions occur in a number or regions of the Na^+ channel involved in inactivation of the Na^+ current. Mormyr, mormyrid; Gymnot, gymnotiform. (A) Schematic illustration of the Na^+ channel. (B) S4–S5 linker in domain II; (C) S4–S5 linker in domain III; (D,E) different parts of the inactivation 'ball' or hinged lid in the loop between domains III and IV. Amino acid sequences below the dashed line were used in the 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 in human Na^+ channels cause diseases. 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. Reproduced with permission (Zakon et al., 2006).

analysis on *Nav1.4b*, which is still expressed in the muscle in both groups of weakly electric fish, and found that there was no difference in the rates of evolution in this gene between electric and non-electric fish (M. Arnegard, D.J.Z., Y.L. and H.H.Z., unpublished observations). Likelihood-based analyses (PAML) support the contention that these changes are the result of positive selection. In these analyses, there was a difference in the distribution of changes at the codon level in *Nav1.4a* of some of the electric fish that were not observed in *Nav1.4a* of non-electric species (or *Apteronotus leptorhynchus*). As implemented, these tests identified branches in the tree (i.e. taxa) that showed elevated rates of non-synonymous substitutions, but they did not have the power to identify specific sites at which positive selection might have occurred.

EOD pulses of various species often vary in duration, and this might 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 known to be critical for inactivation (Fig. 3). The final step in inactivation is believed to occur when the highly conserved intracellular loop connecting domains III and IV rotates into position in the cytoplasmic side of the channel and binds to the S4–S5 linkers from domains II–IV, thereby occluding the conduction of Na^+ ions through the channel (Kellenberger et al., 1977; Kellenberger et al., 1997; Popa et al., 2004). Despite the persistence of conserved amino acids in these regions across ~500 million years of evolution (tunicates–vertebrates), we noted changes in key amino acids in these sites in both groups of weakly electric fish. In the single mormyrid that we studied, we noted amino acid substitutions at two key residues in the domain III–IV loop 'inactivation ball' (Fig. 3D) whereas in the gymnotiforms we noted substitutions in the domain III S4–S5 linker (Fig. 3C), 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. 3B) 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 in the figure mutations that have been noted in the human clinical literature and associated with muscular, cardiac or neurological disease (Wei et al., 1999; Bendahhou et al., 2002; Splawski et al., 2002; Tan et al., 2003; Tian et al., 2004; Wang et al., 2004; Berkovic et al., 2004). These mutations flank (Fig. 3B,C,E) or occur at the same (Fig. 3C) 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. 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).

Future directions

Pinpointing likely amino acid changes that underlie the evolution of Na^+ channel genes is a big step in understanding the evolution of electric signaling in electric fish. However, understanding how these substitutions actually alter the biophysical properties of the Na^+ currents can only be approached by site-directed mutagenesis and expression of channels.

A second intriguing direction is investigating the molecular events that led to the loss of *Nav1.4a* expression from muscle and, even more interesting, how the genes that are expressed in the EO come to recognize the novel phenotype of the EO. This analysis can be commenced by cloning and sequencing upstream regulatory regions of *Nav1.4a* in a number of species in which the gene is still expressed in muscle (apteronotids and non-electric outgroups) and those in which it is lost (most electric fish) to determine whether there are any radical alterations or losses of particular transcription factor binding sites.

Apteronotid electric fish probably use a different Na^+ channel gene since *Nav1.4a* is not expressed in the CNS (Y.L. and H.H.Z., unpublished observations). Identification of the Na^+ channel genes that are expressed in the apteronotid pacemaker or electromotorneurons by PCR would be a profitable first step. Once

candidate genes are identified, a similar analysis to the one described here could be performed.

In an analogous manner to that in which EOs have evolved from muscle in fish, specialized muscles for the generation of species-specific acoustic communication signals have evolved multiple times in teleosts. These muscles generate sounds by the rapid compression and relaxation of the swimbladder at rates exceeding 100 Hz. It would be interesting to test whether a similar pattern of compartmentalization and specialization of either *Nav1.4a* or *Nav1.4b* has occurred in these specialized sound-producing muscles.

Na^+ channels have associated subunits called β subunits that modify the biophysical properties of the Na^+ channel proper. In addition, the repolarization of the action potential is through K^+ channels. It will be intriguing to determine whether other ion channels evolved in parallel with Na^+ channels.

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