Old gene duplication facilitates origin and diversification of an innovative communication system—twice

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The genetic basis of parallel innovation remains poorly understood due to the rarity of independent origins of the same complex trait among model organisms. We focus on two groups of teleost fishes that independently gained myogenic electric organs underlying electrical communication. Earlier work suggested that a voltage-gated sodium channel gene (*Scn4aa*), which arose by whole-genome duplication, was neofunctionalized for expression in electric organ and subsequently experienced strong positive selection. However, it was not possible to determine if these changes were temporally linked to the independent origins of myogenic electric organs in both lineages. Here, we test predictions of such a relationship. We show that *Scn4aa* co-option and rapid sequence evolution were tightly coupled to the two origins of electric organ, providing strong evidence that *Scn4aa* contributed to parallel innovations underlying the evolutionary diversification of each electric fish group. Independent evolution of electric organs and *Scn4aa* co-option occurred more than 100 million years following the origin of *Scn4aa* by duplication. During subsequent diversification of the electrical communication channels, amino acid substitutions in both groups occurred in the same regions of the sodium channel that likely contribute to electric signal variation. Thus, the phenotypic similarities between independent electric fish groups are also associated with striking parallelism at genetic and molecular levels. Our results show that gene duplication can contribute to remarkably similar innovations in repeatable ways even after long waiting periods between gene duplication and the origins of novelty.

evolutionary novelty | ion channel | independent species radiations | gymnotiforms | mormyroids

The evolution of novelty is a key process in the diversification of life, yet investigating genetic changes associated with novelty remains one of evolutionary biology's greatest challenges (1, 2). Regressive losses of complex traits have received much attention (3, 4), yet such cases offer limited insights into the constructive evolution of complex novel traits. It has long been suspected that gene duplication is an important source of genetic raw material for evolutionary novelty (5). However, the innovations that have been linked to gene duplication are typically simple phenotypes, such as the modification of protein function (6) rather than the de novo construction of a complex trait (7). Comparing relationships between a single gene duplicate and multiple independent gains of the same complex trait has not been possible previously in any system. Here, we test whether a duplicated voltage-gated Na^+ channel gene directly contributed to independently derived organs of electrical communication in teleost fishes.

Although distantly related, African mormyroid and South American gymnotiform fishes independently gained myogenic electric organs, which produce electrical communication signals in both groups (Fig. S1). This novel organ is a key innovation in communication (8) that has directly contributed to the parallel evolutionary diversification of mormyroids and many gymnotiforms (9, 10). Adult myogenic electric organ (EO_{myo}) is developmentally derived from skeletal muscle (SM) and shows striking similarities in structure and function between groups (11, 12). In gymnotiforms and mormyroids, EO_{myo} is composed of many electrocytes that fire simultaneously to produce each electric organ discharge (EOD) (13). A different kind of electric organ derived from neurons (neurogenic EO, or EO_{neuro}) is not relevant to our study because it is restricted to a single group within gymnotiforms (14). The much larger number of taxa possessing EO_{myo} exhibit an impressive degree of variation in EODs, which function as communication signals. Species producing pulses or waves are present in both mormyroids and gymnotiforms, and EOD duration varies from less than 200 μs to more than 30 ms among species (Fig. S1). Discharge of the electric organ requires voltage-gated Na^+ channels, and the dramatic signal variation observed among electric fishes depends on properties of this same class of ion channel (13, 15, 16).

A fish-specific whole-genome duplication (WGD) is conservatively estimated to have occurred 226–316 million years ago, with teleosts radiating soon thereafter (17, 18). More than 100 million years after the WGD, gymnotiform and mormyroid electric fishes arose independently from nonelectrogenic ancestors (17, 19–22). Due to the WGD, nonelectrogenic teleosts possess two *Scn4a* paralogs that are expressed in SM (23, 24). *Scn4aa* and *Scn4ab* code for voltage-gated Na^+ channel α-subunits $Na_v1.4a$ and $Na_v1.4b$, respectively. Each contains functional regions responsible for activation and inactivation of transmembrane Na^+ current (Fig. 1).

Results of a previous study suggested that *Scn4aa* was lost from SM, became expressed in EO_{myo}, and subsequently experienced a change in selective forces at some point during the radiation of each electric fish group (24). These results raised the hypothesis that *Scn4aa* directly contributed to the parallel origins of adult EO_{myo}. This hypothesis makes three predictions that were not testable previously. It predicts (*i*) that co-option of *Scn4aa* by EO_{myo} (i.e., loss of expression from SM and gain of expression by EO_{myo}) and the change in selection acting on *Scn4aa* coincided with each origin of this complex trait; (*ii*) that any changes in the selective forces acting on *Scn4aa* were specific to this paralog, rather than part of a larger pattern of changes affecting many genes in electric fishes, including the other paralog with un-

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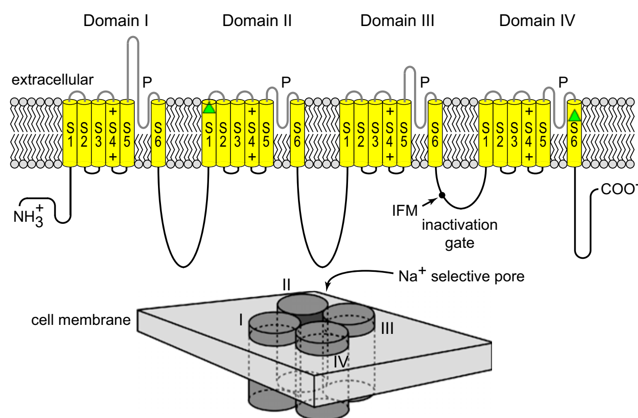

Fig. 1. Voltage-gated Na^{+} channel α-subunit. (*Upper*) Transmembrane folding diagram. Each domain contains six segments (yellow) with intracellular (black) and extracellular (gray) loops. Green triangles bound the investigated sequence. S4 of each domain contains positively charged residues that serve as voltage sensors. P-loops between S5 and S6 form the outer pore. An inactivation gate in the linker between domains III and IV mediates fast inactivation via a conserved binding particle (IFM across many species). (*Lower*) Representation of the α-subunit in the cell membrane.

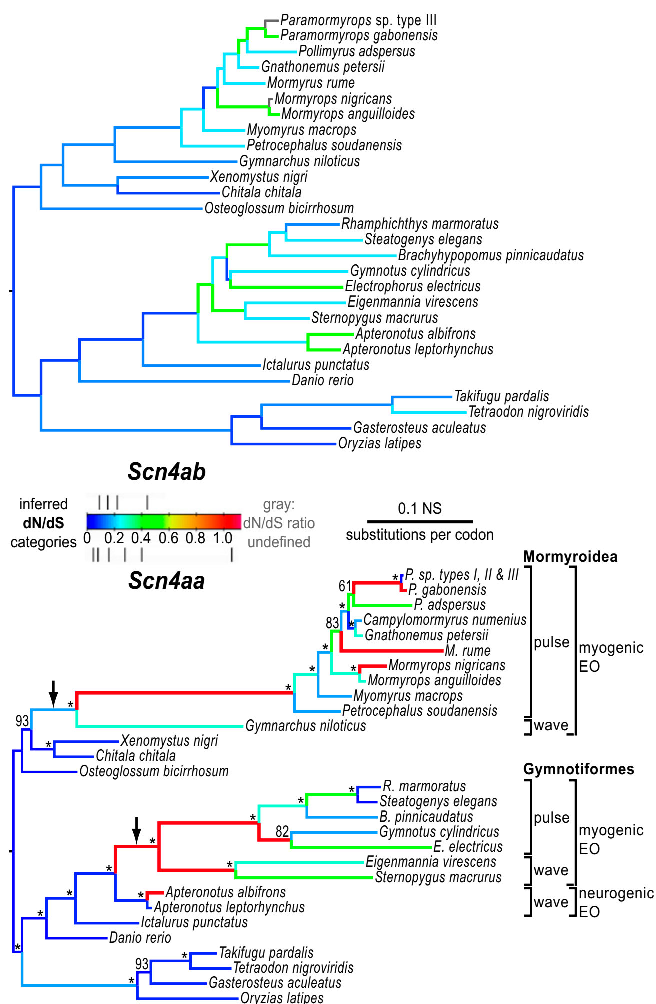

Fig. 2. Selection on Na^{+} channel paralogs along branches of the teleost phylogeny. Shown are optimal categorizations of branches into selective categories (tick marks along heat scale). Branches assigned to a given dN/dS category share the same color. Deeper blue indicates strongly constrained lineages, whereas hotter colors indicate lineages with elevated dN/dS. Following branch categorization, dN/dS category values and branch lengths were re-estimated in HyPhy allowing dN/dS variation across sites. Branch lengths are drawn proportional to expected numbers of nonsynonymous substitutions per codon. A single tree topology inferred from the dataset *Concat* was used for both genes, but bootstrap support values (%) are indicated in only one tree (asterisks: 100%). Trees were rooted for display purposes according to accepted fish relationships, with length of the branch on which the root falls split equally on each side. Arrows indicate the most parsimonious branches in which *Scn4aa* was co-opted for exclusive expression in EO_{myo}.

changed expression (*Scn4ab*); and (*iii*) that selection should have targeted regions of *Scn4aa* with functional consequences for electrocyte action potentials and, therefore, for variation in the resulting EOD waveform (25, 26). Here, we test all three predictions by collecting sequence and expression data for the electric fish species required to evaluate this hypothesis and by conducting an analysis of changes in the selective forces on *Scn4ab* as well as a high-resolution analysis of selection across functional regions of $Na_v1.4a$.

Results

Changes in Paralog Expression in Electric Fish Relative to Other Teleosts. Testing whether changes in *Scn4aa* expression coincided with two independent origins of adult EO_{myo} requires a robust phylogeny on which to infer the timing of these events. Although a sound knowledge of mormyroid phylogeny has been acquired using genetic data (9, 22, 27, 28), the overall gymnotiform phylogeny has remained less resolved (14, 19, 29, 30). Thus, we inferred phylogenetic relationships among the teleosts that we investigated using four datasets: all nucleotide positions of both paralogs concatenated ("*Concat*"), all positions of each gene separately, and only third-codon positions of the concatenated data set ("*Concat3rd*").

The phylogenetic relationships consistently generated by each dataset agree with accepted relationships among nonelectric fish and mormyroid species (*SI Text*), as well as a recently published phylogeny for pulse-type gymnotiforms (31). Our results (summarized in Fig. 2) strongly suggest a single origin of an EO_{myo} group of gymnotiforms, which are sister to an EO_{neuro} group (also Fig. S2). Similar to mormyroids, this EO_{myo} gymnotiform lineage then split into two natural groups, one consisting of all pulse species and the other of all wave species (Fig. S1). Taken individually, *Scn4aa* and *Scn4ab* (Fig. S3) each yield the same broad gymnotiform relationships as recovered using *Concat* (Fig. S2). The same groups are also strongly supported by *Concat3rd*, suggesting no misleading effect of positive selection (Fig. S2).

Within this phylogenetic framework, we analyzed the expression of both Na^{+} channel genes in SM and EO_{myo} by reverse transcriptase-PCR. Extending previous results (24), the nonelectrogenic species that we examined expressed both paralogs in SM (Fig. 3). Moreover, we found loss of *Scn4aa* expression in SM and its gain by EO_{myo} in all electric fish species possessing this novel organ as adults, including the most basal species. These data allowed us to infer the timing of *Scn4aa* co-option relative to both origins of EO_{myo} (Fig. 2, arrows), which supports the hypothesis that *Scn4aa* played a formative role in this parallel evolution of novelty.

Changes in Selection on *Scn4aa* and *Scn4ab* Across the Teleost Phylogeny. We estimated the selective forces that acted upon both paralogs near the origins of EO_{myo}, yielding additional evidence consistent with the first prediction of our hypothesis. The ratio of the rates of nonsynonymous-to-synonymous nucleotide substitutions (dN/dS) provides a measure of the selection pressures that acted on a sequence over a given period, with low values indicating constraint and increases in value indicating relaxation of constraint or positive selection. We conducted

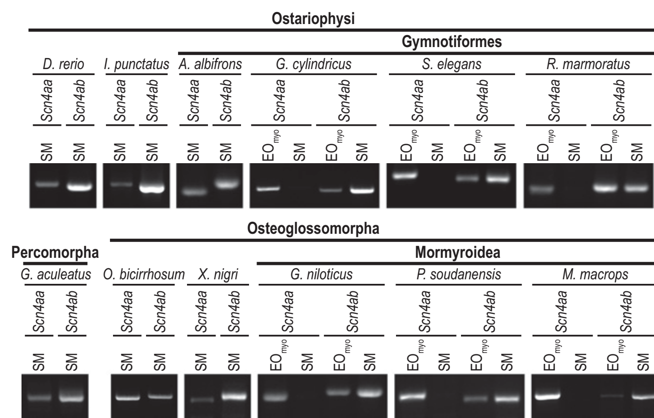

Fig. 3. *Scn4aa* and *Scn4ab* expression in EO_{myo} and SM of electric fish compared with nonelectrogenic relatives. (*Upper*) Three gymnotiform species with EO_{myo} (*Right*) compared with one gymnotiform lacking EO_{myo} in adults and two nonelectrogenic ostariophysan fishes (*Left*). (*Lower*) Three mormyroid species compared with two nonelectrogenic osteoglossomorphs and a more distantly related percomorph. Note the loss of *Scn4aa* expression (i.e., lack of bands) in SM and the gain of expression (presence of bands) in EO_{myo} for all electric fish possessing adult EO_{myo}.

a maximum-likelihood (ML) analysis of the patterns of dN/dS variation (32) across all branches of the phylogeny. During or very soon after gene co-option, *Scn4aa* exhibited greatly elevated average dN/dS values. In support of the second prediction of our hypothesis, the paralog (*Scn4ab*), which remains expressed in muscle in nonelectrogenic teleosts (and is also often expressed in EO_{myo}), exhibited no dramatic change in selection pressures (Fig. 2). The highest dN/dS value inferred for any lineages in either paralog (dN/dS = 1.065, shown in red in Fig. 2) occurs only in the *Scn4aa* tree. Importantly, the average dN/dS for *Scn4aa* immediately after its co-option by EO_{myo} represents more than a 10-fold increase relative to preceding lineages. Increased dN/dS values also occur in some terminal mormyroid branches in the *Scn4aa* tree (Fig. 2), which are known for particularly rapid rates of signal differentiation (8, 33).

Striking Parallelism in Selective Forces Acting on Regions of *Scn4aa* Underlying Ion Channel Kinetics. The hypothesis that *Scn4aa* contributed to the origin and diversification of myogenically derived electrical communication predicts that selection should have targeted regions of this paralog with functional consequences for EOD variation. We also found evidence in support of this third prediction. We developed an ML test to locate windows of 10 codons in the *Scn4aa* alignment that experienced a significant shift in selective forces over the entire evolutionary history of adult EO_{myo} following the co-option of *Scn4aa* by this organ. To allow the detection of such shifts, the test uses the selective pattern estimated across all lineages connecting our nonelectric species as a "reference," representing the pattern of constraint expected along the molecule when *Scn4aa* continues to be expressed in SM. To increase power, all nonelectric species were pooled into a single subalignment, which was then compared with both the gymnotiform and mormyroid alignments. Results of these tests show a visually striking pattern of parallelism between gymnotiforms and mormyroids (Fig. 4). If we make the assumption that the individual tests of window significance are independent in gymnotiforms and mormyroids (an assumption that is partially violated; *SI Text*), then a χ^2 test suggests more coincidence of significant windows than expected by chance (13 significant windows in gymnotiforms, 19 in mormyroids, and 7 in both; $P = 0.00477$).

Parallel changes occurred in a number of functionally important motifs. For example, we discovered changes in the extracellular S3–S4 loops of domains II and III in pulse mormyroids and pulse gymnotiforms (Fig. 4). The S3–S4 loop is attached to the S4 voltage sensor (Fig. 1), which influences the kinetics of channel activation (35–37). Changes in this region are expected to affect the rising phase of electrocyte action potentials (13, 15), likely modifying EOD duration.

Voltage-gated Na^+ channels inactivate when a gate in the intracellular DIII/DIV linker (Fig. 1) rotates around a glycine hinge on one side of the gate and a hinge composed of multiple prolines on the other, placing a key hydrophobic motif of three amino acids [isoleucine-phenylalanine-methionine (IFM)] against the inner mouth of the channel. Many changes to such residues likely have significant effects on the duration of Na^+ channel-dependent spikes (38, 39), such as those that compose EODs (Fig. S1) (24, 40). In all pulse mormyroids in our $Na_v1.4a$ alignment, we observed loss of an amino acid adjacent to the glycine hinge, alteration of IFM to the less hydrophobic CFM (cysteine-phenylalanine-methionine), and loss of a serine that, when phosphorylated, slows channel inactivation (Fig. 5). These changes occurred in the most basal pulse mormyroids, which make very brief EODs (Fig. S1) (28); they then remained fixed during the subsequent radiation of this group. Throughout the $Na_v1.4a$ alignment, other such "invariable re-

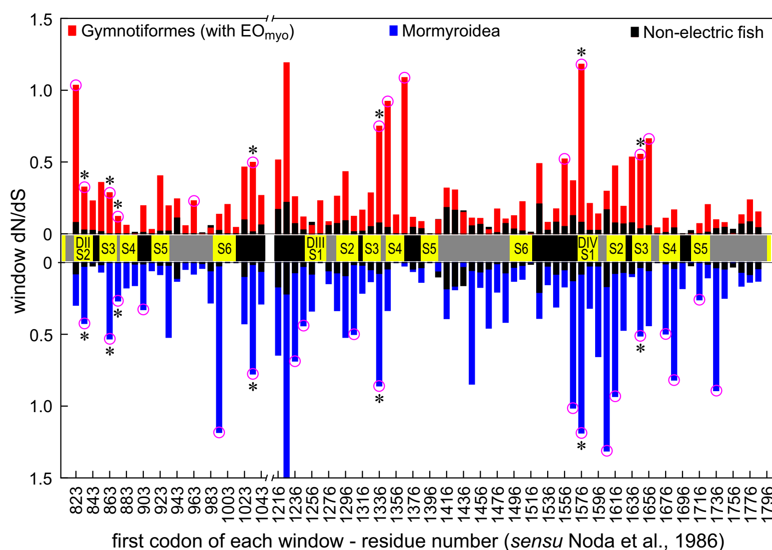

Fig. 4. Pattern of selection across *Scn4aa*. Per-window dN/dS estimates (window size: 10 codons) for *Scn4aa*: red, gymnotiforms with adult EO_{myo}; blue, mormyroids, all of which have adult EO_{myo}; black, nonelectric fish (smaller values plotted in front such that all values are visible). Black bars are mirrored because the same set of nonelectric fish species was compared with each group of electric fish. Color scheme of middle axis matches channel schematic in Fig. 1. Lower axis provides standard residue numbers from electric eel (34). The breakpoint spans excluded hyper-variable sequence. dN/dS estimates in pink circles are significantly different from nonelectric fish; asterisks indicate windows that are significantly different in both electric fish groups. The clipped dN/dS estimate for mormyroids in the second window after the breakpoint is 2.56 (nonsignificant).

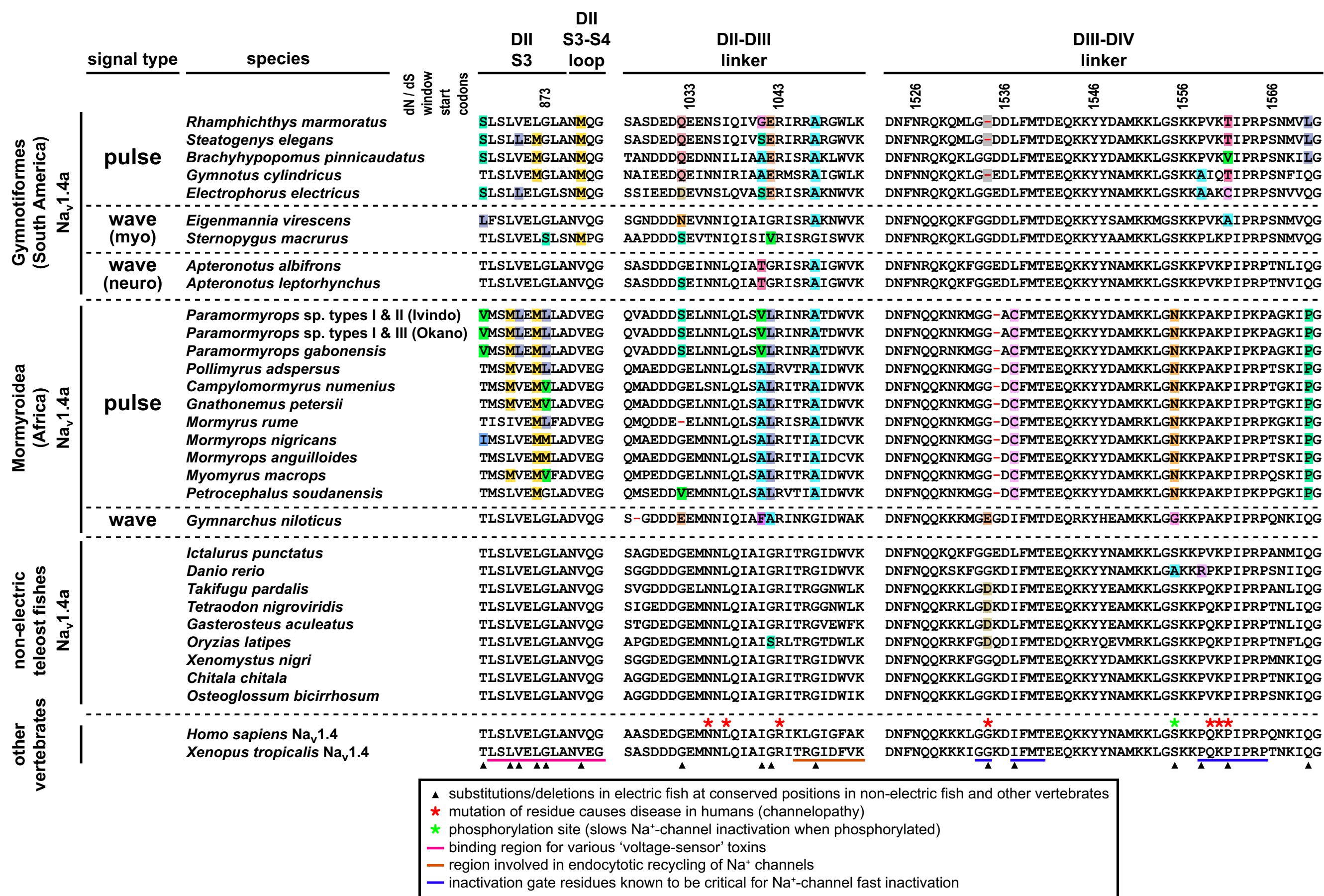

Fig. 5. Functionally important regions of Na$_v$1.4a exhibiting amino acid substitutions in electric fish at otherwise conserved sites. Positions conserved across most vertebrates but variable in electric fish are indicated by black triangles below the sequences. Variable residues at conserved sites are shown by differently colored shadings for each amino acid. Deletions are shown as red hyphens. Colored asterisks and lines next to the human and *Xenopus* sequences indicate residues with critical roles in channel gating, human channelopathies, or neurotoxin function (*Inset*).

placement" substitutions (7) are also apparent in pulse mormyroids and pulse gymnotiforms (e.g., Fig. 5); these kinds of substitutions are thought to result from brief episodes of directional selection (7). In other organisms, alteration of the Na$^+$ channel's glycine hinge has been implicated in deleterious conditions, such as myotonia in humans (41) and the "out cold" paralysis phenotype in *Drosophila* (42).

Pulse gymnotiforms also exhibit amino acid substitutions in the same channel region, yet these changes have occurred at proline residues forming the inactivation gate's other hinge (Fig. 5). In addition to basal pulse mormyroids, some pulse gymnotiforms have rapidly inactivating Na$^+$ currents and brief duration pulses. Our results suggest that amino acid substitutions in the S3–S4 loops of domains II and III, and in the inactivation gate, are perhaps associated with the evolution of rapid pulse-type signals in both mormyroids and gymnotiforms.

Finally, we found numerous substitutions in both lineages in the linker between domains II and III (Fig. 5) in a region that includes a key motif for Na$^+$ channel endocytosis (43) and where deleterious cardiac and neurological mutations localize in humans (44, 45). The Na$^+$ current of EO$_{myo}$ (i.e., EOD amplitude) varies in magnitude over circadian cycles and as a result of social stimulation in some gymnotiforms, due to hormone-dependent modulation of constitutive Na$^+$ channel cycling (46). Mormyroids do not show circadian rhythms in EOD amplitude, but EOD amplitude in some mormyroid species might be homeostatically regulated to compensate for large changes in water conductivity that occur seasonally in some regions of Africa (47). It is possible that this is also achieved by the modulation of constitutive Na$^+$ channel cycling in mormyroids. The signature of evolutionary change in and around this motif in both electric fish groups likely has implications for *Scn4aa* Na$^+$ channel trafficking in EO$_{myo}$.

Discussion

In this study, we found evidence in support of three key predictions made by the hypothesis that *Scn4aa* was involved in the constructive evolution of adult EO$_{myo}$. First, we demonstrated altered *Scn4aa* expression during both independent origins of EO$_{myo}$, as well as large increases in dN/dS during or immediately after the origin of this novel organ. Release from constraint and positive selection both can contribute to increases in dN/dS. *Scn4aa* almost certainly experienced some relaxation from constraint when it was lost from SM and co-opted by EO$_{myo}$ because it was no longer under purifying selection for contractile muscle function (40). Nevertheless, the elevated dN/dS values that we found at the base of each electric fish radiation suggest the presence of extensive positive selection because these values are averaged across all amino acid positions, many of which are necessarily highly constrained in voltage-gated Na$^+$ channels specific to any tissue type (23, 25, 26, 34). For example, across our entire *Scn4aa* sequence alignment, 395 of 823 amino acid sites are invariant (Fig. S4). In all likelihood, positive selection on some residues must have been strong to result in more than a 10-fold increase in average dN/dS, given a background of constraining selection on many other sites. Moreover, we detected no similar increase in dN/dS for *Scn4ab*, the paralog that remains expressed in SM (second prediction). We also found a striking

degree of parallelism between electric fish groups in the spatial clustering of selective forces in functional regions related to channel gating and electric signal variation (third prediction). Taken together, our results strongly suggest that *Scn4aa* directly contributed to the origins and diversification of a novel electrical communication system.

Sodium channel duplication may have been necessary for the convergent evolutionary pathways that eventually led to independent origins of adult EO_{myo}. The large number of substitutions that we found in functional regions of the α-subunit likely would have been impossible with an unduplicated Na^+ channel that remained both expressed in SM and critical to contractile muscle function. Consistent with this view, substitutions at many $Na_v1.4a$ positions appear adaptive in electric fish while producing channelopathic phenotypes in tetrapod paralogs (e.g., Fig. 5). In contrast to the adult EO_{myo}, larval myogenic electric organs are found in many electric fish lineages, including the apteronotid gymnotiforms that only possess EO_{neuro} as adults, although the resulting larval EODs of electric fish appear to exhibit less variation among species than adult EODs (11, 30). Evolution of larval myogenic electric organs may not have required co-option of *Scn4aa* because the two apteronotid species examined show no loss of *Scn4aa* expression in SM (Fig. 3).

Scn4aa co-option into adult EO_{myo} occurred more than 100 million years after its origin by gene duplication. Typically, the average half-life before duplicate gene silencing occurs is on the order of 4 million years (48). Therefore, it is likely that at least some functional divergence happened between *Scn4aa* and *Scn4ab* in muscle in nonelectric teleosts; this would have helped to retain the expression of both paralogs in the SM of all of these fishes over a long "preservation phase" of paralog evolution (49) before any subsequent myogenically derived innovation. Currently, however, no data that relate to the functional divergence of the paralogs in nonelectric teleosts are available. Apparently, *Scn4aa* did not evolve to become essential to muscle function in all teleosts because its loss from SM was not prohibited in electric fishes.

Electroreception is thought to have arisen twice in teleosts: once at the base of the siluriform lineage (gymnotiforms + catfishes) and once in the lineage uniting mormyroids and Old World knifefishes (20, 50, 51). Electroreception initially appeared in the form of passive, ampullary electroreceptors, with high-frequency tuberous electroreceptors evolving later in subgroups contained within these lineages (52). Remarkably, preexisting genetic variation—in the form of an old, duplicated Na^+ channel gene (*Scn4aa*)—was co-opted into a novel signal-generating organ (adult EO_{myo}) in only these two teleost lineages possessing electroreception. Although the same genetic variation was present in all other teleost lineages, it was not recruited for novelty as it was in the electric fish groups, probably owing to the lack of preexisting electroreception. Replicated neofunctionalization of *Scn4aa* during the independent origins of adult EO_{myo} in unrelated fish groups illustrates that gene duplication can contribute to strikingly similar innovations even after extremely long waiting periods between gene duplication and the origins of novelty. Thus, ancient gene duplication can provide long-standing genetic variation for innovations arising much later. Moreover, the novel traits to which delayed co-option contributes can have far-reaching evolutionary significance despite potentially lengthy waiting periods between duplication and the origins of complex traits. In the weakly electric fish system, the myogenic electric organ is a key evolutionary innovation that has contributed twice to the radiation of species in independent lineages.

Materials and Methods

More detailed descriptions of all methods are provided in the *SI Text*. We collected and electrically recorded mormyroids in the Republic of the Congo and Gabon in July and August 2006. Additional electric and nonelectric fish species were obtained through the aquarium trade or by donation from other scientists (Table S1). Tissue samples (EO_{myo} and SM) were preserved in RNAlater (Ambion) and stored on ice in the field or at −80 °C in the laboratory. Published sequences from GenBank for additional teleost species are also included in our analyses.

We isolated RNA from homogenized SM and EO_{myo} by guanidinium thiocyanate–phenol–chloroform extraction. Extracted mRNA was reverse transcribed and amplified by PCR. Resulting cDNAs were cloned into *Escherichia coli*. We sequenced the inserts (partial transcripts of *Scn4aa* and *Scn4ab*) in both directions using an ABI 3100 sequencer (Applied Biosystems). *Scn4aa* and *Scn4ab* expression patterns were assayed by first using paralog- and species-specific primers to reverse-transcribe 0.5 μg of total mRNA from SM and 0.5 μg of total mRNA from adult EO_{myo} of species possessing this organ. The resulting cDNA was amplified by PCR, also with species- and paralog-specific primers (Tables S2 and S3). Reactions used cycling conditions optimized to obtain strong signal while avoiding saturation. Simultaneous use of primers for *Scn4aa* and *Scn4ab* (separate reactions) on mRNA extracts from the same individuals provided internal controls for quality of both mRNA and primers.

Methods for aligning sequences and inferring phylogenetic relationships are described in the *SI Text*. After finding evidence of significant variation in dN/dS over the history of both *Scn4aa* and *Scn4ab*, we applied the GABranch method (32) using HyPhy version 1.0 to investigate how this variation was distributed across branches of our phylogenetic tree. The optimal branch categorization scheme for each gene was then combined with a more realistic codon substitution model to estimate final branch lengths and dN/dS values for each category. Next, we developed a ML-based test to locate regions of the gene that had experienced significant shifts in dN/dS after the origins of EO_{myo}. We applied this test to successive, nonoverlapping windows of 10 codons. For each window, we used a likelihood-ratio test (with sequential Bonferroni correction) to compare selective forces acting over the evolutionary history of the nonelectrogenic taxa to those of each EO_{myo} clade.

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