A Novel Na⁺ Channel Splice Form Contributes to the Regulation of an Androgen-Dependent Social Signal

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Na⁺ channels are often spliced but little is known about the functional consequences of splicing. We have been studying the regulation of Na⁺ current inactivation in an electric fish model in which systematic variation in the rate of inactivation of the electric organ Na⁺ current shapes the electric organ discharge (EOD), a sexually dimorphic, androgen-sensitive communication signal. Here, we examine the relationship between an Na⁺ channel (Na_v1.4b), which has two splice forms, and the waveform of the EOD. One splice form (Na_v1.4bL) possesses a novel first exon that encodes a 51 aa N-terminal extension. This is the first report of an Na⁺ channel with alternative splicing in the N terminal. This N terminal is present in zebrafish suggesting its general importance in regulating Na⁺ currents in teleosts. The extended N terminal significantly speeds fast inactivation, shifts steady-state inactivation, and dramatically enhances recovery from inactivation, essentially fulfilling the functions of a β subunit. Both splice forms are equally expressed in muscle in electric fish and zebrafish but Na_v1.4bL is the dominant form in the electric organ implying electric organ-specific transcriptional regulation. Transcript abundance of Na_v1.4bL in the electric organ is positively correlated with EOD frequency and lowered by androgens. Thus, shaping of the EOD waveform involves the androgenic regulation of a rapidly inactivating splice form of an Na⁺ channel. Our results emphasize the role of splicing in the regulation of a vertebrate Na⁺ channel and its contribution to a known behavior.

Key words: androgen; electric organ; neuroethology; oocyte; sodium channel; splice variant

Introduction

Na⁺ channels are composed of an Na⁺ channel α subunit, and one or more β subunit. Na⁺ channels may also be spliced (Sarao et al., 1991; Thackeray and Ganetzky, 1995; Kerr et al., 2004; Raymond et al., 2004; Wood, 2005). The consequences of Na⁺ channel splicing have been studied in invertebrates (Tan et al., 2002; Du et al., 2006), but little investigated in vertebrates (Dietrich et al., 1998; Akopian et al., 1999; Ou et al., 2005). It is not clear what role Na⁺ channel splicing plays in the nervous system. We have been studying the functional consequences of Na⁺ channel splicing in the electric organ (EO) of an electric fish where Na⁺ currents play a key role in shaping a social signal. The waveform of this signal is related to the rate of inactivation of the Na⁺ current providing a functional context in which to interpret the role of splicing.

The electric organ discharge (EOD) of the weakly electric fish *Sternopygus macrurus* is a regular series of rounded pulses approximating a sine wave (see Fig. 1). EOD frequency is set by a brainstem pacemaker whereas the duration of each pulse depends on the membrane properties of the cells of the EO, the electrocytes. Subtle variation in the discharge conveys information about the species, sex, and individual identity of the emitter

(Hopkins, 1974). Ion currents of the EO are tightly regulated to achieve this level of precision (Ferrari et al., 1995; McAnelly and Zakon, 2000). Males emit long EOD pulses at low frequencies and their electrocyte Na⁺ currents inactivate slowly, females emit short EOD pulses at high frequencies and their electrocyte Na⁺ currents inactivate rapidly, and the properties of juvenile EODs and Na⁺ currents are intermediate. Androgens lower EOD frequency, broaden the EOD pulse, and slow the rate of Na⁺ current inactivation in the electrocytes (Ferrari et al., 1995).

Two Na⁺ channel α subunit (Na_v1.4a and Na_v1.4b) orthologs of the mammalian muscle Na⁺ channel gene *Na_v1.4* (Lopreato et al., 2001) and a β subunit (β 1) are expressed in the *Sternopygus* EO (Zakon et al., 2006; Liu et al., 2007). Androgenic regulation of the β subunit accounts for some of the variation in this social signal (Liu et al., 2007).

Here, we examine the role of the Na⁺ channel α subunit Na_v1.4b in shaping the EOD. We found that Na_v1.4b is spliced; both splice forms are equally represented in muscle but one splice form (Na_v1.4bL) predominates in the EO. Na_v1.4bL possesses a novel first exon encoding a 51 aa N-terminal extension that significantly affects fast and steady-state inactivation and recovery from inactivation, functionally substituting for a β subunit. Na_v1.4b is threefold higher in the EO of fish with rapidly inactivating Na⁺ currents (typically females) than in those with slowly inactivating Na⁺ currents (typically males). Transcript abundance is lowered twofold by androgens. Androgenic regulation of this Na⁺ channel gene and the predominant expression of its novel rapidly inactivating splice form in the EO aid in shaping a social signal.

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Materials and Methods

Experimental animals. Gold-lined black knife-fish (*Sternopygus macrurus*) were housed in aquaria in the laboratory at ~25°C and on a 12 h light/dark cycle. All fish used in this study were sexually immature adults of both sexes. EOD frequencies were measured with an amplifier (Grass P-15) and a multimeter (Fluke) on the frequency counter setting.

For harvesting EOs only, each fish was removed from its aquarium, placed on a counter-top in a net, and its tail was rapidly cut with scissors. The fish was immediately returned to its aquarium to recover. When multiple tissues were taken, fish were killed with 1.0% phenoxyethanol, and brain, muscle, and heart were removed.

Zebrafish (*Danio rerio*) were obtained from a local vendor and temporarily housed in the laboratory aquaria. Tissues (brain, muscle, and heart) were taken after fish were killed with 1.0% phenoxyethanol. Rat leg muscle was obtained from the laboratory of Dr. Hitoshi Morikawa at the University of Texas at Austin. These procedures were in accordance with the University of Texas Institutional Animal Care and Use Committee protocols.

Molecular biology. Total RNA and genomic DNA were isolated as described previously (Lopreato et al., 2001). Total RNA (10 µg) from each tissue type was used to perform 5' and 3' rapid amplification of cDNA ends (RACE) with the FirstChoice RLM-RACE kit (Ambion). The proximal promoter sequence were identified by 5' rapid amplification of genomic ends (RAGE) following the method described previously (Liu and Baird, 2001).

To examine the relative abundance of smNa_v1.4a and smNa_v1.4b (sm, *Sternopygus macrurus*) in EO, one-step reverse transcriptase (RT)-PCRs were set up with 100 ng of pooled total RNA isolated from three medium-EOD-frequency fish and primers designed to amplify a region of the loop II–III in both genes. Reactions were stopped after every third cycle between 24 and 39 cycles. RT-PCR products were analyzed on a 2% agarose gel.

To examine the relative abundance of smNa_v1.4bL and smNa_v1.4bS, 90 ng of total RNA isolated from pooled EO and muscle was used in 20 µl one-step PCRs. Primers designed specifically for either exon were used to amplify exon 1L and exon 1S. Reactions were stopped after 24, 27, 30 and 33 cycles, and analyzed on a 1.5% agarose gel.

Real-time quantitative RT-PCR was performed as described previously (Liu et al., 2007).

Immunoprecipitation and Western blot. Two antibodies were used in this study. One polyclonal rabbit antibody (Na1-NT) was generated and affinity-purified by Sigma-Genosys against the peptide NRTAH-VKKSEKLKLW. The other polyclonal rabbit antibody (pan-Na channel antibody) was against a conserved region in all Na⁺ channels (Upstate Biotechnology).

Electric organ, brain, and muscle from four *Sternopygus* were quickly dissected, pooled, and homogenized in ice-cold lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100) freshly mixed with protease inhibitors (Roche). Cell lysates were incubated for 2 h and centrifuged at 10,000 × g for 10 min to remove the insoluble fraction. Cell lysate containing 1 mg of solubilized protein was cleared with 100 µl of protein A-agarose (Upstate Biotechnology), then mixed with 5 µg of Na1-NT antibody and incubated overnight. The sample was added with 50 µl of protein A-agarose again, incubated for 2 h, collected by 1 min centrifugation, washed three times with ice-cold lysis buffer, and finally resuspended in 50 µl 2× Laemmli sample buffer. All incubation and centrifugation steps were performed at 4°C. Immunoprecipitated samples were used in Western blot probed with pan-Na channel antibody as described previously (Liu et al., 2007).

Figure 1. Relationship between the EOD and Na⁺ current inactivation kinetics. **A**, EOD frequency is determined by the firing rate of pacemaker neurons in the pacemaker nucleus. The ~50 pacemaker neurons are electrotonically coupled and fire simultaneously. Each action potential travels down to spinal motor neurons (not depicted) and these innervate the electrocytes, the cells of the electric organ. There is a 1:1 relationship between the firing rate of the pacemaker neurons and the EOD frequency as each descending action potential initiates a pulse from the electric organ. **B**, The duration of each pulse of the EOD is determined by the membrane properties of the electrocytes. Males generate long-duration pulses at low frequencies (top left), females generate short-duration pulses at high frequencies (bottom left). EOD pulse duration depends on the duration of the electrocyte action potentials (note stylized action potential riding on current injection, center), whose duration is mostly determined by the rate of inactivation of the electrocyte Na⁺ current (right). [This figure is reprinted from Liu et al. (2007), their Fig. 1].

To compare the smNa_v1.4bL expression semiquantitatively, pan-Na channel antibody was used to immunoprecipitate all Na⁺ channels from the tails of four high-frequency fish and four low-frequency fish with the method above. Immunoprecipitated samples were pooled into two groups and 10 µl from each group were loaded on 7.5% Tris-HCl SDS-PAGE gels in duplicates. One gel was stained with Coomassie blue (Bio-Rad) and the other was used in Western blot probed with Na1-NT antibody with 1:2500 primary antibody and 1:100,000 secondary antibody and treated with Supersignal West Femto Chemiluminescent substrate (Pierce). SDS-PAGE gels and Western blot film were scanned and analyzed semiquantitatively with ImageJ software available at <http://rsb.info.nih.gov/ij/>.

Expression of Na_v1.4 genes in *Xenopus* oocytes. The full-length sequence of smNa_v1.4bL was cloned by PCRs and ligated into a vector derived from pGEMHE (Liman et al., 1992). smNa_v1.4bS was obtained by cutting the beginning sequence of smNa_v1.4bL including the first start codon out of the plasmid. The P30-deletion mutant was made by site-directed mutagenesis with QuikChange XL Mutagenesis kit (Stratagene). All plasmids constructed in this study were linearized by *Spe*I and used as templates to synthesize cRNA using T7 mMessage mMachine *in vitro* transcription kit (Ambion).

Electrophysiological experiments were performed as described previously (Liu et al., 2007). Typical Na⁺ currents were obtained by presenting a series of voltage-clamp steps (−60 to +35 mV, 50 ms) from a holding potential of −90 mV. Current–voltage (*I*–*V*) relationships were plotted. Conductance was calculated by $G = I/(V_m - V_{rev})$ and normalized conductance–voltage curves were fit with a Boltzmann equation. Steady-state inactivation curves were obtained by presenting a test pulse (50 ms, −10 mV) after a series of conditioning pulses (−100–0 mV, 50 ms) with intervals of 1 s for smNa_v1.4b and 10 s for hNa_v1.4 at −90 mV to allow full recovery from inactivation. Currents were normalized and fit with a Boltzmann equation. The time constant of fast inactivation (τ_h) was tested by a depolarization step from −90 to −10 mV. τ_h was extracted from a single exponential fit over the range where the current decayed from 90 to 10%. Recovery from fast inactivation was studied by two pulses protocol. Cells were prepulsed to −10 mV for 50 ms to inactivate all of the current, and then recovery potential at −90 mV with increasing recovery durations were applied before the test pulse to −10 mV (50 ms) to assay the fraction of current recovered. Normalized peak current amplitudes were plotted and fitted to a single exponential function and the time constant was determined. Data analysis was performed using a combination of Clampfit 8.2 (Molecular Devices) and Origin 7.0 (OriginLab).

Figure 2. Identification of a novel Na_v1.4 with an extended N terminus in *Sternopygus*. **A**, RT-PCR showed alternative splicing patterns in the electric organ (E) and skeletal muscle (M) of *Sternopygus macrurus* and in the skeletal muscle of the zebrafish (*Danio rerio*) and the rat (*Rattus norvegicus*). The DNA ladder (L) is in base pairs. **B**, Schematic illustration of the alternative splice pattern of Na_v1.4 in the three species. Each block represents an exon with the assigned name marked above. The size of each intron and exon is marked below if known.

All values are presented as mean ± SEM. Statistical differences were derived from one-way ANOVA using SPSS (SPSS). Linear correlation between gene expression and EOD frequency was done in Origin 7.0. A summary of the results of all statistical tests is given in supplemental Tables 2–4 (available at www.jneurosci.org as supplemental material).

Results

Identification of the cDNA sequence of a novel Na_v1.4 with an extended N terminus

Partial sequence of smNa_v1.4b, one of the two mammalian Na_v1.4 homologs, was identified previously (Lopreato et al., 2001). Using RT-PCR and 5' and 3' RACE to obtain the full-length sequence of smNa_v1.4b, we observed two transcripts of this gene in EO and muscle of *Sternopygus* (Figs. 1, 2A). Interestingly, the longer band showed an extraordinary dominance in the EO, in contrast to comparable abundances of the two transcripts in the muscle. Further cloning and sequencing of the genomic sequence around the beginning of smNa_v1.4b revealed that this gene has two exclusive first exons that we named 1S and 1L according to their sizes, separated by at least 1.2 kb (Fig. 2B). The

most significant difference between the two alternative first exons is that exon 1L contains a start codon but exon 1S does not (Fig. 2B); thus, the two mRNA transcripts will be translated into two different proteins. One protein (smNa_v1.4bL, translated from exon 1L) has an extra 51 aa segment in the N terminus, which makes its N terminus ~50% longer than the other (smNa_v1.4bS), of which the translation starts in the second exon. Structural analyses (Chou and Fasman, 1974; Garnier et al., 1978) predict a helix in the middle of this extended N-terminal segment of Na_v1.4b; this helix contains six positively charged amino acids and it is adjacent to a possible proline hinge (Fig. 3). The extra 51 aa N-terminal segment in smNa_v1.4bL is composed of 41% nonpolar and 59% polar and charged residues. Hydrophilicity predictions suggest that this segment is located inside the cell.

We wished to determine whether this pattern of splicing is unique to this species, found more generally in teleosts, or widely observed in other vertebrates so we examined the mRNA splicing pattern of Na_v1.4b in the zebrafish (*Danio rerio*) and Na_v1.4 in the rat (*Rattus norvegicus*). In the zebrafish skeletal muscle, the pattern of two transcripts is very similar to that of *Sternopygus* (Figs. 2, 3): exon 1L also contains a start codon, the extra segment of N terminus is translated from exon 1L, and the helical structure and multiple positively charged residues in this helix all show high similarities to those in *Sternopygus* (Fig. 3). In rat muscle, we also identified two rNa_v1.4 transcripts (Fig. 2A), but in a different pattern: both transcripts include a distant exon 1 but one transcript splices out a proximal cassette exon 1A (Fig. 2B, supplemental Table 1, available at www.jneurosci.org as supplemental material).

Neither the distant first exon nor the proximal cassette exon contains a start codon; thus, the two transcripts will be translated into identical protein products, although the presence of the cassette exon may still affect the translation process. The transcript containing the cassette exon is novel. Its abundance seems fairly low in the leg muscle. The exact locations of the exons and introns in zebrafish and rat were identified in their genomes (supplemental Table 1, available at www.jneurosci.org as supplemental material). Notably, the distance between exon 1S and 1L is 34.4 kb, far longer than the average intron size in zebrafish (0.2 kb) and other animals (Vinogradov, 1999; Mattick and Gagen, 2001).

Immunoprecipitation and Western blot confirm the existence of smNa_v1.4bL protein

Bioinformatic analysis of start codons on NetStart 1.0 prediction server (<http://www.cbs.dtu.dk/services/NetStart/>) ranked the start codon in exon 1L and the first start codon in exon 1S with the second and ninth highest scores of all 56 predicted start codons in the whole sequence, suggesting that both of these start

Figure 3. Extended N terminus of smNav_{1.4bL}. **A**, Alignment of translated N-terminal amino acid sequences of Na_v1.4b in *Sternopygus macrurus* (smNav1.4bL) and the zebrafish *Danio rerio* (drNav1.4bL), the other EO-expressing Na⁺ channel gene from *S. macrurus* (smNav1.4a), and their human ortholog (hNav1.4). Residues with similar functionality are shaded. Round dots mark the positively charged residues in the predicted helical region of the extended N terminus of smNav_{1.4bL}. A square marks the proline residue immediately after the helix and mutated in this study. **B**, Immunoprecipitation of Na⁺ channel with N-terminal specific antibody followed by labeling with a pan-Na⁺ channel antibody on Western blots confirms the translation of the extended N terminus and the expression of the long splice form of smNav_{1.4bL} in *Sternopygus* muscle and EO.

Figure 4. Properties of hNav_{1.4}, smNav_{1.4bS}, and smNav_{1.4bL} expressed in *Xenopus* oocytes. **A**, Averaged peak current traces show that smNav_{1.4bL} inactivates faster than smNav_{1.4bS} and both are faster than their human ortholog hNav_{1.4}. **B**, The three channels show no significant difference in voltage-dependent activation. **C**, smNav_{1.4bL} shows a leftward shift of steady-state inactivation compared with the other two channels. Results of hNav_{1.4} and smNav_{1.4bS} in this figure and Figure 5A–D were published previously by Liu et al. (2007) and replotted with smNav_{1.4bL} for comparison.

Table 1. Biophysical properties of expressed Na⁺ channels

	hNav _{1.4}	smNav _{1.4bS}	smNav _{1.4bL}	smNav _{1.4bL} -P30Δ
Activation V _{1/2} (mV)				
α only	−28.66 ± 3.3, n = 8	−28.53 ± 1.51, n = 10	−26.86 ± 0.70, n = 15	−27.99 ± 0.98, n = 17
α + β1L	−24.60 ± 1.35, n = 24	−30.30 ± 0.99, n = 14	−28.07 ± 1.42, n = 8	−26.90 ± 0.80, n = 7
α + β1S	−25.69 ± 1.17, n = 16	−31.16 ± 1.38, n = 9	−28.74 ± 1.18, n = 16	N/A
Inactivation V _{1/2} (mV)				
α only	−48.35 ± 2.37, n = 11	−43.01 ± 1.48, n = 21	−58.98 ± 0.94, n = 15	−53.91 ± 0.78, n = 21
α + β1L	−52.76 ± 0.92, n = 24	−59.22 ± 1.33, n = 10	−61.85 ± 1.15, n = 7	−53.56 ± 1.73, n = 11
α + β1S	−52.02 ± 0.93, n = 16	−56.18 ± 1.19, n = 24	−61.03 ± 1.31, n = 13	N/A
Inactivation τ _h (ms)				
α only	9.04 ± 0.18, n = 26	2.63 ± 0.10, n = 14	1.31 ± 0.05, n = 14	2.45 ± 0.19, n = 26
α + β1L	1.23 ± 0.08, n = 23	1.81 ± 0.15, n = 12	0.78 ± 0.03, n = 7	1.72 ± 0.16, n = 15
α + β1S	2.09 ± 0.14, n = 21	1.95 ± 0.11, n = 22	1.08 ± 0.08, n = 23	N/A
Recovery τ (ms)				
α only	N/A	825.8 ± 74.3, n = 6	2.99 ± 0.28, n = 14	2.63 ± 0.27, n = 6
α + β1L	N/A	1.68 ± 0.17, n = 12	1.39 ± 0.14, n = 7	1.10 ± 0.05, n = 5
α + β1S	N/A	1.60 ± 0.18, n = 4	1.61 ± 0.30, n = 7	N/A

p values from ANOVA between groups are listed in supplemental Tables 2–4 (available at www.jneurosci.org as supplemental material).

codons have a high probability of being translation initiation sites.

To confirm that this novel N terminus is translated and part of the mature Na⁺ channel, we generated a polyclonal antibody against the α-helix (Fig. 3A) in this region. We used this antibody to immunoprecipitate solubilized proteins from EO and muscle, then probed them with a pan-Na channel antibody (the epitope is identical to smNav_{1.4b} sequence) in a Western blot. The presence of a band at ~280 kDa (Fig. 3B) confirms that the smNav_{1.4bL} splice form is indeed translated in EO and muscle.

Figure 5. $\beta 1$ subunits differentially modulate smNav1.4bL and smNav1.4bS. **A, B.** Both alternatively spliced $\beta 1$ subunits speed up the inactivation (**A**) and negatively shift the steady-state voltage dependence of inactivation (**B**) of smNav1.4bS. **C.** The $\beta 1L$ subunit slightly but significantly speeds up the inactivation of smNav1.4bL, whereas $\beta 1S$ has no effect. **D.** $\beta 1$ subunits do not shift the voltage dependence of steady-state inactivation of smNav1.4bL. **E.** smNav1.4bS shows extraordinarily slow recovery from inactivation, but the recovery is much faster when coexpressed with a $\beta 1$ subunit (for clarity, only $\beta 1L$ is illustrated). **F.** In contrast, smNav1.4bL shows fast recovery from inactivation with or without a $\beta 1$ subunit.

The extended N terminus influences Na⁺ current inactivation

To examine whether the extended N terminus contributes to Na⁺ channel function, we constructed plasmids containing full-length sequences of smNav1.4bL and smNav1.4bS and injected cRNA into *Xenopus* oocytes. We compared the activation and inactivation voltage dependencies (as determined by $V_{1/2}$ values from a Boltzmann equation) and time constants of fast inactivation and rate of recovery from inactivation (as determined by fits with a single exponential function) of smNav1.4bL, smNav1.4bS, and their human ortholog hNav1.4.

The three channels did not differ in their voltage dependence of activation (Fig. 4B, Table 1). smNav1.4bS and hNav1.4 showed similar voltage dependences for steady-state inactivation. However, smNav1.4bL inactivated at significantly more negative membrane potentials than the other two channels ($p < 0.01$).

smNav1.4bS inactivated significantly more rapidly than hNav1.4, but smNav1.4bL inactivated more rapidly than both of these ($p < 0.001$) (Fig. 4A, Table 1). In the absence of a β subunit, Na_v1.4bS recovered from inactivation slowly (Fig. 5E, Table 1). Surprisingly, even without a β subunit, smNav1.4bL recovered from inactivation very rapidly (~ 350 times faster than smNav1.4bS) (Fig. 5F, Table 1). Because the only difference between Na_v1.4bS and Na_v1.4bL is the extended N-terminal segment, the increase in the speed of fast inactivation, the negative shift in voltage dependence of steady-state inactivation, and the significant enhancement of recovery from inactivation must be caused by the extended N-terminal segment. These are all attributes that are conferred on Na⁺ currents by coexpression with a β subunit.

$\beta 1$ subunits have only a small effect on smNav1.4bL

Because the extended N-terminal segment appeared to substitute for a β subunit, we reasoned that the response of the Na_v1.4bL splice form to coexpression with a β subunit would be attenuated or absent. Because the EO evolved from skeletal muscle and the $\beta 1$ subunit is the only auxiliary subunit found in skeletal muscle, we coexpressed the α subunits with the $\beta 1$ subunit. The $\beta 1$ subunit of teleosts itself has two alternative splice forms found recently by us and two other groups (Chopra et al., 2007; Fein et al., 2007; Liu et al., 2007), and we coexpressed each α subunit splice form with both $\beta 1$ subunit splice forms from *Sternopygus*.

The coexpression of either $\beta 1$ subunit had no effect on voltage dependence of activation of any of the channels. Coexpression of either $\beta 1$ splice variant with smNav1.4bS [as in the study by Liu et al. (2007)] significantly shifted steady-state inactivation curve leftward by ~ 20 mV ($p < 0.001$), sped up τ_h ($p < 0.001$), and drastically increased the rate of recovery from inactivation ($p < 0.001$) (Fig. 5, Table 1). Indeed, the addition of a $\beta 1$ subunit to smNav1.4bS caused it to behave much like smNav1.4bL alone. Coexpression of either β subunit with smNav1.4bL had no effect on steady-state inactivation. Coexpression with the $\beta 1L$ subunit sped up τ_h ($p < 0.05$) and recovery from inactivation ($p < 0.01$). The $\beta 1S$ subunit had no significant effects on τ_h or recovery from inactivation. Thus, the N terminus substitutes for and attenuates the response to the $\beta 1$ subunit.

Deletion of a proline strongly affects inactivation kinetics

The rigid structure of proline often forms a sharp bend in a peptide chain. Several prolines are located between the two predicted α helices in the N terminus of smNav1.4bL (Fig. 2A), including a proline (P30) immediately after the predicted helix in the ex-

tended N-terminal segment of smNav_v1.4bL; this proline is conserved in zebrafish. In an initial attempt to understand the function of the extended N-terminal sequence, this proline was deleted from smNav_v1.4bL by site-directed mutagenesis. This is expected to sharply divert the orientation of the positively charged helix.

Not surprisingly, deletion of this proline had no effect on the voltage dependence of activation (Fig. 6B, Table 1). However, the proline deletion slowed the inactivation speed of smNav_v1.4bL almost twofold ($p < 0.001$), making it statistically indistinguishable from smNav_v1.4bS (Fig. 6A, Table 1). The voltage dependence of steady-state inactivation was shifted rightward (Fig. 6C), but was not significantly different from wild type smNav_v1.4bL. The proline deletion had no effect on the rapid recovery from inactivation of smNav_v1.4bL (Fig. 6D). The proline deletion, therefore, dissociated the processes of fast inactivation and recovery from inactivation.

Similar to wild-type smNav_v1.4bL, co-expression of β 1L with mutant P30 deletion sped τ_h ($p < 0.05$) and recovery from inactivation ($p < 0.05$), but had no significant effect on voltage dependence of activation and inactivation (Table 1).

Tissue distribution pattern of smNav_v1.4b transcripts

5' RACE of Na_v1.4b in *Sternopygus* suggested a preferential expression of exon 1L in the EO, despite the longer size of its amplicon and, therefore, presumably lower amplification efficacy in PCRs (Fig. 2A). To further compare the expression level of each transcript between EO and muscle, we performed semiquantitative RT-PCR with primers specifically designed for exon 1L and 1S and total RNA isolated from pooled tissues of three medium-EOD-frequency (~90 Hz) fish. The same amount of total RNA from each tissue was used in RT-PCRs and amplified for 24, 27, 30, and 33 cycles. Because of possibly different amplification efficacies of different primer pairs, comparisons can only be made on the same transcript between tissue types, but not on different transcripts in the same tissue. Figures 2A and 7A show that smNav_v1.4bL is expressed in EO and muscle at comparable levels, whereas smNav_v1.4bS is strongly repressed in EO.

smNav_v1.4a and smNav_v1.4b are expressed in electric organ at comparable levels

We next compared the expression of total smNav_v1.4b with smNav_v1.4a in the EO. One-step RT-PCRs were set up with primers designed to amplify a shared region, but with different lengths, in smNav_v1.4a and smNav_v1.4b (519 and 450 bp, respectively). Total RNA was isolated from the same sample of EOs from three medium-EOD-frequency fish as above. Reactions were stopped every third cycle between 24 and 39 cycles and loaded on an agarose gel (Fig. 7B). The results showed smNav_v1.4a (top band) and smNav_v1.4b (bottom band) have comparable abundance in fish with midfrequency EODs.

Figure 6. Deletion of a proline in the extended N terminal partially abolishes the difference between smNav_v1.4bL and smNav_v1.4bS. **A**, Representative current traces show that the proline deletion slows the inactivation of smNav_v1.4bL. **B**, The three channels show no significant difference in voltage-dependent activation. **C**, The deletion of the proline shifts the voltage-dependent inactivation toward the direction of smNav_v1.4bS. **D**, The deletion of proline does not significantly change the recovery from inactivation.

mRNA level of smNav_v1.4bL correlates with EOD frequency

We then used real-time quantitative RT-PCR to examine the mRNA levels of smNav_v1.4a (that does not appear to be spliced), total smNav_v1.4b, and the smNav_v1.4bL splice form (levels of Na_v1.4bS were too low in all fish for accurate detection with a one-step PCR procedure) in samples of EO from a number of fish across the EOD frequency range to test whether the levels of their mRNA vary with EOD frequency. The amplification of the most variable region in these two Na⁺ channels, the domain II-III loop, was examined to assay mRNA levels of smNav_v1.4b and smNav_v1.4a for maximum specificity.

The mRNA level of smNav_v1.4a showed no significant difference between high- (>100 Hz; 0.96 ± 0.14) and low-EOD-frequency (<80 Hz; 1.00 ± 0.13 , low-frequency group normalized to 1.0) fish when fish were grouped into two frequency classes, nor did the mRNA level of smNav_v1.4a correlate with EOD frequency (Fig. 8A). However, the total mRNA level of smNav_v1.4b was significantly higher in high- (1.39 ± 0.13) than in low-EOD-frequency (1.00 ± 0.05) fish and the normalized mRNA level of smNav_v1.4b positively correlated with EOD frequency (Fig. 8B).

Primers were designed to exon 1L of Na_v1.4bL to specifically measure its abundance. Low-EOD-frequency fish had significantly lower levels of smNav_v1.4bL mRNA than high-EOD-frequency fish (1.00 ± 0.13 ; high, 2.71 ± 0.49) and the mRNA level of Na_v1.4bL positively correlated with the EOD frequency (Fig. 8C). The low level of Na_v1.4bS mRNA in the EO across the EOD frequency range suggests it plays little or no role in determining the characteristics of the Na⁺ current in the EO (although it is likely to be important in muscle).

Differences in Na_v1.4bL were confirmed on the protein level using a pan-Na⁺ channel antibody to immunoprecipitate all Na⁺ channel protein from the EO and then probing the

Figure 7. Semiquantitative RT-PCR reveals the expression profiles of Na⁺ channels in muscle and electric organ of *Sternopygus*. **A**, RT-PCR with primers specific to smNav1.4bL or smNav1.4bS. The same amount of total RNA from the electric organ and skeletal muscle was loaded as template. One-step RT-PCRs were set up identically but stopped after different numbers of cycles. Comparison is made between tissue types for each splice form, showing that smNav1.4bL (L) has a comparable abundance in muscle and electric organ whereas smNav1.4bS (S) is very low in the electric organ. **B**, smNav1.4a and smNav1.4b have a comparable abundance in the electric organ of midfrequency EOD (~90 Hz) fish. One-step RT-PCRs with primers in conserved regions were set up identically but stopped after different numbers of cycles. Products with expected sizes (519 bp, top band, from smNav1.4a and 450bp, bottom band, from smNav1.4b) were separated and shown in a 2% agarose gel. Comparisons made between the two genes show that they have comparable abundance in a sample of fish with a midrange EOD frequency.

Western blot with the N-terminal extension-specific antibody (Fig. 8D).

DHT affects expression of sodium channels in electric organ

DHT lowers the EOD frequency of *Sternopygus*, broadens the electrocyte action potential, and slows down the inactivation of Na⁺ current in electrocytes (Ferrari et al., 1995). We next examined whether DHT influences the expression of the two Na_v1.4 orthologs. We implanted fish intraperitoneally with DHT-filled SILASTIC capsules that significantly elevated serum DHT compared with the control group, which received empty capsules (DHT implant, 9.8 ± 2.7 ng/ml; control, 0.52 ± 0.50 ng/ml; $p < 0.0001$).

The EOD frequency of DHT-implanted fish began decreasing 3 d after the implant and was statistically significant from the controls after 7 d (control group, 123.15 ± 8.96 Hz; DHT group, 100.86 ± 3.90 Hz; t test, $p = 0.03$). We dissected the fish tails 18 d after the implant when the EOD frequency of the DHT group stabilized and still significantly differed from the control group (control group, 125.00 ± 9.94 Hz; DHT group, 83.92 ± 1.88 Hz; $p = 0.0004$). Real-time quantitative RT-PCR showed that the total smNav1.4b mRNA as well as mRNA of the Na_v1.4bL splice form were lowered (Fig. 9) (Na_v1.4b total: control group, 1.00 ± 0.13 Hz; DHT group, 0.55 ± 0.05 Hz; $p = 0.003$; smNav1.4bL: control group, 1.00 ± 0.21 Hz; DHT group, 0.53 ± 0.05 Hz; $p = 0.01$). The mRNA level of smNav1.4a was not affected by DHT (control group, 1.00 ± 0.16 Hz; DHT group, 0.91 ± 0.04 Hz; $p = 0.46$).

To locate the where DHT might act on genomic DNA, the intron between exon 1L and exon 2 and the genomic DNA sequence 1.2 kb

upstream of exon 1L were cloned by PCR and 5' RAGE. Transcriptional factor analysis on a bioinformatics server (Genomatix) identified a putative androgen receptor (AR) binding site at 702–720 bp in the intron between exon 1L and exon 2.

Discussion

A novel alternatively spliced N terminus in an Na⁺ channel

5' RAGE revealed two alternative first exons of smNav1.4b, one of which possesses a novel predicted start site that extends the N terminus by 51 aa. Recognition of the predicted novel N terminal by a specific antibody confirmed that this start site is used for translation. The same pattern of exons, including the start site, was observed in the zebrafish, suggesting that this variant may be common to other teleosts. We observed a different set of alternative exons in the 5' end of the rat ortholog (Na_v1.4) but, as is true of other Na⁺ channels with transcriptional variants at the 5' end, these were in the untranslated region (Shang and Dudley, 2005). Alternative splicing has been observed at a variety of sites in the coding region of Na⁺ channels (Sarao et al., 1991; Schaller et al., 1992; Plummer et al., 1997; Dietrich et al., 1998; Wang et al., 2003), but our finding is the first example of splicing at the N terminal. Alternative splicing of the N terminal has been noted in calcium-activated potassium channels (*slo*) in *Drosophila* (Becker et al., 1995; Bohm et al., 2000), *shaker*-type K_v channels (Stocker et al., 1990; French et al., 2005), and L-type Ca²⁺ channels (Blumenstein et al., 2002). N-terminal splicing may be a general mechanism for regulation of ion channels.

The novel splice form influences Na⁺ current inactivation

The N terminus extension causes drastic changes in multiple aspects of the inactivation of the current: it inactivates ~50% faster, there is a –16 mV shift in the $V_{1/2}$ of steady-state inactivation, and there is a strikingly faster rate of recovery from inactivation. These are the same properties that are conferred on an Na⁺ channel α subunit by coexpression with a β subunit. Thus, the extended N terminus not only influences inactivation, but it functionally substitutes for a β subunit.

The C terminus influences the rate of inactivation of Na⁺ channels (Mantegazza et al., 2001; Cormier et al., 2002). An extended N terminus may be unique to teleosts, but it raises the question as to whether the “normal” N terminus of the Na⁺ channel participates in inactivation. Surprisingly, this has not been studied in detail, but a few lines of evidence suggest that it is likely. Biochemical evidence indicates that the N and C termini interact (Sun et al., 1995; Zhang et al., 2000). The phosphorylation of a serine in the N terminus (S36) (Tateyama et al., 2003) of the cardiac Na⁺ channel (Na_v1.5) influences the inactivation of Na⁺ current, and this appears to be attributable to an interaction of this site with a phosphorylated residue in the C terminus. This serine is not conserved across Na⁺ channels, so it may be unique to this channel type. Finally, missense mutations in the N terminus of the brain-expressed Na⁺ channel Na_v1.1 are correlated with various types of epilepsy (Mulley et al., 2005) and a mutation in the N terminus of the heart-expressing Na_v1.5 is correlated with long QT syndrome (Priori et al., 2002). None of these mutations have been studied in expression systems, so it is unknown how they affect the Na⁺ current.

We do not know how the fish's extended N terminus interacts with the Na⁺ channel. The N terminus of a mammalian (rNa_v1.4) Na⁺ channel interacts with the C terminus by electrostatic forces with two helices at 13–30 (N terminus) and 1716–1737 (C terminus) (Zhang et al., 2000). Perhaps the extended N terminus interferes with such an electrostatic interaction, thereby

affecting inactivation. The basic residues are condensed in the predicted α -helical region of the extended N terminal (6 of 17 aa between 14 and 31), thus they may compete with the basic residue-rich region in the C terminus (6 of 22, 1716–1737 in hNa_v1.4). Several prolines located between the two helices of the N terminus may act as hinges to facilitate the movement of N terminus into position. Indeed, deletion of the proline closest to the helix from the smNa_v1.4bL by site-directed mutagenesis significantly perturbed the effect of the N terminus on inactivation rate, although it only modestly effected steady-state inactivation and had no effect on recovery from inactivation. This dissociation of entry into and recovery from fast inactivation by the proline mutation is supported in the literature in which various manipulations of Na⁺ channels influence one process but not the other (Dib-Hajj et al., 1997).

Expression of Na⁺ channel splice forms in muscle and electric organ

Exon 1L of Na_v1.4b is predominant in the EO, whereas the two exons are expressed equally in muscle in electric fish and zebrafish. How is the expression of 1L enhanced, or more likely, the expression of 1S repressed? One possibility is tissue-specific transcriptional factors that regulate transcription initiation. We observed multiple transcription initiation sites of smNa_v1.4b transcripts, which might be the downstream targets for tissue-specific regulations. Search for promoter sites on a bioinformatics server (http://www.fruitfly.org/seq_tools/promoter.html) revealed only one putative promoter at 850 bp upstream of exon 1L, probably because of the limited number of fish specific promoter sequences in the database. Further bioinformatics study and quantitative measurements are needed to address this question.

Androgenic regulation of sodium channel expression

Systemic administration of DHT decreases the mRNA levels of total Na_v1.4b transcript as well as the Na_v1.4bL splice form. The slopes of the lines indicating the relationship between total Na_v1.4b and EOD frequency, and Na_v1.4bL and EOD frequency, are similar (Fig. 8B,C), and the extent of suppression of total Na_v1.4b mRNA and mRNA of the Na_v1.4bL splice form by DHT are similar (Fig. 9B), suggesting that DHT regulates levels of Na_v1.4b but not its splicing.

Steroids enhance or repress gene expression via nuclear receptors (Chew and Gallo, 1998; Walters and Nemere, 2004). The steroidal repression of gene expression can involve several complex mechanisms (Dobrzycka et al., 2003). One possibility is that the DHT-AR complex may bind in the vicinity of the Na_v1.4b gene and recruit histone deacetylase and nucleosome remodeling complexes to repress gene expression. We performed 5' RAGE and identified ~1 kb genomic DNA sequence upstream of exon 1L and the intron sequence between exon 1L and exon 2 and located a putative AR binding site in the intron. Because this intron is contained in transcription products of both Na_v1.4bL

Figure 8. The expression of smNa_v1.4a, smNa_v1.4b, and the smNa_v1.4bL splice variant versus EOD frequency. Measurements are by RT-PCR and normalized to 18S RNA. **A**, mRNA level of smNa_v1.4a shows no significant correlation with each individual's EOD frequency. **B**, **C**, mRNA levels of smNa_v1.4b and its major splice variant smNa_v1.4bL are significantly higher in fish with high than with low EOD frequencies and positively correlated with EOD frequency. **D**, Protein samples from the EO of fish with high or low EOD frequencies were immunoprecipitated by a pan Na⁺ channel antibody, loaded on SDS-PAGE gels, and stained with either Coomassie blue (bottom) or blotted with the antibody against smNa_v1.4bL extended N terminus (top, slight difference in apparent molecular weight because of "smiling" on the gel). The comparison shows that a greater proportion of the total Na⁺ channel protein is Na_v1.4bL in fish with higher EOD frequencies. CTL, Control.

and Na_v1.4S, AR binding here may abort the transcription of both splice forms. Notably, this putative AR binding site is not found in the homologous 2.3 kb intron in zebrafish. An antibody against AR of *Sternopygus* is needed for experimental confirmation (such as chromatin immunoprecipitation) of the association between AR and this site, which may help reveal the mechanism of DHT repression on the Na_v1.4b gene.

The relationship between EOD pulse duration and Na⁺ channel expression

The systematic variation in EOD pulse duration is caused by coregulated variation in the activation kinetics of a delayed rectifier and the inactivation kinetics of the Na⁺ current (Ferrari et al., 1995; McAnelly and Zakon, 2000). The rate of inactivation of the electrocyte Na⁺ current varies systematically with the most rapid inactivation in mature females (τ = 0.5–1.0 ms) to the slowest in males (τ = 4.0–4.7 ms) (Ferrari et al., 1995).

Figure 10 illustrates schematically how the transcript abundance of Na_v1.4a, Na_v1.4bL (Na_v1.4bS levels are so low that we do not believe they play a role in the EO), and the total levels of β 1 are expressed in the EO as a function of EOD waveform. Fish with the shortest EOD pulse durations (and highest EOD frequencies) have the highest levels of total β 1 subunit (Liu et al., 2007) and smNa_v1.4bL, whereas smNa_v1.4a is expressed at a constant level. We have not been able to express Na_v1.4a in *Xenopus* oocytes, but we propose that Na_v1.4a possesses slow inactivation kinetics as it is the predominant channel expressed in male electrocytes and the Na⁺ current of males inactivates slowly.

Figure 9. DHT lowered the EOD frequency and the expression of smNav1.4b. **A**, DHT implants lowered the EOD frequency over a period of 18 d. The EOD frequency was significantly different by 7 d after the DHT implant. **B**, Real-time quantitative RT-PCR shows that the expression of smNav1.4b and its splice variant Nav1.4bL are lowered by DHT implants. There is no significant change in the expression of smNav1.4a. [The data in **A** have been published previously in and are adapted from Liu et al. (2007), their Fig. 7A]. CTL, Control.

We hypothesize that the graded increase in the rate of inactivation of Na⁺ current in fish with ever-shorter EOD pulses derives from two factors. First, the increasing ratio of rapidly inactivating smNav1.4bL channels to (hypothetically) slowly inactivating Nav1.4a channels in the total population of Na⁺ channel α subunits would increase the overall speed of Na⁺ current inactivation. Second, increases in the total level of the β 1 subunit would likely cause Na⁺ currents to inactivate more rapidly. Fish have two β 1 splice forms that have nearly identical effects on Na⁺ currents (Fein et al., 2007; Liu et al., 2007). Thus, we consider only total levels of β 1. β 1 subunits have a slight effect on Nav1.4bL as these currents already inactivate rapidly. However, as the total level of the β 1 subunit rises with respect to Nav1.4a, a greater number of Nav1.4a channels will have an associated β 1 subunit and will inactivate more rapidly. This would be most likely if the affinity of the β 1 subunit were greater for Nav1.4a than Nav1.4bL. Future experiments will allow us to test this hypothesis.

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Figure 10. A hypothesis for molecular regulation of the rate of inactivation of the Na⁺ current in the *Sternopygus* electrocyte. **A**, Schematic illustration of how mRNA levels of smNav1.4a, smNav1.4bL, and the β 1 subunit vary with systematic variation in EOD waveform and electrocyte Na⁺ current. **B**, We hypothesize that smNav1.4a inactivates slowly. In this study, we show that smNav1.4bL activates rapidly. We hypothesize that two factors regulate the rate of inactivation: (1) as the ratio of smNav1.4bL to smNav1.4a increases, the average rate of inactivation of the population of Na⁺ channels will be faster; (2) because β 1 subunits speed the rate of inactivation of Na⁺ currents, the increase in levels of β 1 will speed both currents, especially Nav1.4a.

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