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Title: Contribution of the Na^+/K^+ Pump to Rhythmic Bursting, Explored with Modeling and Dynamic Clamp Analyses

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Author Questionnaire

1. **Microscopy**: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **Y**, **Wild M5 with binocular video port. We lack a video camera, but this port works very well with both the dissecting microscope (M5) and the electrophysiology microscope (M5).**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as possible.

   Videographer: Authors will capture on the day of the shoot

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **41**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Ricardo Erazo Toscano**: Dynamic clamping can modify or introduce any membrane current into a neuron. We introduce and modify a Na/K pump and persistent Na currents into a living heart interneuron of a leech **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Ricardo Erazo Toscano**: Using a dynamic clamp allows complete control with a key stroke of the dynamics and amplitude of any current introduced into a neuron in real time **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Ronald Calabrese**: The development of real-time interactive systems, such as the dynamic clamp, underpins all BMI research **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.4. **Ronald Calabrese**: All aspects of cellular studies on the electrical activity of neurons and networks can potentially benefit from the use of dynamic clamp **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.5. **Gennady Cymbalyuk**: The main challenges of this technique are developing a real-time model of the neuron and calibrating the dynamic clamp adjustments to the neuron selected for study **[1]**.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Isolated Ganglion 7 Preparation

2.1. For isolation of the ganglion 7 from a leech nerve cord, fill a black, resin-lined dissecting dish with about 1 centimeter of chilled saline supplemented with sodium chloride, 4 potassium chloride, calcium chloride, D-glucose, and HEPES (heeps) in deionized water **[1]** and pin a cold-anesthetized leech dorsal side up in the chamber **[2]**.

2.1.1. WIDE: Talent filling dish, with solution container visible in frame

2.1.2. Leech being pinned

2.1.2B Added shot: A CU OF THE PINNING

2.2. Use a stereomicroscope at a 20x magnification with oblique light guide illumination **[1]** and 5-millimeter spring scissors to make a longitudinal cut at least 3 centimeters long through the body wall in the rostral third portion of the body **[2]**.

2.2.1. Talent placing dish under microscope

2.2.2. SCOPE: Incision being made

2.3. Use pins to pull apart the body wall tissue to expose the internal organs **[1-TXT]** and vacuum the blood to get a better view **[added 2]**. Isolate an individual mid-body ganglion 7 **[3-TXT]**.

2.3.1. SCOPE: Tissue being pinned **TEXT: Use fire-polished Pasteur pipette to remove any stored blood meal**

2.3.2. Added shot: SCOPE: Vacuum the blood to see better.

2.3.3. SCOPE: Ganglion being isolated **TEXT: 7th free segmental ganglion caudal to brain**

2.4. Using sharp number 5 forceps to help guide the cutting and to hold the sinus, use the scissors to open the sinus in which the nerve cord resides, taking care to split the sinus dorsally and ventrally **[1]**.

2.4.1. SCOPE: Sinus being opened/split *Videographer: Important step*

2.5. Keeping the sinus attached to each of the two bilateral nerve roots that emerge from the ganglion, cut the rostral and caudal connective nerve bundles to remove the ganglion from the body **[1]** and cut the roots lateral to where they emerge from the sinus **[2]**.

2.5.1. SCOPE: Shot of strips being left attached, then bundles being cut

2.5.2. SCOPE: Roots being cut

2.6. Use old, blunted number 5 forceps to secure the sinus strips and loose tissue with shortened minuten insect pins ventral side up in clear, resin-lined Petri dishes **[1]** **[2]**.

2.6.1. SCOPE: Strips being secured. Videographer NOTE: Shot on a cam as well 'pouring into dish'

2.6.1 B. SCOPE shot.

2.6.2. Added shot: SCOPE: Old, blunted number 5 forceps being used.

2.7. Pin the rostral and caudal connectives as far from the ganglion as possible **[1]** and securely pin the roots **[2]**.

2.7.1. SCOPE: Ganglion being pinned *Videographer: Important step*

2.7.2. SCOPE: Roots being pinned

2.8. Increase the magnification of the stereomicroscope to 40x or greater **[1]** and adjust the oblique illumination such that the neuronal cell bodies can be easily observed on the ventral surface of the ganglion just below the perineurium **[2]**.

2.8.1. SCOPE: Higher magnification being selected Videographer NOTE: 2.8.1 and 2.8.2 are shot with scope and a cam. Use the B version of the shot

2.8.2. SCOPE: Illumination being adjusted

2.9. Using microscissors, begin cutting the loose perineal sheath between the roots on one side of the ganglion, continuing the cut laterally to the other side and making sure to keep the scissor blades superficial without harming the neuronal cell bodies directly beneath the sheath **[1]**.

2.9.1. SCOPE: Sheath being cut *Videographer: Important/difficult step*

2.10. Make a similar superficial cut caudally from the lateral cut along the midline **[1]** and use fine number 5 forceps to pull one caudolateral flap of sheath away from the ganglion at a time to allow each to be excised **[2]**.

2.10.1. SCOPE: Cut being made

2.10.2. SCOPE: Flap being pulled and cut

2.11. When both flaps have been removed, both heart interneurons should be exposed **[1]**. Place the dish in the recording setup **[2]** and superfuse the sample with saline at a 5-milliliter/minute flow rate at room temperature **[3]**.

2.11.1. SCOPE: Shot of heart interneurons

2.11.2. Talent placing dish into setup

2.11.3. Sample being superfused

3. Leech HN Identification and Recording

3.1. To identify the interneurons on the recording setup, select a 50-100x magnification with dark field illumination below **[1]** and locate an HN7 (H-N-seven) neuron of the bilateral pair by its canonical location at the posteriolateral position in midbody ganglion seven **[2]**.

3.1.1. WIDE: Talent selecting magnification

3.1.2. SCOPE: HN7 neuron being located Videographer NOTE: See the still image with the neuron(s) circled. *Video Editor: Please do the same (encircle the neurons) in the video*

3.2. Next, use a micromanipulator to position a sharp microelectrode filled with 2-molar potassium acetate and 20-millimolar potassium chloride microelectrode very near the target cell body **[1]** and use a neurophysiological electrometer to continuously observe the recorded potential **[2]**.

3.2.1. SCOPE: Electrode being positioned *Videographer: Important step*

3.2.2. SCREEN: 3_2_2.mp4. 0:00-0:05. Shot of electrometer readout

3.3. Set this potential to zero millivolts **[1]** and penetrate the neuron with the microelectrode, using the manipulator to slowly drive the electrode along its long axis **[2]**.

3.3.1. Talent setting potential

3.3.2. SCOPE: Neuron being penetrated *Videographer: Important step*

3.4. Use a 100-millisecond electrometer buzz function until a negative shift in membrane potential **[1]** and vigorous spiking activity is observed **[2]**.

3.4.1. SCOPE: Buzz function being delivered *Videographer: Important step*
Videographer NOTE: Shot on a cam, unslated

3.4.2. SCREEN: 3_4_2.mp4. 0:02-0:06. Shot of vigorous spiking activity in electrometer readout

3.5. Set the electrometer to at least 3 kilohertz in discontinuous current-clamp mode to simultaneously record the membrane potential while passing the current to the neuron **[1]**.

3.5.1. Talent setting electrometer

3.6. Use an oscilloscope to monitor the setting of the electrode **[1]** and use a steady current injector to inject a steady current of minus 0.1 nanoamps for 1-2 minutes to stabilize the recording **[2]**.

3.6.1. SCREEN: 3_6_1.mp4. 0:00-0:05. Shot of oscilloscope readout

3.6.2. SCREEN: 3_6_3.mp4. 0:40-0:55. Shot of recording being stabilized *Video Editor: please add as inset in 3.6.2.*

3.7. The HN7 neuron can be identified by its characteristic spike shape and weak bursting activity **[1]**.

3.7.1. SCREEN: 3_7_1.mp4. 0:10-0:23. Shot of readout with spike shape *Video Editor: Feel free to speed the footage*.

4. Dynamic Clamp Conductance and Current Implementation and Variation

4.1. For dynamic clamp conductance and current implementation, open a dynamic clamp software program custom-built for the digital signal processing board **[1]** and, while the model is running, set the maximal pump current to 0.1-0.2 nanoamps and gradually increase the maximal conductance of the persistent sodium current until regular bursting ensues **[2]**.

4.1.1. WIDE: Talent opening software, with monitor visible in frame

4.1.2. SCREEN: 4_1_2.JPG. *Video Editor: Emphasize pop up box*

4.2. Systematically co-vary these currents in 0.1-nanoamp increments for the maximal conductance and 1-nanosecond increments for the persistent sodium current and assess the effects of these increases on the spike frequency, interburst interval, burst duration, and burst period **[1]**.

4.2.1. SCREEN: 4_2_1.mp4. 0:09-0:22, 1:00-1:23. Values being increased, shot of burst characteristic changes *Video Editor: Feel free to speed up the footage as required*.

4.3. Hold the maximal conductance of the sodium current at a specific fixed value and sweep in 1-nanoamp increments over a range of maximal pump currents to support regular bursting activity before increasing the fixed value of the sodium conductance by 1 nanosiemen and sweeping over a second range of maximal pump currents **[1]**.

4.3.1. SCREEN: 4_3_1.mp4. 0:13-0:22, Maximal conductance of sodium current at fixed value, and maximal pump current being changed, then maximal conductance of sodium current at new fixed value, then maximal pump current being changed

4.4. For each implemented parameter pair, collect data containing at least 8 bursts so that reliable average measures of the spike frequency, interburst interval, burst duration, and burst period can be made **[1]**.

4.4.1. SCREEN: 4_4_1.mp4: Data being collected *Video Editor: please emphasize spike frequency, IBI, BD, and burst period when mentioned as possible*

4.5. Then collect data from several additional neurons as just demonstrated to generate a composite graph **[1]**.

4.5.1. LAB MEDIA: Figure 5

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.4.1., 2.7.1., 2.9., 3.2., 3.4., 3.5.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
2.9 depends heavily on steady hands, lots of practice, and good illumination (2.8.)

Results

5. Results: Representative HN Membrane Potential Induction and Analysis

5.1. Using this model **[1]**, the outward pump current oscillates throughout the burst cycle as the internal concentration of sodium around a baseline level **[2]**. This pump current contributes to burst termination during the burst phase **[3]**.

5.1.1. LAB MEDIA: Figure 2
5.1.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize Ipump row*
5.1.3. LAB MEDIA: Figure 2 *Video Editor: please emphasize [Na+]i row*

5.2. The hyperpolarization produced by the pump current activates hyperpolarization-activated inward current during the interburst interval **[1]**.

5.2.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize Ih row*

5.3. The real-time heart interneuron model indicates that the persistent sodium current in heart interneurons **[1]** contributes to much of the sodium entry strongly affecting the internal concentration of the sodium and thus the pump current **[2]**.

5.3.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize Ip row*
5.3.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize Ipump and [Na+]i rows*

5.4. Because the persistent sodium current is active at relatively negative membrane potentials, it opposes the pump current during both the burst and interburst intervals **[1]**.

5.4.1. LAB MEDIA: Figure 2

5.5. As illustrated, robust bursting is restored in tonically active heart interneurons **[1]** by the co-addition of persistent sodium and outward currents with a dynamic clamp **[2]**.

5.5.1. LAB MEDIA: Figure 4
5.5.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize Ip and Ipump rows*

5.6. Preliminary results indicate a strong complicated interaction between the two currents, which can be further explored using the model **[1]**.

5.6.1. LAB MEDIA: Figure 5 *Video Editor: please sequentially add/emphasize data lines*

Conclusion

6. Conclusion Interview Statements

6.1. **Ricardo Erazo Toscano**: A successful experiment depends on a good de-sheathing of the ganglion and a carefully directed driving, penetrating, and buzzing of the microelectrode **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.7, 2.9, 2.10)

6.2. **Ricardo Erazo Toscano**: We augment the dynamic clamp by implementing a Na-dependent pump current that is calculated by estimating the intracellular concentration of an ion. Such estimates can be used to inject any ion-dependent current into a neuron **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera