A highly-polarized excitable cell separates sodium channels from sodium-activated potassium channels by more than a millimeter.

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Running Head: Electrocyte morphology and ion channel localization
ABSTRACT

The bioelectrical properties and resulting metabolic demands of electrogenic cells are determined by their morphology and the subcellular localization of ion channels. The electric organ cells (electrocytes) of the electric fish *Eigenmannia virescens* generate action potentials (APs) with Na\(^+\) currents >10 microamperes and repolarize the AP with Na\(^+\)-activated K\(^+\) (K\(_{Na}\)) channels. To better understand the role of morphology and ion channel localization in determining the metabolic cost of electrocyte APs, we used two-photon 3D imaging to determine the fine cellular morphology and immunohistochemistry to localize the electrocytes' ion channels, ionotropic receptors, and Na\(^+\)/K\(^+\) ATPases. We found that electrocytes are highly polarized cells ≈1.5 mm in anterior-posterior length and ≈0.6 mm in diameter, containing approximately 30,000 nuclei along the cell periphery. The cell's innervated posterior region is deeply invaginated and vascularized with complex ultrastructural features while the anterior region is relatively smooth. Cholinergic receptors and Na\(^+\) channels are restricted to the innervated posterior region, while inward rectifier K\(^+\) channels and the K\(_{Na}\) channels that terminate the electrocyte AP are localized to the anterior region, separated by >1 millimeter from the only sources of Na\(^+\) influx. In other systems submicron spatial coupling of Na\(^+\) and K\(_{Na}\) channels is necessary for K\(_{Na}\) channel activation. However, our computational simulations showed that K\(_{Na}\) channels at a great distance from Na\(^+\) influx can still terminate the AP suggesting that K\(_{Na}\) channels can be activated by distant sources of Na\(^+\) influx and overturning a long-standing assumption that AP-generating ion channels are restricted to the electrocyte's posterior face.
INTRODUCTION

Action potentials (APs) are transient changes in membrane voltage that are typically initiated by inward Na\(^+\) current (I\(_{Na}\)) and terminated by outward K\(^+\) current (I\(_{K}\)). These currents are driven by ionic concentration gradients across the cell membrane (Bean, 2007) and transmembrane Na\(^+\)/K\(^+\) ATPases consume energy to restore the ionic gradients after each AP. Total Na\(^+\) influx during the AP determines ATPase activity and therefore the resulting metabolic cost of the AP (Attwell and Laughlin, 2001; Howarth et al., 2012; Niven and Laughlin, 2008). Any temporal overlap of inward I\(_{Na}\) and outward I\(_{K}\) reduces energy efficiency, but this overlap is often necessary in fast-spiking cells where the need to maintain brief APs requires that I\(_{K}\) begins while I\(_{Na}\) is still active to terminate the AP quickly. This incomplete inactivation of Na\(^+\) channels during AP repolarization can result in the entry of twice as much Na\(^+\) as the theoretical minimum, significantly reducing energy efficiency at high AP frequencies (Carter and Bean, 2009).

The weakly electric fish *Eigenmannia virescens* generates electric organ discharges (EODs) to navigate and communicate in darkness (Hopkins, 1974). Because they generate APs at steady frequencies of 200-600Hz (Scheich, 1977) with underlying Na\(^+\) currents that can exceed 10 microamperes (Markham et al., 2013), the electric organ cells (electroctyes) create extremely high metabolic demands (Lewis et al., 2014). High rates of ATP hydrolysis by the Na\(^+\)/K\(^+\) ATPases are therefore necessary to remove Na\(^+\) from the cell between APs. The simultaneous APs of \(>1000\) electroctyes during each EOD further magnify the metabolic cost of signal production and as a result EOD amplitude is highly sensitive to metabolic stress (Reardon et al., 2011).

*E. virescens* electroctyes are large cells >1mm in length, each innervated by spinal motoneurons at a cholinergic synapse on the morphologically complex posterior membrane (Fig.
1B) (Schwartz et al., 1975). Only the posterior membrane generates an AP with the anterior membrane presumed to be electrically passive (Bennett, 1961, 1971). These findings together led to a long-standing assumption that the ion channels responsible for producing the AP are restricted to the posterior region of the cell. Electrocytes express an inwardly rectifying K\textsuperscript{+} current, a voltage-gated Na\textsuperscript{+} current and a Na\textsuperscript{+}-activated K\textsuperscript{+} (K\textsubscript{Na}) current that terminates the AP. The expression of K\textsubscript{Na} channels instead of voltage-gated K\textsuperscript{+} (K\textsubscript{v}) channels increases AP energy efficiency by reducing the overlap of I\textsubscript{Na} and I\textsubscript{K} during the electrocyte AP (Markham et al., 2013).

Early biochemical studies identified Na\textsuperscript{+}/K\textsuperscript{+} ATPases expressed on both the anterior and posterior membranes (Denizot, 1982), but the spatial distribution of the cholinergic receptors and ion channels is not yet known. A full account of the electrocyte's fine morphology and distribution of ionotropic receptors and ion channels is a crucial first step toward understanding the interplay of the ionic currents that determine the spatiotemporal dynamics of intracellular Na\textsuperscript{+} concentrations which regulate K\textsubscript{Na} channel activation, ATPase activity, and ultimately the metabolic burden of EOD production.

We therefore used confocal laser-scanning fluorescence microscopy and immunohistochemistry to identify the electrocyte's fine 3D structure and the subcellular localization of their cholinergic receptors, ion channels, and ion transporters. We found that cholinergic receptors and Na\textsuperscript{+} channels were restricted to the innervated posterior region, while inward rectifier K\textsuperscript{+} channels and K\textsubscript{Na} channels were localized over 1 mm away in the anterior region. Confirming earlier results (Denizot, 1982), Na\textsuperscript{+}/K\textsuperscript{+} ATPases were densely expressed on both the posterior and anterior membranes. These findings are unexpected because K\textsubscript{Na} channels that terminate the electrocyte AP and the anterior-membrane Na\textsuperscript{+}/K\textsuperscript{+} ATPases are separated by more than a millimeter from the only sources of Na\textsuperscript{+} influx that would activate them. Our
computational simulations of electrocyte APs and Na\textsuperscript{+} dynamics confirm that K\textsubscript{Na} channels, even at a distance of more than 1 mm from Na\textsuperscript{+} influx, can terminate the electrocyte AP and maintain high-frequency firing. This discovery is particularly surprising in light of data from other systems where micron-scale spatial coupling of K\textsubscript{Na} and Na\textsuperscript{+} channels is necessary for K\textsubscript{Na} channel activation (Budelli et al., 2009; Hage and Salkoff, 2012).

**MATERIALS AND METHODS**

**Animals**

Fish were wild-caught male and female *Eigenmannia virescens* (Glass Knife-fish). Animals were from tropical South America, obtained from tropical fish importers and ranging in size from 12 to 19 cm. Fish were housed in groups of 4-10 in 40-liter or 10-liter tanks in a recirculating aquarium system at 28 ± 1 °C with water conductivity of 200-500 μS/cm. The EOD of *E. virescens* is a sinusoidal wave with a frequency of 250-600 Hz (Fig. 1F). Each positive-going pulse of the wave is a single EOD (Fig. 1D,E), and these occur at regular intervals under the control of a medullary pacemaker nucleus (Fig. 1A,B).

We harvested electric organ (EO) tissue from *E. virescens* by cutting off a small (1-2 cm) piece of the narrow tail filament, consisting of only soft tissue and composed almost entirely of the EO (Fig. 1C). This brief procedure lasts less than 15 seconds and is performed without anesthesia because induction and recovery from immersion anesthesia are more harmful to the fish than the tail-clip itself. The tail and EO regenerate within two months.

To obtain mouse brains for validating antibodies used in the present study, five C57BL/6J mice (The Jackson Laboratory, stock #664) were deeply anesthetized by isoflurane inhalation (5% (v/v) isoflurane in oxygen), then decapitated and brains were quickly removed and frozen in liquid nitrogen.
All methods were approved by the Institutional Animal Care and Use Committee of The University of Oklahoma, and complied with the guidelines given in the Public Health Service Guide for the Care and Use of Laboratory Animals.

**Confocal Imaging and 3D reconstructions**

**Microinjection**

We harvested a ≈2 cm section of the tail, removed the overlying skin, and pinned the exposed EO tissue in a Sylgard® (Dow Corning Corporation) coated petri dish containing normal saline (114 mM NaCl, 2 mM KCl, 4 mM CaCl₂·2H₂O, 2 mM MgCl₂·6H₂O, 2 mM HEPES, and 6 mM glucose; pH to 7.2 with NaOH). Temperature of the preparation was stable at room temperature (22±1°C). Rhodamine B or Alexa Fluor® 594 dextran (10,000MW) (Life Technologies) were prepared as a 1% w/v solution in water. Precipitate within the dextran solution was removed by centrifugation at 12,000 × g for 5 min. Microinjections were performed using an automatic nanoliter injector ("Nanoject II”, Drummond Scientific Company). Micropipettes for injection were drawn from borosilicate glass capillaries (Drummond Scientific Company) with a laser micropipette puller (Model P-2000, Sutter Instruments). We injected 13.8 nL dextran solution into the cytoplasm of 4-5 electrocytes in each sample of EO with the constant injection speed of 23 nL/sec. The EO tissue with Rhodamine B or Alexa Fluor® 594 dextran-injected electroctyes was then held in normal saline at room temperature (22±1°C) for 15 minutes until the dextran fully diffused into injected electrocytes. We then proceeded immediately to image the live cells directly, or fix and section the tissue before mounting and imaging.
**Vibratome sectioning**

We fixed the Alexa Fluor® 594 dextran-injected EO tissue in 2% paraformaldehyde buffered with 1× phosphate-buffered saline (PBS) overnight at 4°C, and washed six times for 15 min each in 1× PBS. The EO tissue was then embedded in 3% agar, trimmed and glued to a vibratome chuck with cyanoacrylate. The chuck was mounted on a vibratome (Leica Series 1000) and 100-μm sections were cut and mounted on microscope slides with VectaShield® with DAPI (Vector Laboratories).

**Confocal imaging**

Live electrocytes were imaged in situ on a Leica® TCS SP8 laser scanning confocal microscope, with a Coherent Chameleon mode-locked Ti:sapphire laser and a 25x/0.95NA dipping objective. The images were acquired as serial sections through the entire electrocyte using intensity compensation via increasing detector gain. The entire electrocyte was imaged using a 2×4 tiled scan with a 30% overlap between adjacent images. The images were then rendered using a 3D shaded projection in Avizo® Fire 8.0.1 (FEI Visualization Sciences Group).

Fixed electrocyte sections were imaged on a Leica® TCS SP8 laser scanning confocal microscope, using a 63x/1.3NA glycerol objective with a correction collar and an argon laser, 488nm laser line to observe tissue autofluorescence, and a DPSS 561nm laser line to excite the Alexa® 594. The images were acquired via serial sections with a voxel size of 160nm × 160nm × 160nm and intensity compensation via increasing laser output. The images were then rendered using Imaris® x64 7.6.5 (Bitplane). Electrocyte nuclei were counted by determining the number of DAPI-stained nuclei colocalized with Alexa 594 within an image series from the anterior end, posterior end, or cell body, and then extrapolating to the total observed volume of each electrocyte region.
**Western Blot**

EOs and mouse brains were isolated from animals and flash frozen in liquid nitrogen. Tissues were ground into fine powder using a pre-chilled pestle in a mortar filled with liquid nitrogen. 15 mg mouse brain and 100 mg *E. virescens* EO tissue powder were dissolved in 1 mL 1× NuPAGE® LDS sample buffer (Life Technologies) containing 2.5 % (v/v) 2-mercaptoethanol (Amresco), then heated at 70°C for 10 min. After heating, protein samples were centrifuged at 17,000 × g for 10 min to remove DNA. The collected supernatants were run on a NuPAGE® Novex® 4-12% Bis-Tris protein gradient gel (Life Technologies) then transferred onto a PVDF membrane using an iBlot® dry blotting system (Life Technologies). Membranes were blocked in 1× Tris-buffered saline (TBS) containing 0.1% Tween20 (Sigma-Aldrich) (TBST) and 5% nonfat dry milk for 1 h at room temperature under agitation. After blocking, membranes were sequentially incubated with primary antibodies diluted in TBST containing 5% nonfat dry milk and 0.02% Sodium Azide (Sigma-Aldrich) including rabbit polyclonal anti-kcnt1 (Aviva Systems Biology) 1:200, rabbit polyclonal anti-Pan Na, (Alomone Labs) 1:200, and mouse monoclonal antibody against the α subunit of Na⁺/K⁺ ATPase (a5, developed by D.M. Fambrough (Lebovitz et al., 1989), and obtained from the Developmental Studies Hybridoma Bank (DSHB) at University of Iowa) 1:1000 at room temperature for 2 h under agitation. After primary antibody incubation, membranes were washed three times for 5 min with TBST. Then membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology) diluted in TBST containing 5% nonfat dry milk (1:5000 and 1:2000 for goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP respectively) for 1 h at room temperature. After washing, proteins on the membrane were detected using the Amersham™ ECL™ prime western blotting detection reagent (GE Healthcare) and imaged with a Chemi Doc™ XRS+
imaging system (Bio-Rad). Exposure time was selected manually depending on the observed signal intensity. The membranes stained with polyclonal anti-Pan Na, and mouse monoclonal antibody against the α subunit of Na⁺/K⁺ ATPase were cut to separate the EO and mouse brain strips then exposed separately due to the large difference in signal intensity between E. virescens EO and mouse brain. Molecular weights of the detected protein were determined by loading Precision Plus Protein™ Kaleidoscope™ standards (Bio-Rad) together with protein samples into the same gel. Final processing of the images was performed with ImageJ-win64 version 1.44B (National Institutes of Health).

**Immunohistochemistry**

Sections of EO were embedded completely in OCT compound, flash frozen in liquid nitrogen-chilled isopentane (Sigma-Aldrich) and stored at -80°C until further processing. Serial longitudinal sections (15-20 µm thick) were cut at -25°C using a cabinet cryostat (Leica CM 1900), mounted on gelatin-subbed slides and air-dried overnight at room temperature. Tissue sections were then fixed in 4% paraformaldehyde (Electron Microscopy Sciences) buffered with 1×PBS for 20 min, subsequently washed three times for 5 min each in 1× PBS containing 0.05% Tween-20 (Sigma-Aldrich) (PBST) and blocked in PBST containing 2% bovine serum albumin (BSA) and 5% goat normal serum (Jackson ImmunoResearch) for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies diluted in PBST. After primary antibody incubation, tissue sections were washed as above, and incubated with Alexa Fluor® 488 or 594-conjugated secondary antibodies (Jackson ImmunoResearch) 1:200 diluted in PBST for 1 h at room temperature in a humidified chamber. Sections were then washed and air dried. Slides were mounted using VectaShield® with DAPI (Vector Laboratories) and kept in the dark at 4°C.
A rabbit polyclonal antibody (1:100) against an intracellular epitope of Na,1.x channels (Anti-Pan Na+, obtained from Alomone Labs) was used to label voltage-gated Na⁺ channels. A mouse monoclonal antibody (1:100) against the α subunit of Na⁺/K⁺ ATPase (α5, developed by D.M. Fambrough (Lebovitz et al., 1989), and obtained from the Developmental Studies Hybridoma Bank (DSHB) at University of Iowa) was used to label Na⁺/K⁺ ATPase. A rat antibody (1:10) against the muscle-type acetylcholine nicotinic receptor (mAb 35, developed by J. Lindstrom (Tzartos et al., 1981), and obtained from DSHB at University of Iowa) was used to label acetylcholine receptors. A mouse monoclonal antibody (1:100) against neurofilament-associated antigen (3A10, developed by T. Jessel and J. Dodd, and obtained from DSHB at University of Iowa) was used to label axon terminals. A rabbit polyclonal antibody (1:200) against KNa channel (anti-Kcnt1, obtained from Aviva Systems Biology) was used to label KNa channels. A mouse monoclonal antibody (1:100) against inward-rectifier K⁺ channel 6.2 (B-9, obtained from Santa Cruz Biotechnology), was used to label ATP-sensitive inward rectifier K⁺ channels.

Immunohistochemistry slides were imaged on a Zeiss ApoTome.2 microscope with 5X/0.16NA and 10X/0.45NA dry objectives. Images were acquired with a Zeiss AxioCam MRm camera, then processed by Zeiss AxioVision Rel.4.8. We used structured illumination to create optical sections of our fluorescent samples. Final processing of the images was performed with ImageJ-win64 version 1.44B (National Institutes of Health).

Computational Simulations

We modeled the *E. virescens* electrocyte as a three-compartment cell consisting of an active posterior compartment, a passive central compartment, and an active anterior compartment. We used the Hodgkin-Huxley formalism to simulate ionic currents and changes in
membrane voltage. We also applied a simplified model of Na\(^+\) entry, diffusion, and pumping to simulate changes in Na\(^+\) concentrations in the three compartments. Simulated cholinergic synaptic current was applied only to the posterior compartment. The capacitance for the posterior compartment was 48.0 nF and the anterior-compartment capacitance was 18.0 nF. We based these values on the surface areas of the posterior 0.35 mm and anterior 0.2 mm of the electrocyte, respectively, with surface areas determined from confocal 3D reconstructions of electrocytes. The central-compartment capacitance was 18 nF, estimated as the surface area of a cylinder 0.95 mm long and 0.6 mm in diameter, dimensions determined by our imaging data. Differential equations were coded in Matlab (Mathworks, Inc. Natick MA) and integrated using Euler’s method with integration time steps of 5 \times 10^{-8} \text{ sec}. All model parameters are shown in Table 1.

The passive central compartment’s current balance equation included only passive leak (I\(_L\)) fixed at 5 \mu S, and coupling to the two active compartments:

\[
C_m \frac{dV_c}{dt} = -I_L + g_w(V_a - V_c) + g_w(V_p - V_c) \quad (1)
\]

where \(g_w\) is the coupling conductance, fixed at 322 \mu S.

The current balance equations for the posterior and anterior active compartments were, respectively:

\[
C_m \frac{dV_p}{dt} = I_{Syn}(t) - I_{Na} - I_L + g_w(V_c - V_p) \quad (2)
\]

\[
C_m \frac{dV_a}{dt} = -I_{KNa} - I_R - I_L + g_w(V_c - V_a) \quad (3)
\]
where $I_{\text{Syn}}$ represents synaptic current, $I_{\text{Na}}$ is $\text{Na}^+$ current, $I_{\text{KNa}}$ is the $\text{Na}^+$-activated $\text{K}^+$ current, and $I_R$ is the inward rectifier $\text{K}^+$ current. For all three compartments, $I_L$ is the leak current, which was given by Equation 4.

$$I_L = \bar{g}_L(V + 95) \quad (4)$$

The posterior-compartment synaptic current, $I_{\text{Syn}}$, was given by Equation 5

$$I_{\text{Syn}} = \bar{g}_{\text{Syn}} g_{\text{Syn}(t)}(V_p - 15) \quad (5)$$

where the time series $g_{\text{Syn}(t)}$ was a series of 10 alpha waveforms generated using the discrete time equation:

$$g_{\text{Syn}(n+2)} = 2 \left( 1 - \frac{T}{\tau} \right) g_{\text{Syn}(n+1)} - \left( 1 - \frac{T}{\tau} \right)^2 g_{\text{Syn}(n)} + \left( \frac{T}{\tau} \right)^2 x(n) \quad (6)$$

(Graham and Redman, 1993) where $T$ is the integration time step and $\tau$ is the time constant. The binary series $x(n)$ specified the onset times of the synaptic inputs, and the resulting time-series $g_{\text{Syn}(n)}$ was normalized such that $0 \leq g_{\text{Syn}(n)} \leq 1$.

The equation for $I_{\text{Na}}$ was divided into a transient component ($I_{\text{NaT}}$) and a persistent component ($I_{\text{NaP}}$) as in Equations 7 and 8,

$$I_{\text{NaT}} = \bar{g}_{\text{Na}} m^3 (1 - \gamma) h (V_p - E_{\text{Na}}) \quad (7)$$

$$I_{\text{NaP}} = \bar{g}_{\text{Na}} m^3 \gamma (V_p - E_{\text{Na}}) \quad (8)$$

where $0 < \gamma < 1$. $E_{\text{Na}}$, the $\text{Na}^+$ equilibrium potential, was allowed to vary with changing $\text{Na}^+$ concentrations in the posterior compartment ($\text{Na}_P$) according to the equation $E_{\text{Na}} = 25.7 \ln(114/[\text{Na}_P])$, assuming 114 mM extracellular $\text{Na}^+$ and temperature of 25 °C.
The anterior-compartment K⁺ currents were given by equations 9 and 10.

\[ I_{KNa} = \bar{g}_{KNa} n^4 s^4 (V_a + 95) \]  
(9)

\[ I_R = \bar{g}_R \left( \frac{1}{1 + \exp(\eta_R (V_m + 110))} \right) (V_a + 95) \]  
(10)

The gating variables \( m, n, \) and \( h \) in Equations 7 - 9 are given by Equations 11-13 where \( j = m, n, \) or \( h. \)

\[ \frac{d j}{dt} = \alpha_j (1 - j) - \beta_j (j) \]  
(11)

\[ \alpha_j = k_{\alpha j} \exp (\eta_{\alpha j} V) \]  
(12)

\[ \beta_j = k_{\beta j} \exp (\eta_{\beta j} V) \]  
(13)

We modeled the Na⁺-dependence of \( I_{KNa} \) with the gating variable, \( s, \) which is determined by the Na⁺ concentration in the bulk cytoplasm in the anterior compartment (Naₐ), according to Equation 14.

\[ \frac{d s}{dt} = k_t [Na_A] (1 - s) - k_b s \]  
(14)

We modeled Na⁺ concentrations in each compartment based on the compartmental volumes which were \( 4.2 \times 10^7 \mu m^3, 2.7 \times 10^8 \mu m^3, \) and \( 1.7 \times 10^7 \mu m^3 \) for the posterior, central, and anterior compartments, respectively. The posterior and anterior compartment volumes were measured from our 3D reconstructions of electrocytes, while the central compartment volume was estimated as the volume of a cylinder 0.95 mm long and 0.6 mm in diameter. The initial Na⁺ concentration in all three compartments was 15 mM. The equation for Na⁺ concentration in the posterior compartment was:
where \( p \) represents sodium leak and \( q \) is moles of \( Na^+ \) ions entering through the cholinergic receptors and \( Na^+ \) channels, given by Equation 16:

\[
q = \frac{dt(2I_{Syn} + I_{Na})10^{-12}}{eL}
\]

wherein the integrated \( Na^+ \) current, \( dt(2I_{Syn} + I_{Na}) \) in \( nA*ms \), is multiplied by \( 10^{-12} \) to yield Coulombs, divided by the elementary charge on a monovalent cation, \( e \), to yield number of \( Na^+ \) ions, and divided by Avogadro’s constant, \( L \), to yield moles of \( Na^+ \). \( I_{Syn} \) was multiplied by 2 in Equation 16 to account for \( Na^+ \) entry associated with \( I_{Syn} \) where \( g_{Syn} \) arises from cholinergic receptors, assuming the \( Na^+ \) permeability is twice that of the \( K^+ \) permeability. Diffusion of \( Na^+ \) between compartments is governed by \( \delta \) (the diffusion rate constant), the difference in \( Na^+ \) concentration between the compartments, and the ratio of their volumes (\( \lambda \)). \( Na^+ \) removal is modeled by the fractional pumping rate \( b_P \), representing the rate at which \( Na^+ \) is pumped out of the posterior compartment to the extracellular space.

The central compartment's \( Na^+ \) concentration was affected only by diffusion to and from the posterior and anterior compartments as in Equation 17.

\[
\frac{d[Na_{C}]}{dt} = \delta([Na_P] - [Na_C]) \frac{\lambda_P}{\lambda_C} - \delta([Na_C] - [Na_A]) \frac{\lambda_C}{\lambda_A}
\]

The posterior compartment \( Na^+ \) concentration was given by Equation 18

\[
\frac{d[Na_P]}{dt} = \delta([Na_C] - [Na_A]) \frac{\lambda_C}{\lambda_A} - b_A[Na_A]
\]
which includes diffusion to and from the central compartment as well as $b_A$ which gives the fractional rate at which $\text{Na}^+$ is pumped from the anterior compartment to the extracellular space.

**RESULTS**

**Gross electrocyte morphology**

In *E. virescens*, the EO runs longitudinally along the body and extends into the caudal tail filament (Fig. 1A). Several rows of electrocytes are densely packed in the EO (Fig. 1C). We injected Rhodamine B dextran (10,000 MW) into single electrocytes within an isolated section of EO, allowed 15 minutes for the dextran to fully diffuse, then imaged the cell on a Leica® TCS SP8 laser scanning confocal microscope. 3D reconstructions of these cells showed that electrocytes are large cylinder-like cells approximately 1.5 millimeter in length and 600 µm in diameter (Fig. 2A). The electrocyte's surface structure was not uniform, and based on differences in surface structure we divided the cell into three regions: the posterior face, the main body, and the anterior face (Fig. 2A, supplementary movie 1). The space between adjacent electrocytes along the rostral-caudal axis is ≈30 µm, and the posterior face of each electrocyte is surrounded by the anterior face of the adjacent cell (Fig. 2B).

The surface of the posterior face was densely occupied by narrow invaginations that extended longitudinally into the cell approximately 300 µm, resulting in a pronounced increase in membrane surface area (Fig. 2C, supplementary movie 2). The anterior face usually contained three large lobes with smaller papillary extensions. Additionally, a network of capillaries was observed embedded immediately underneath the ruffled anterior membrane (Fig. 2D, Fig. 3B2, supplementary movie 3). In contrast to the posterior and anterior faces, the surface of the main body is relatively smooth, with an array of spherical structures just beneath the membrane (Fig. 2E).
Electrocytes are multi-nucleated cells formed by the fusion of skeletal myocytes during development (Bennett, 1970; Unguez and Zakon, 1998a, 2002; Zakon and Unguez, 1999) resulting in a syncytium with nuclei localized to the surface of the electrocyte (Machado, et al., 1976). Our images of DAPI stained nuclei colocalized with Alexa Fluor® 594 within our 3D reconstruction confirmed that the nuclei of *E. virescens* electrocytes are densely distributed in a thin layer near the membrane (Fig. 2F; see Fig. 3A for colocalization). The combination of whole cell 3D imaging and high-resolution imaging of smaller electrocyte sections in the present study allowed us to estimate the number of nuclei within a single electrocyte by two distinct methods, both of which indicated that each electrocyte has approximately 30,000 nuclei.

The first method we used was to create an isosurface around the whole-cell image of the electrocyte (Fig. 2A). This allowed us to measure the surface area of the entire electrocyte, which was $4.5 \times 10^6 \ \mu m^2$. We then counted the number of nuclei per unit surface area in a single high-resolution image of the cell body, and found that there were 220 nuclei per $2.8 \times 10^4 \ \mu m^2$ of cell surface area. This method thereby yielded $4.5 \times 10^6 \ \mu m^2 \left( \frac{220 \ \text{nuclei}}{2.8 \times 10^4 \ \mu m^2} \right) = 35,000$ nuclei in the electrocyte.

To validate the above estimation, we used an alternate approach to estimating the number of nuclei within an electrocyte by modelling the electrocyte with simple geometries, with the anterior end and cell body approximated as a hollow cylinder with a paraboloid scooped out at the anterior end, and the posterior end as a solid paraboloid. The anterior end had a cupped shape, with nuclei along the surface, so the surface area was approximated as a paraboloid with a measured radius of $365 \ \mu m$ and a depth of $144 \ \mu m$, giving a surface area of $4.8 \times 10^5 \ \mu m^2$. We then counted the number of nuclei in a single high resolution image of the anterior region with $1.5 \times 10^4 \ \mu m^2$ of surface area and found 170 nuclei, giving a total of
4.8 × 10^5 μm^2 \left( \frac{170 \text{ nuclei}}{1.5 \times 10^4 \text{ μm}^2} \right) = 5.4 \times 10^3 \text{ nuclei in the anterior end. The central compartment end was approximated as a hollow cylinder with a measured length of 1.0 \times 10^3 \text{ μm and a measured diameter of 600 μm. Therefore, the total number of nuclei on the cell body would be } (600\text{μm})(π)(1000\text{μm}) \left( \frac{220 \text{ nuclei}}{2.8 \times 10^4 \text{ μm}^2} \right) = 1.5 \times 10^4 \text{ nuclei in the central compartment. For the posterior end, since the invaginations penetrate back to the cell body resulting in nuclei being located throughout the posterior end, we modelled the posterior end as a solid paraboloid. The paraboloid had a measured length of 590 μm and a radius of 215 μm giving a total volume of 8.6 \times 10^7 \text{ μm}^3. We then measured the number of nuclei in a 7.0 \times 10^6 μm^3 image. To ensure that we only measured electrocyte nuclei, we only counted nuclei that were colocalized with Alexa Fluor® 594. In the sample volume, we counted 1000 nuclei. Extrapolated over the whole posterior end, there would therefore be 8.6 \times 10^7 \text{ μm}^3 \left( \frac{1000 \text{ nuclei}}{1.2 \times 10^4 \text{ μm}^3} \right) = 1.2 \times 10^4 \text{ nuclei in the posterior region. Taken together, these quantities sum to 32,000 nuclei per electrocyte, which is in close concordance with our estimate based on the first method.}

**Fine structure of the electrocyte**

We investigated the fine structure of the posterior and anterior faces by imaging 100-μm thick serial sections of a paraformaldehyde-fixed EO sample that contained a single target electrocyte filled with Alexa Fluor® 594 dextran. Electrocytes, vasculature, and pigment cells within the EO also emit a broad autofluorescence spectrum when excited by a 488 nm laser line. We took advantage of this autofluorescence to image tissue adjoining the Alexa Fluor® 594 dextran injected cell.
Our images of the posterior face showed that the surface of each invagination contained many small spine-like structures approximately 50 μm in length. The spines terminate in an enlarged sphere approximately 20 μm in diameter (Fig. 3A). Blood vessels occupy the space between electroctyes with the majority penetrating into the invaginations of the posterior face, while a smaller number contact the anterior face (Fig. 3 A2 and B2, respectively). Capillaries occupy much of the space within each posterior-face invagination, and the spines within the invaginations are largely enveloped by these capillaries. The fine structure of the anterior face (Fig. 3B) is generally less complex than the posterior face, with the unique feature that capillaries appear to reside within enclosed, tube-like structures proximal to the anterior face membrane (Fig. 3B2). An additional and striking difference between the anterior and posterior faces is that the posterior-face membrane is densely occupied by vesicles, which are exceedingly less abundant on the anterior face (Fig. 3C,D).

**Subcellular localization of cholinergic receptors, ion channels, and ion transporters**

In gymnotiforms, electrocyte APs are controlled by a medullary pacemaker nucleus via spinal electromotor neurons that innervate each electrocyte (Fig. 1A). Labeling of spinal nerves with the antibody 3A10 against neurofilament-associated antigen (Unguez and Zakon, 1998b) showed that only the posterior face is innervated and the innervation occurs throughout the posterior face (Fig. 4A). We also found that acetylcholine receptors were clustered only on the posterior face (Fig. 4B). Given that the cholinergic synapses are restricted to the posterior membrane, we hypothesized that the other ion channels would be localized also on the posterior membrane.

We labeled voltage-gated Na⁺ channels (Naᵥ) with a pan-Naᵥ antibody raised against an epitope identical in all isoforms of Naᵥ1 and found that Naᵥ channels were expressed only on the
electrocyte's posterior face (Fig. 4C). To ensure these signals are not from the innervating axons, we costained Na\textsubscript{v} channels with 3A10 and found no colocalization between them (Fig. 4D). The expression pattern of acetylcholine receptors and Na\textsubscript{v} channels indicates that the posterior face is the only entrance site for Na\textsuperscript{+} influx and the site of AP initiation.

Based on electrophysiological and molecular evidence that electrocytes express inward-rectifier and K\textsubscript{Na} channels (Markham et al., 2013), we immunolabeled both channels and to our surprise, found that both are localized only to the anterior face (Fig. 4E, F). Immunolabeling of Na\textsuperscript{+}/K\textsuperscript{+} ATPase alpha subunits showed that these are found on both the anterior and posterior faces, another unusual arrangement given that the sources of Na\textsuperscript{+} influx are restricted to the posterior membrane (Fig. 4G).

Given the counterintuitive spatial separation of the Na\textsuperscript{+} channels, K\textsubscript{Na} channels, and Na\textsuperscript{+}/K\textsuperscript{+} ATPases, we performed western blot analyses to ensure that these antibodies indeed specifically labeled the proteins of interest. Western blot of *E. virescens* EO and mouse brain whole-cell lysate labeled with polyclonal anti-Na\textsubscript{v} detected specific bands of approximately 250 kDa (the predicted molecular weight of Na\textsubscript{v} channels) in both tissues (Fig. 4H). Similarly, polyclonal anti-\textsc{kcnt}1 (K\textsubscript{Na}) and monoclonal anti Na\textsuperscript{+}/K\textsuperscript{+} ATPase-\textalpha subunit labeled bands of approximately 130 kDa and 100 kDa, respectively, in both EO and mouse brain (Fig. 4H). These molecular weights correspond with the predicted molecular weights of K\textsubscript{Na} channels and Na\textsuperscript{+}K\textsuperscript{+} ATPase-\textalpha subunits.

**Numerical simulations of electrocyte function**

Given the unusually large separation of K\textsubscript{Na} channels from potential Na\textsuperscript{+} sources revealed by our imaging data, we used computational simulations of electrocyte action potentials and Na\textsuperscript{+} dynamics to test whether the high-frequency firing typical of *E. virescens* electrocytes could be
reproduced in a model cell where the spatial arrangement of ion channels matched our imaging
data. Our model cell had three compartments, an active posterior compartment, a passive central
compartment, and an active anterior compartment. The passive properties of these compartments
were guided by morphological measurements of their volumes and membrane areas. We
estimated the coupling conductances between compartments based on our measurements of the
electrocyte’s length and diameter.

The posterior compartment had linear leak current, synaptic current via cholinergic
receptors, and voltage-gated Na+ current (I_L, I_{Syn}, and I_{Na}, respectively). The central
compartment had only I_L, and the anterior compartment had I_L, inwardly rectifying K+ current (I_R)
and KNa current (I_{KNa}). Unlike previous simulations of I_{KNa} where the Na+ sensitivity of the
current arises from direct access to a localized persistent Na+ current (Brown et al., 2008;
Markham et al., 2013), we modeled I_{KNa} here such that its Na+ sensitivity was determined by the
Na+ concentration in the bulk cytoplasm of the anterior compartment. Our model also simulated
changes in intracellular Na+ in each compartment based on Na+ influx resulting from a static Na+
leak, I_{Syn} and I_{Na} in the posterior compartment, a fractional Na+ pumping rate in both the
posterior and anterior compartments, and Na+ diffusion between all three compartments.

In the full model with all currents present according to the parameters in Table 1, the
model cell maintained repetitive firing in response to 500 Hz synaptic stimulation (Fig. 5A).
During these action potentials, I_{Na} reached peak currents of approximately 15 μA, consistent with
earlier experimental results (Lewis et al., 2014), and I_{KNa} exhibited current magnitudes exceeding
5 μA (Fig. 5B) suggesting an important role in shaping the cell's firing pattern. Na+
concentrations in the three compartments did vary in response to Na+ influx, pumping, and
diffusion, but only over a range of 1-2 mM (Fig. 5C). As a result, the fractional activation of
IK_{Na} due to Na\(^+\) concentrations in the anterior compartment remained fairly steady around 0.7 throughout the action potential train.

To evaluate whether IK_{Na} is a necessary component of AP repolarization in the model cell, we made a model cell with all of the same parameters as in Fig. 5A-C, but set the conductance for IK_{Na} to zero. This model cell could not maintain repetitive firing at 500Hz (Fig. 5D-E) and instead remained in a state of depolarized oscillation. These results provide evidence that IK_{Na} in our model cell is necessary for AP repolarization and plays a role in AP termination even when the K_{Na} channels are at a great distance from transient Na\(^+\) sources.

DISCUSSION

The most striking finding from this study is the extreme compartmentalization of ion channels and ion transporters across vast distances in *E. virescens* electrocytes. The K_{Na} channels that repolarize the electrocyte AP and a substantial portion of the Na\(^+\)/K\(^+\) ATPases responsible for removing Na\(^+\) after each AP are separated by more than a millimeter from the only identified sources of Na\(^+\) influx.

The presence of K_{Na} channels at such a great distance from Na\(^+\) sources raises the important question of how these channels are activated during the AP. In previous work with K_{Na} channels from other taxa, channel activation required intracellular Na\(^+\) concentrations ([Na\(^+\)]) that far exceed those normally found in bulk cytoplasm (Dryer et al., 1989; Kameyama et al., 1984; Yuan et al., 2003). In mammalian neurons, K_{Na} channels are clustered within just a few μm of Na\(^+\) channels in microdomains that allow localized elevation of Na\(^+\) concentrations sufficient to activate K_{Na} channels without changing [Na\(^+\)] in the bulk cytoplasm (Budelli et al., 2009; Hage and Salkoff, 2012). However, we show here that Na\(^+\) and K_{Na} channels are
separated by great distances in *E. virescens* electrocytes, suggesting that $K_{Na}$ channels in *E. virescens* electrocytes do not require proximal sources of Na$^+$ influx.

One hypothesis as to how $K_{Na}$ channels are activated in electrocytes experience a significant increase in [Na$^+$]$_i$ during each AP or during sustained high-frequency firing, a distinct possibility given the magnitude of $I_{Na}$ during the AP. We found a high density of Na$^+$/K$^+$ ATPases on the anterior face consistent with earlier biochemical data (Denizot, 1982), suggesting that Na$^+$ influx from the posterior face ultimately increases [Na$^+$]$_i$ in the anterior region. Our computational simulations suggest that only small changes in [Na$^+$]$_i$ occur during high-frequency firing in *E. virescens* electrocytes. This suggests instead a second hypothesis, that the electrocyte's $K_{Na}$ channels are more sensitive to [Na$^+$]$_i$ than most other $K_{Na}$ isoforms identified to date. Indeed, our simulated $I_{KNa}$ was based on Na$^+$-dependent rate constants that created partial activation with Na$^+$ concentrations of approximately 15 mM. Multiple factors determine the Na$^+$ sensitivity of $K_{Na}$ channels. Single amino-acid substitutions can shift the Na$^+$ sensitivity of $K_{Na}$ channels over a range of 200 mM (Zhang et al., 2010). Given the elevated rates of evolution for other ion channels in gymnotiform electrocytes (Zakon et al., 2006; Zakon et al., 2008) it seems possible that $K_{Na}$ channels also could have undergone evolutionary changes that would enhance Na$^+$ sensitivity. Additionally, intracellular factors such as NAD$^+$ can modulate the Na$^+$ sensitivity of $K_{Na}$ channels such that the EC$_{50}$ is approximately 17 mM (Tamsett et al., 2009). It is possible that such molecular evolution or functional modulation in *E. virescens* $K_{Na}$ channels could allow their activation at Na$^+$ concentrations in the 15 mM range. These possibilities can only be directly addressed through cloning and heterologous expression of *E. virescens* electrocyte $K_{Na}$ channels.
Our numerical simulations in the present study suggest that the electrocyte \( K_{Na} \) channels remain in a state of partial activation during repetitive firing, rather than responding to transient increases in \( [Na^+] \), as concluded in earlier work (Markham et al., 2013). If electrocyte \( K_{Na} \) channels indeed are maintaining a steady level of activation during normal repetitive firing, this raises the question of what function the channels' Na\(^+\) sensitivity serves in this system. Further experimental investigation and computational simulations of Na\(^+\) dynamics in the electrocyte are needed to clarify the functional significance of \( K_{Na} \) channels' Na\(^+\) sensitivity in *E. virescens* electrocytes.

Additionally, the finding that ion channels are expressed on both the anterior and posterior electrocyte faces contradicts a long-standing assumption, originating with some of the earliest electrophysiological studies of *E. virescens* electrocytes, that only the innervated face is active (Bennett, 1961, 1971) and that all ion channels which produce the AP should therefore be localized to the posterior face (e.g., Assad et al., 1998). The presence of \( K_{Na} \) and inward-rectifier K\(^+\) channels on the electrocyte anterior membrane indicates that both the posterior and anterior faces of the electrocyte are involved in AP production.

A second important finding here is the sheer magnitude and complexity of the electrocyte's morphology. Our two-photon live-cell and fixed-tissue imaging of *E. virescens* electroctyes extends earlier electron microscopy studies (Schwartz et al., 1975) that first reported the 2D morphological polarization of these cells. Our 3D reconstructions of *E. virescens* electrocytes also show that they are extremely large multi-nucleated cells with striking differences in the ultrastructural features of the posterior and anterior faces. Because of the electrocytes' large diameter, the laser scanning confocal microscope could not resolve the submicron detail of the medial region of the cells, preventing us from obtaining a detailed 3D
reconstruction of this region of the electrocyte. However, we observed a discrete lower boundary
of the fluorescence on the medial side of the electrocyte at a depth of 600 µm, suggesting that the
cell is 600 µm across on both the lateral-medial axis and the ventral-dorsal axis.

The deep invaginations of the posterior region create a significant expansion of
membrane surface area in a comparatively small volume. This membrane area is almost entirely
occupied by vascularization on the extracellular surface, and vesicles on the intracellular surface.
It is perhaps possible that some of the fibrillary structures we identified as capillaries were
instead portions of the innervating spinal nerves, but this is unlikely because blood vessel walls,
unlike nerve, contain autofluorescent elastin and collagen (Deyl et al., 1980) and the narrowest
vessels we observed were ≈3 µm in diameter with a hollow center. Additionally, the vessels
branched from a large central hollow tube that was almost 20 µm in diameter (Fig. 3B2), making
it extremely unlikely that these are axons. The dense vascularization of the posterior face is
likely necessary to provide efficient nutrient supply and waste removal consistent with reports
that high concentrations of mitochondria are present in the posterior region (Schwartz et al.,
1975). We hypothesize that the densely packed vesicles on the posterior membrane are
associated with constitutive trafficking of Na\(^+\) channels and Na\(^+\)/K\(^+\) ATPases. In a related
electric fish, Na\(^+\) channels are constitutively cycled into and out of the electrocyte membrane,
and upregulation of channel exocytosis by hormonal factors can increase I\(_{\text{Na}}\) magnitude by more
than 50% within minutes (Markham et al., 2009), as has been reported for \(E.\) \(virescens\)
(Markham et al., 2013).

The proliferation of membrane surface area on the posterior face also provides a substrate
for high numbers of Na\(^+\)/K\(^+\) ATPases which are expressed throughout the posterior membrane.
Active transport of Na\(^+\) by the Na\(^+\)/K\(^+\) ATPase occurs at the rate of \(\approx 10^3\) ions per second
while selective ion channels pass $10^7$ to $10^8$ ions per second (Morais-Cabral et al., 2001). In *E. virescens* electrocytes $\approx 6 \times 10^{10}$ Na$^+$ ions enter the electrocyte with each AP (Lewis et al., 2014) with only one millisecond between APs at 500 Hz. Accordingly, efficient removal of Na$^+$ during the brief interspike interval would depend on extremely high densities of Na$^+/K^+$ ATPases. The extensive posterior-face membrane area also would increase membrane capacitance and decrease resistance (assuming a constant membrane resistivity). This combination of high capacitance and low resistance would increase current flow during the AP (Schwartz et al., 1975) and the tuning of resistance relative to capacitance would determine the membrane time constant potentially influencing AP duration (Mills et al., 1992).

The complex organization of the posterior face in *E. virescens* electrocytes contrasts sharply with the morphology of the anterior face which is relatively featureless with sparse vascularization and few detectable vesicles. Within the relatively simple organization of the anterior face, K$_{Na}$ channels, inward-rectifier K$^+$ channels, and Na$^+/K^+$ ATPases were densely and apparently evenly distributed across the membrane surface. The paucity of anterior-face exocytotic vesicles suggests that K$_{Na}$ channels are perhaps not cycled or trafficked in the same manner as Na$^+$ channels on the posterior-face. These results are consistent with our earlier studies of the hormonal regulation of ionic currents in *E. virescens* electrocytes. Application of adrenocorticotropin hormone (ACTH) increased the magnitudes of I$_{Na}$ and I$_{KNa}$. The increase in I$_{Na}$ was a direct effect of ACTH application regulating vesicular trafficking of Na$^+$ channels, but the increased conductance of K$_{Na}$ channels was found to be a secondary effect of the hormone-induced increase in I$_{Na}$ magnitude (Markham et al., 2013).

The bioelectrical properties of all excitable cells are determined by their morphology and the subcellular localization of ion channels. Electrocyte morphology is an important determinant
of species-specific and individual-specific EOD waveforms (Bass, 1986; Gallant et al., 2011; Hopkins et al., 1990; Mills et al., 1992) and the subcellular distributions and densities of ionic currents also help determine EOD waveform (Ferrari and Zakon, 1993; Markham and Zakon, 2014; Shenkel and Sigworth, 1991). Some of the ion channels, ionotropic receptors, and ion transporters responsible for electrocytes' biophysical properties have been localized in other gymnotiform and mormyriform electric fish (Cuellar et al., 2006; Gallant et al., 2011; Liu et al., 2007). The present work is, to our knowledge, the first comprehensive presentation of detailed electrocyte morphology together with subcellular localization of all ionic mechanisms responsible for an electrocyte's electrical excitability. It is of course possible that additional key membrane proteins are present but not yet detected. Of particular concern is the possibility that a second undetected isoform of $K_{\text{Na}}$ channel is expressed in proximity to the voltage gated $Na^+$ channels. We believe this is unlikely because an earlier study detected only a single isoform of $K_{\text{Na}}$ channel in *E. virescens* electrocytes by RT-PCR, the KCNT1/Slack isoform (Markham et al., 2013), supporting the conclusion that our immunolabeling in the present study detected all $K_{\text{Na}}$ channels expressed in electrocytes. Moreover, the present immunolabeling study detected ion channels corresponding to all known ionic currents in *E. virescens* electrocytes, and these ionic currents are sufficient to reproduce completely the electrical behavior of these cells as shown by our computational simulations in this study and in earlier work (Markham et al., 2013).

Ultimately, the energetic demands of electrocyte APs and the ability to maintain firing rates exceeding 500 Hz throughout the animal's lifespan stem from the spatiotemporal dynamics of $Na^+$ entry during the AP and the subsequent $Na^+$ removal within a millisecond by the $Na^+/K^+$ ATPases. For these peripheral excitable cells the metabolic cost of AP generation is likely a major force governing their biophysical properties as is the case for central neurons (Hasenstaub
et al., 2010). Future studies on the temporal and spatial dynamics of Na\(^+\) entry and removal in electrocytes will be necessary for understanding how the ion channels and Na\(^+\)/K\(^+\) ATPases coordinate to maintain high firing rates while managing the extremely large inward Na\(^+\) currents. Further, additional exploration of the interaction between electrocyte Na\(^+\) channels and K\(_{Na}\) channels will likely lead to new insights on the many important roles that K\(_{Na}\) channels play in excitable cell physiology (Bhattacharjee and Kaczmarek, 2005).
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REFERENCES


Table 1. Parameter values for the electrocyte model

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FIGURE LEGENDS

Figure 1. The electric organ discharge in *E. virescens*. (A) The electric organ discharge (EOD) is produced by the coordinated action potentials (APs) of the electrocytes in the electric organ (EO). A medullary pacemaker nucleus controls the electrocyte APs via spinal electromotor neurons which innervate the electrocytes. (B) Simplified schematic of an electrocyte. These are large cells, greater than 1 millimeter in length, innervated by a cholinergic synapse on the posterior end of the cell. Activation of the cholinergic synapse initiates the AP when sodium enters the cell via voltage-gated sodium channels. The cell geometry causes the Na\(^{+}\) current to move along the rostral-caudal body axis. (C) A section of EO from the tail, with skin removed to expose the electrocytes, which are densely packed within the EO. A single electrocyte is outlined in black. (D) The simultaneous APs of all electrocytes in the EO generate current that moves forward toward the head, following a return path through the water to the tail. By convention, current moving toward the head is measured as positive (upward). (E) A single EOD waveform corresponds to one cycle of APs in the EO. (F) The EOD waveform from a fish with EOD frequency of ≈500 Hz.

Figure 2. Gross morphology of *E. virescens* electrocytes. (A) 3D reconstruction from serial confocal scanning through a live electrocyte injected with Rhodamine B dextran (10,000 MW). Arrow indicates site of dextran injection. Darkened horizontal and vertical lines (arrowheads) are artifacts caused by the image tile overlap. Full 3D image is shown in Supplemental Movie 1. (B) A segmented autofluorescence image of the junction between two adjoining electrocytes with the posterior face of one electrocyte on the left (cyan) and the anterior face of the adjacent
Figure 3. Fine structure of *E. virescens* electrocytes. (A) 3D reconstruction of membrane papillae within a single invagination from serial confocal scans through a fixed 100 μm-thick section of EO tissue. A1 shows the intracellular structure identified by injection of Alexa Fluor® 594 dextran (10,000 MW). Arrows point to the spines projecting from membrane papillae. In A2, tissue autofluorescence reveals that the spines shown in A1 are wrapped by capillaries. DAPI stains the multiple nuclei in A3. Images taken in channels A1-A3 opaquely superimposed in A4. (B) 3D reconstruction of the junction between adjacent electrocytes from serial confocal scans through a 100 μm-thick section of fixed EO tissue. B1 shows the anterior face of the electrocyte injected with Alexa Fluor® 594 dextran. Blood vessels occupy the space between adjacent electrocytes in B2 and arrows indicate the capillaries penetrating the anterior face. DAPI labels nuclei in B3. Images taken in channels B1-B3 are merged in B4. (C) The posterior face membrane is mostly occupied by vesicles. C1 is an optical section from the serial confocal scans of the posterior face shown in (A1). The region outlined in white in C1 is enlarged in C2. Arrows
indicate vesicles. (D) Few vesicles are found on the surface of the anterior face. D1 is an optical
section from the serial confocal scans of the anterior face shown in (B1). Region outlined in
white in D1 is enlarged in D2. Green circular structures in C-D are nuclei. Arrows indicate
vesicles.

Figure 4. Western blot analysis and immunohistochemistry staining of acetylcholine
receptors, ion channels and Na\(^+\)/K\(^+\) ATPases in *E. virescens* electrocytes. (A-G) Expression
pattern of acetylcholine receptors, ion channels and Na\(^+\)/K\(^+\) ATPases. (A) Only the posterior face
(right) is innervated by spinal electromotor neurons. The axons of innervating neurons are
labeled with 3A10 (green). (B) Acetylcholine receptors (green) are clustered only on the
posterior face (right). (C) Voltage-gated Na\(^+\) channels (red) are localized only on the posterior
face (right). (D) An electrocyte is co-labeled with 3A10 (green) and voltage-gated Na\(^+\) channels
(red). (E) Na\(^+\)-activated K\(^+\) (K\(_{\text{Na}}\)) channels (red) are only expressed on the anterior face (left). (F)
ATP-sensitive inward rectifier K\(^+\) channels (red) are localized on the anterior face (left). (G)
Na\(^+\)/K\(^+\) ATPases (green) are expressed in both anterior and posterior faces. DAPI (blue) labels
the electrocyte’s nuclei in A-G. The region outlined in white in A-G1 is enlarged in A-G2. (H)
western blot analysis to confirm the specificity of rabbit polyclonal anti-Na\(_{\text{v}}\) (left), rabbit
polyclonal anti-kcnt1(middle) and mouse monoclonal anti-\(\alpha\) subunit of Na\(^+\)/K\(^+\) ATPase (right) in
*E. virescens* electric organ and mouse brain. In electric organ, a band around 250 kDa was labeled
with anti-Na\(_{\text{v}}\), which is slightly larger than that in mouse brain (left). Anti-kcnt1 detected a band
around 130 kDa in electric organ which is slightly smaller than that in mouse brain (middle). A
band at 100 kDa was detected with anti- \(\alpha\) subunit of Na\(^+\)/K\(^+\) ATPase in both electric organ and
mouse brain (right).
Figure 5. Computational simulations of electrocyte action potentials in a model electrocyte with K\textsubscript{Na} channels (A-D) and without K\textsubscript{Na} channels (E-G). (A) Membrane potential of the posterior (red), central (black), and anterior (blue) compartments of a model cell with K\textsubscript{Na} during a train of 10 action potentials elicited at 500 Hz by simulated synaptic conductances. (B) Time course of the Na\textsuperscript{+} current (I\textsubscript{Na}), K\textsubscript{Na} current (I\textsubscript{KNa}) and synaptic current (I\textsubscript{Syn}) during the action potential train shown in A. (C) Timecourse of internal Na\textsuperscript{+} concentrations in the posterior (red), central (black), and anterior (blue) compartments during the action potential train shown in Panel A. The different initial Na\textsuperscript{+} concentrations reflect the equilibrium of resting Na\textsuperscript{+} leak, pumping, and diffusion rates as shown in D. (D) Posterior membrane potential and time course of internal Na\textsuperscript{+} concentrations in the posterior (red), central (black), and anterior (blue) compartments during the same simulation shown in A, but on an expanded timescale that shows the initial changes in Na\textsuperscript{+} concentrations as the Na\textsuperscript{+} leak, pumping, and diffusion processes reach equilibrium. (E) Membrane potential of the posterior (red), central (black), and anterior (blue) compartments of a model cell without K\textsubscript{Na} during a train of 10 action potentials elicited at 500 Hz by simulated synaptic conductances. (F) Time course of the Na\textsuperscript{+} current (I\textsubscript{Na}), K\textsubscript{Na} current (I\textsubscript{KNa}) and synaptic current (I\textsubscript{Syn}) during the action potential train shown in E. (G) Time course of internal Na\textsuperscript{+} concentrations in the posterior (red), central (black), and anterior (blue) compartments during the action potential train shown in E. The different initial Na\textsuperscript{+} concentrations reflect the equilibrium of resting Na\textsuperscript{+} leak, pumping, and diffusion rates as shown in D.
Dextran  |  Autofluorescence  |  DAPI  |  Merge
--- | --- | --- | ---
A1  |  A2  |  A3  |  A4
B1  |  B2  |  B3  |  B4
C1  |  C2  |  D1  |  D2

30 μm