Journal of Visualized Experiments

Contribution of the Na+/K+ pump to rhythmic bursting, explored with modeling and dynamic clamp analyses.

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61473R2
Full Title:	Contribution of the Na+/K+ pump to rhythmic bursting, explored with modeling and dynamic clamp analyses.
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Additional Information:	
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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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KEYWORDS:
leeches, modulation, Invertebrate Central Pattern Generator CPG, Na^+/K^+ pump, h-current, persistent Na^+ current

SUMMARY:
Presented here is a method for investigation of the roles of the Na^+/K^+ pump and persistent Na^+ current in leech heart interneurons using dynamic clamp.

ABSTRACT:
The Na^+/K^+ pump, often thought of as a background function in neuronal activity, contributes an outward current (I_{pump}) that responds to the internal concentration of Na^+ ($[Na^+]_i$). In bursting neurons, such as those found in central pattern generator (CPG) neuronal networks that produce rhythmic movements, the $[Na^+]_i$ and therefore the I_{pump}, can be expected to vary throughout the burst cycle. This responsiveness to electrical activity, combined with independence from membrane potential, endow I_{pump} with dynamical properties not common to channel-based currents (e.g., voltage- or transmitter-gated or leak channels). Moreover, in many neurons, the pump's activity is modulated by a variety of modulators, further expanding the potential role of I_{pump} in rhythmic bursting activity. This paper shows how to use a combination of modeling and dynamic clamp methods to determine how I_{pump} and its interaction with persistent Na current influence rhythmic activity in a CPG. Specifically, this paper will focus on a dynamic clamp protocol and computational modeling methods in heart interneurons of medicinal leeches.

INTRODUCTION:

Heartbeat in leeches is driven by a CPG consisting of 9 bilateral pairs of heart interneurons (HNs) distributed across as many mid-body segmental ganglia. At the core of the CPG are mutually inhibitory pairs of interneurons located in the 3rd and 4th segmental ganglia that form half-center oscillators (HCOs) (**Figure 1A**). These neurons continue to burst when synaptically isolated pharmacologically using bicuculline[1]. Others, such as the pair in the 7th segmental ganglia (the focus of this protocol), are also bursters, capable of producing bursting activity when synaptically isolated. They are not mutually connected and receive only descending input, and thus are easily isolated by severing the ganglion from the rest of the nerve cord. This independent bursting activity is sensitive to introduced leak current caused by penetration with sharp microelectrodes for recording, but is vigorously burst when recorded with loose patch methods[1].

Both individual HN neurons and HN HCOs (Hodgkin-Huxley-based single isopotential compartment models of HN neurons containing all experimentally identified voltage-gated and synaptic currents) have been modeled, and all the burst characteristics of the living system have been successfully captured[2]. Myomodulin, an endogenous neuropeptide in leeches, markedly decreases the period (T) of the burst rhythm of isolated HN neurons and HN HCOs. This modulator acts to increase h-current (hyperpolarization-activated inward current, I_h) and to decrease I_{pump}[3]. This observation led to the exploration of how I_{pump} interacts with I_h, and how their co-modulation contributes to the rhythmic activity of HN neurons. Activation of the pump by increasing $[Na^+]_i$ (using the ionophore monensin) speeds the HN burst rhythm in both HN HCOs and isolated HN neurons[4]. This speed-up was dependent on I_h—when I_h was blocked (2 mM Cs^+), the burst period was not altered by this method of pump activation; however, the burst duration (BD) was curtailed, and the interburst interval (IBI) increased in both HN HCOs and isolated HN neurons[4].

For this protocol, all the currents of a living HN(7) neuron, including the pump current, I_{pump}, are incorporated in the HN model as follows:

$$C\frac{\mathrm{d}V}{\mathrm{d}t} = -\left(I_{\mathrm{Na}} + I_{\mathrm{P}} + I_{\mathrm{K1}} + I_{\mathrm{K2}} + I_{\mathrm{KA}} + I_{\mathrm{h}} + I_{\mathrm{CaF}} + I_{\mathrm{CaS}} + I_{\mathrm{Leak}} + I_{\mathrm{pump}}\right) \quad (1)$$

where C is the membrane capacitance (in nF), V is the membrane potential (in V), t is time (in s). Detailed ionic current descriptions and equations have been described elsewhere[2,4]. The complete HN model neuron runs in real time (**Figure 2**). The software will be made available on GitHub upon publication and will be suitable to run on the digital signal processing board described in the **Table of Materials**. Here, the focus of enquiry is the Na^+/K^+ pump current (I_{pump}) and the voltage-gated currents contributing significant Na^+ flux: a fast Na^+ current (I_{Na}) and a persistent Na^+ current (I_P). The maximal conductances of these currents are $\bar{g}_{Na}$ and $\bar{g}_P$, respectively. The Na^+/K^+ pump exchanges three intracellular Na^+ ions for two extracellular K^+ ions, thus producing a net outward current. Importantly, it pumps 3 times as much Na^+ out of the neuron as this current indicates, which is important for calculating the intracellular Na^+ concentration.

The Na^+/K^+ pump current depends on intracellular Na^+ concentrations and is expressed by the following sigmoidal function:

$$I_{\text{pump}} = \frac{I_{\text{pump}}^{\max}}{1+\exp\left(\frac{[\text{Na}]_{\text{ih}}-[\text{Na}]_{\text{i}}}{[\text{Na}]_{\text{is}}}\right)} \quad (2)$$

where $[Na]_i$ is the intracellular Na^+ concentration, $I_{\text{pump}}^{\max}$ is the maximal Na^+/K^+ pump current, $[Na]_{ih}$ is the intracellular Na^+ concentration for the half-activation of the Na^+/K^+ pump, and $[Na]_{is}$ the sensitivity of the Na^+/K^+ pump to $[Na]_i$. $[Na]_i$ builds as a result of the Na^+ influxes carried by I_P and I_{Na} and is diminished by the Na^+ efflux of the Na^+/K^+ pump. The contribution of I_h and I_{Leak} to the total Na^+ flux is small and is not considered in the real-time model.

$$\frac{\mathrm{d}[Na]_i}{\mathrm{d}t} = -\frac{I_{\text{P}} + I_{\text{Na}} + 3\,I_{\text{pump}}}{\text{vF}} \quad (3)$$

where, v is the volume (~6.7 pL) of the intracellular Na^+ reservoir, F is Faraday's constant, and the extracellular Na^+ concentration is kept constant.

Voltage-gated and leak conductances have been differentiated—these respond to membrane potential–from the pump current, which is regulated by the calculated intracellular Na^+ concentration ($[Na^+]_i$). $[Na^+]_i$ is built up through Na^+ entry via the fast Na^+ current (I_{Na}) that produces action potentials (spikes) and the persistent Na^+ current (I_P) that provides the depolarization to support spiking. $[Na^+]_i$ is, in turn, reduced by the action of the pump through the extrusion of Na^+. Baseline living HN values of $\bar{g}_P$ (5nS) and $\bar{g}_{Na}$ (150 nS) have been assumed to take account of any added dynamic clamp $\bar{g}_P$.

The goal of the protocol described here is to manipulate I_{pump} precisely and reversibly in real time to discover how it interacts with voltage-gated currents (persistent Na^+ current in the current protocol) to control rhythmic bursting in single HNs. To accomplish this goal, dynamic clamp was used, which artificially introduces, upon command, a precise amount of any current that can be calculated as the model is running. This method has advantages over pharmacological manipulation of the pump, which affects the entire tissue, can have off-target effects that are often hard to reverse, and cannot be precisely manipulated. Dynamic clamp[5,6] reads the voltage of a recorded neuron in real time (**Figure 1B**) and calculates and injects, in real time, the amount of any current based on model equations and the set values of any $\bar{g}_x$ or I_x^{max}. Similar methods can easily be applied to any neuron that can be recorded intracellularly. However, parameters will have to be rescaled to the neuron chosen, and the neuron should be isolated from synaptic inputs, e.g., pharmacologically.

PROTOCOL:

NOTE: Invertebrate animal experimental subjects are not regulated by the NIH or Emory and Georgia State Universities. All measures were nevertheless taken to minimize the suffering of the leeches used in this work.

1. Prepare isolated ganglion 7 from the leech nerve cord

1.1. Maintain leeches *Hirudo verbana* in artificial pond water (containing 0.05% w/v of sea salt) diluted in de-ionized water at 16 °C on a 12:12 light-dark cycle.

1.2. Prepare the leeches for dissection by cold-anesthetizing them in a bed of crushed ice for >10 min until immobile.

1.3. Fill a black, resin-lined dissecting dish to a depth of ~1 cm with chilled saline containing 115 mM NaCl, 4 mM KCl, 1.7 mM $CaCl_2$, 10 mM D-glucose, and 10 mM HEPES in de-ionized water; pH adjusted to 7.4 with 1 M NaOH. Pin the leech dorsal side up in the black resin-lined chamber (at least 20 cm x 10 cm with a depth of at least 2 cm above the resin that is at least 2 cm thick).

1.4. Under a steromicroscope at 20x magnification with oblique light guide illumination, make a longitudinal cut at least 3 cm long with 5 mm spring scissors through the body wall in the rostral 1/3rd portion of the body. Use pins to pull aside the body wall and expose the internal organs.

NOTE: Any stored blood meal can be removed by suction with a fire-polished Pasteur pipette.

1.5. Isolate an individual mid-body ganglion 7 (seventh free segmental ganglion caudal to the brain).

1.5.1. Open the sinus in which the nerve cord resides using the 5 mm spring scissors. Be sure to split the sinus dorsally and ventrally leave two strips of sinus. Use sharp #5 forceps to help guide the cutting and hold the sinus.

1.5.2. Keep the sinus attached to each of the two bilateral nerve roots that emerge from the ganglion (it adheres tightly to each root) to use these strips of sinus for pinning out the ganglion.

1.5.3. Remove the ganglion from the body by cutting the rostral and caudal connective nerve bundles that link the ganglia (as far from the 7th ganglion as possible) and the sinus strips, and then cut the roots lateral to where they emerge from the sinus.

1.6. Pin (using old blunted #5 forceps) with shortened minuten insect pins, ventral side up, in clear, resin lined Petri dishes. Insert pins in the strips of sinus and loose tissue adhering to the roots and the rostral and caudal connectives, as far from the ganglion as possible.

NOTE: The resin must be no thicker than 3 mm if good illumination from below is to be achieved during recording. Make sure the ganglion is taut, both longitudinally and laterally

1.7. Increase the magnification of the stereomicroscope to 40x or greater, and adjust the oblique illumination so the neuronal cell bodies can be easily seen on the ventral surface of the ganglion just below the perineurium.

1.8. Remove the perineurium of the ganglion (desheath) with microscissors.

1.8.1. Start the desheathing by cutting the loose sheath between the roots on one side, and continue the cut laterally to the other side, making sure to keep the scissor blades superficial and not harm the neuronal cell bodies directly beneath the sheath.

1.8.2. Make a similar superficial cut caudally from the lateral cut along the midline.

1.8.3. Now grab the caudolateral flap of sheath on one side with the fine #5 forceps, pull it away from the ganglion, and cut it off with the microscissors.

1.8.4. Repeat on the other side; this procedure exposes both HN(7) neurons for recording with microelectrodes.

1.9. Place the preparation dish in the recording setup, and superfuse with saline at a flow rate of 50 mL/min at room temperature.

2. Identify and record leech heart interneurons with sharp microelectrodes

2.1. For the duration of the recording of the HN(7) neuron (recordings last between 30 to 60 min), acquire and digitize the intracellular current and voltage traces from a neurophysiological electrometer sampling at rate of 5 kHz with a digital data acquisition (Analog to Digital, A to D) and stimulation (Digital to Analog, D to A) system, and display on a computer screen.

NOTE: Any commercial or custom-built software and A to D/D to A board can be used for data acquisition (A to D). D to A and custom-built software are required for dynamic clamp.

2.2. Under a steromicroscope at 50–100x with dark field illumination from below, tentatively identify an HN(7) neuron of the bilateral pair by its canonical location at the posteriolateral position in midbody ganglion seven.

2.3. Now aim to penetrate the putative HN(7) neuron with a sharp microelectrode filled with 2 M potassium acetate and 20 mM KCl using a micromanipulator.

2.3.1. Place the microelectrode very near the target cell body.

2.3.2. Continuously observe the recorded potential with the electrometer, and set this potential to zero mV before penetrating the neuron.

2.3.3. Penetrate the neuron with the microelectrode, and drive the electrode along its long axis with the manipulator moving slowly. Using the electrometer buzz function, set to 100 ms buzz duration until a negative shift in membrane potential and vigorous spiking activity is observed.

2.4. Set the electrometer in discontinuous current-clamp mode (DCC) ≥ 3 kHz to simultaneously record membrane potential, and pass current with the single microelectrode (capacity compensation set to just below ringing and then dialed back 10%).

2.4.1. Monitor the settling of the electrode during DCC on an oscilloscope.

2.4.2. Inject a steady current of -0.1 nA with the electrometer steady current injector for a minute or two to stabilize the recording.

2.5. Definitively identify the HN(7) neuron by its characteristic spike shape and weak bursting activity (**Figure 1C*i***).

2.6. Perform any data analysis offline after the experiment is completed, and save all data on a disk.

3. Build a real-time HN or another model neuron

3.1. Build custom software using a digital signal processing board (DSB; D to A and A to D) in a desk-top computer to implement in real time the model currents described in[2,4] or different model currents for other neurons or experiments.

3.1.1. Use Hodgkin-Huxley style equations as they are the generally preferred method for representing model currents.

3.1.2. See[7] for a detailed description of the implementation of the real-time HN model and dynamic clamp prior to the addition of the pump current. Refer to the introduction section for the description of the currents, intracellular Na^+ concentration, and conductances in a living HN(7) neuron in the HN model.

4. Implement and vary dynamic clamp conductances/currents

4.1. Use the custom-built dynamic clamp software for the DSB to implement and change in real time dynamic clamp any of the graphical user interface (**Figure 3**) (GUI)-accessible, programmed conductances and currents of the HN real-time model of the HN(7) neuron.

NOTE: As a reminder, $\bar{g}_P$ and I_{pump}^{max}.are the maximal conductance of the persistent Na^+ current (I_P) and the maximal pump current (I_{pump}), respectively.

4.2. Use GUI entry boxes in the software to make changes, as the model is running, in the I_{pump}^{max} (PumpMaxL box) and $\bar{g}_P$ (GpinHNLive box) (**Figure 3**).

NOTE: The GUI input boxes accept typed values, and steps of 0.1 nA are recommended for I_{pump}^{max} and steps of 1 nS are recommended for $\bar{g}_P$.

4.2.1. Add small amounts of I_{pump}^{max} and $\bar{g}_P$ with dynamic clamp to stabilize bursting of the HN(7) neuron, as shown in **Figure 1C*ii***, which is weakened by a microelectrode-induced leak.

NOTE: Sharp microelectrode penetration causes membrane damage that is expressed as increased leak conductance or decreased input resistance.

4.2.2. Start by adding a value of I_{pump}^{max} of 0.1-0.2 nA, which makes up for the microelectrode-induced leak, but depresses excitability, and then gradually increase $\bar{g}_P$, which increases excitability, until regular bursting ensues, usually at $\bar{g}_P$ of ~1–4 nS (**Figure 4A**).

4.3. Systematically co-vary these currents (increments of 0.1 nA for I_{pump}^{max} and 1 nS for $\bar{g}_P$) to the recorded HN(7) neuron with dynamic clamp (**Figure 3**), and assess their effects on burst characteristics: spike frequency (f: the reciprocal of the average of the interspike interval during a burst), interburst interval (IBI: the time between the last spike in one burst to the first spike in the next burst), burst duration (BD: the time between the first spike in a burst and the last spike in a burst), and burst period (T: the time between the first spike in a burst and the first spike in the subsequent burst).

4.3.1. Change the values of I_{pump}^{max} and $\bar{g}_P$, as in the video demonstration, to become familiar with the technique and then venture out.

4.3.1.1. Hold I_{pump}^{max} at a specific fixed value and sweep in 1 nS increments over a range of $\bar{g}_P$ supporting regular bursting activity.

4.3.1.2. Now increase the fixed value of I_{pump}^{max} by 0.1 nA and again sweep over a range of $\bar{g}_P$ supporting regular bursting activity.

4.3.1.3. For each implemented parameter pair, collect data containing at least 8 bursts so that reliable average measures of f, IBI, BD, and T can be made.

4.3.1.4. Continue with sweeps for as long as the neuron remains viable, as assessed by strong spiking and a stable baseline potential of oscillation.

4.3.1.5. Collect data from several neurons (from different animals) to generate a composite graph (**Figure 5**).

REPRESENTATIVE RESULTS:

Modeling with the addition of I_{pump}[4] brought the experimental findings presented in the introduction section into sharper focus and began to explain the pump-assisted mechanism of bursting. The real-time model demonstrated here has been tuned ($\bar{g}_x$ and I_x^{max} parameters chosen) so that it produces regular rhythmic activity falling within the bounds of normal activity as observed in experiments—spike frequency (f), IBI, BD, T)—and continues to produce such activity when the myomodulin-modulated parameters I_{pump}^{max} (the maximal pump current) and $\bar{g}_h$ (maximal conductance of h-current) are varied or co-varied in the model. The parameter values determined can be used as a benchmark or canonical set for modeling experiments. In these model instances, I_{pump} oscillates throughout the burst cycle as $[Na^+]_i$ around a baseline level. I_{pump} contributes to burst termination during the burst phase, and the hyperpolarization it produces activates I_h during the IBI; notice the maximal level of I_h near burst initiation (**Figure 2**).

Although the real-time HN model has all implemented currents[2,4] available for dynamic clamping, the focus here was on I_{pump}^{max} and $\bar{g}_P$, which are available for changes while the model is running in the dynamic clamp GUI (**Figure 3**). Dynamic clamp allows the experimenter to add (or subtract with a negative $\bar{g}_x$ or I_x^{max}) any conductance or current into a neuron artificially that mimics the voltage and ionic dependence of a real conductance or current. Thus, it is possible to fully explore how a particular conductance/current interacts with the endogenous conductances/currents inside cells (**Figure 1**). The real-time HN model indicates that the persistent Na^+ current (I_P) in HN neurons contributes much of the Na^+ entry strongly affecting $[Na^+]_i$ (**Figure 2**) and thus, I_{pump}. Because I_P is active at relatively negative membrane potentials, it opposes I_{pump} even during the IBI.

These observations indicate that it is instructive to explore interactions between I_{pump}^{max} and $\bar{g}_P$ in isolated HN neurons with dynamic clamp as discussed previously[8,9.10]. These experiments (ongoing) are performed with sharp microelectrode recordings in single, synaptically isolated HN(7) neurons (seventh ganglion severed from the nerve cord). To date, these experiments show that robust bursting is restored in tonically active HN neurons (due to microelectrode penetration introduced leak) by co-addition of I_P and I_{pump} with dynamic clamp (**Figure 4**). This is an important observation indicating that a bursting mechanism is available in these neurons (even when leak is compromised) that results from the interaction of I_{pump} and I_P. Preliminary results indicate their strong complicated interaction, which can be explored in the model and experiments (**Figure 5**).

In conclusion, I_{pump} in response to periodic increases in $[Na^+]_i$ during bursting activity contributes to the burst rhythm through burst termination (decreasing BD). The interaction of I_P and I_{pump} constitutes a mechanism that is sufficient to support endogenous bursting activity; this mechanism can reinstate robust bursting in HN interneurons recorded intracellularly in ganglion 7. The interaction between I_P and I_{pump} through $[Na^+]_i$ affects the HN burst period non-monotonically and ensures robustness of autonomous bursting. These conclusions are in line with experiments and modeling in vertebrate systems[11,12].

FIGURE LEGENDS:

Figure 1: Leech heart interneuron electrical activity and implementation of I_{pump} and I_P with dynamic clamp. (**A**) Normal bursting activity simultaneously recorded, extracellularly (top) and intracellularly (bottom), in a leech heartbeat HCO from a third ganglion, a schematic of the recorded neurons and their mutually inhibitory synaptic connections at right. (**B**) Dynamic clamp schematic when recording a HN(7) interneuron in an isolated ganglion 7; note there is no synaptic interaction between the two HN(7) interneurons. (**C*i***) Bursting in a leak-compromised HN(7) interneuron. (**C*ii***) More robust bursting can be produced by adding dynamic clamp I_{pump} (I_{pump}^{max} = 0.1 nA), which makes up for the microelectrode induced leak, but depresses excitability, and $\bar{g}_P$ (1 nS), which increases excitability. Black dashed lines indicate baseline values. Abbreviations: HN = heart interneuron; HCO = half-center oscillator; I_{pump} = outward current; I_P = persistent Na^+ current; $I_{\mathrm{pump}}^{\mathrm{max}}$ = maximal Na^+/K^+ pump current; $\bar{g}_P$ = maximal conductance of the persistent Na^+ current; Vm = membrane potential; $[Na^+]_I$ = internal concentration of Na^+.

Figure 2: Single HN interneuron model showing traces for membrane potential (Vm), I_h, I_{pump}, $[Na^+]_i$, and I_P. Outward hyperpolarizing currents are negative, and inward depolarizing currents are positive. Black dashed lines indicate baseline values. Abbreviations: HN = heart interneuron; I_{pump} = outward current; I_P = persistent Na^+ current; $I_{\text{pump}}^{\max}$ = maximal Na^+/K^+ pump current; I_h = hyperpolarization-activated inward current; $\bar{g}_P$ = maximal conductance of the persistent Na^+ current; $\bar{g}_h$ = maximal conductance of the hyperpolarization-activated inward current; Vm = membrane potential; $[Na^+]_I$ = internal concentration of Na^+.

Figure 3: Graphical user interface of real-time heart interneuron (HN) model and dynamic clamp implemented on a digital signal processing board. Upper Left: Red Math boxes are user-determined parameter boxes for the real-time model, whereas Blue Live boxes are user-determined parameter boxes used in the dynamic clamp. El = the reversal potential of the leak current; Gl = leak conductance; Gh = h-current maximal conductance; Gp = P current maximal conductance; GCaS = slow calcium current maximal conductance; PumpMax = pump maximal current; [GSyn2 maximal synaptic conductance to the respective neuron; ThreshSyn2 spike crossing threshold for mediating a synaptic potential – these used to make a hybrid (living/model) half-center oscillator not illustrated here.]. Lower Left for Dynamic Clamp. At the very left are 5 computed values of dynamic clamp variables: I_{pump} = pump current injected; Ih = h-current injected (not used here); IP = P current injected; NaI = calculated internal Na^+ concentration; Ena = calculated sodium reversal potential. Lower Left for Dynamic Clamp. To the right of the computed variables are 6 user determined parameter boxes: GNa = assumed endogenous fast sodium maximal conductance use to calculate Na^+ flux associated with action potentials; PumpMaxL = maximal pump current to be injected by dynamic clamp; Naih see equation (2); Gh = maximal conductance to determine h-current to be injected by dynamic clamp; Gp = assumed endogenous p maximal conductance use to calculate Na^+ flux associated with endogenous P current; GpinHNLive = maximal conductance to determine P current to be injected by dynamic clamp.

Figure 4: Dynamic clamp analysis of independent HN(7) bursting. Upregulation of $\bar{g}_P$ from (**A**) 4.0 nS to (**B**) 9.0 nS slows down the independent HN burst rhythm. Experimental traces show rhythmic bursting in isolated HN(7) neuron with dynamic clamp. The ranges of oscillation of $[Na^+]_i$ and Vm increase with upregulated $\bar{g}_P$. Traces top to bottom: recorded Vm, injected I_{pump}, calculated $[Na^+]_i$, and injected I_P. Black dashed lines indicate baseline values. Abbreviations: HN = heart interneuron; I_{pump} = outward current; I_P = persistent Na^+ current; $I_{\text{pump}}^{\max}$ = maximal Na^+/K^+ pump current; $\bar{g}_P$ = maximal conductance of the persistent Na^+ current; Vm = membrane potential; $[Na^+]_I$ = internal concentration of Na^+.

Figure 5: Dynamic clamp analysis of independent HN(7) bursting. Upregulation of $\bar{g}_P$ tends to decrease, followed by an increased HN burst period. In individual experiments (points connected by lines) using dynamic clamp, $\bar{g}_P$ values were swept while I_{pump}^{max} was held constant. Colors represent different constant levels of added I_{pump}^{max} used in different experiments. Abbreviations:

HN = heart interneuron; $I_{\text{pump}}^{\max}$ = maximal Na^+/K^+ pump current; $\bar{g}_P$ = maximal conductance of the persistent Na^+ current.

DISCUSSION:

Modeling, dynamic clamp, and the resulting analyses that they enable are useful techniques for exploring how individual and groups of ionic conductance/currents contribute to the electrical activity of neurons (**Figure 1**, **Figure 2**,**Figure 4**, and **Figure 5**). The use of these techniques shows how the Na^+/K^+ pump current (I_{pump}) interacts with voltage-gated currents, particularly the persistent Na^+ current (I_P), to promote robust bursting in the leech heartbeat pattern generator's core HNs. By combining dynamic clamp experiments and modeling, it is possible to test models more directly than is possible with ordinary voltage recording and current clamp techniques. The results gathered from the dynamic clamp experiments (**Figure 5**) will be used to further refine the HN model. The basic method of dynamic clamping demonstrated here can be customized to reflect the properties of any neuron under study if a mathematical model of neuronal currents can be determined with voltage clamp experiments.

Successful completion of the experiments of the type shown here requires careful impalement of an HN or other neuron when using a sharp microelectrode, because strong bursting is curtailed by electrode penetration[1]. (Whole-cell patch recording techniques, which minimize introduced leak, are also applicable to other neurons, but do not work well on leech neurons.) It is critical that the impalement of the HN neuron causes minimal damage to the neuron (added leak), and input resistance should be monitored and must be in the range of 60–100 MOhms for successful experiments[4].

Dynamic clamp is a powerful technique, but it has limitations imposed by neuronal geometry because the artificial conductances are implemented at the site of the recording electrode—usually the cell body—not at the site where rhythm-generating currents are usually localized[5,6,10]. In leech HN neurons, the cell body is electrically close to the integration zone (main neurite) of the neuron where most active currents are localized, and spikes are initiated.

ACKNOWLEDGMENTS:

We thank Christian Erxleben for preliminary dynamic clamp experiments on HN(7) neurons that demonstrated their bursting capabilities. Angela Wenning aided the experiments with expert advice.

DISCLOSURES:

None

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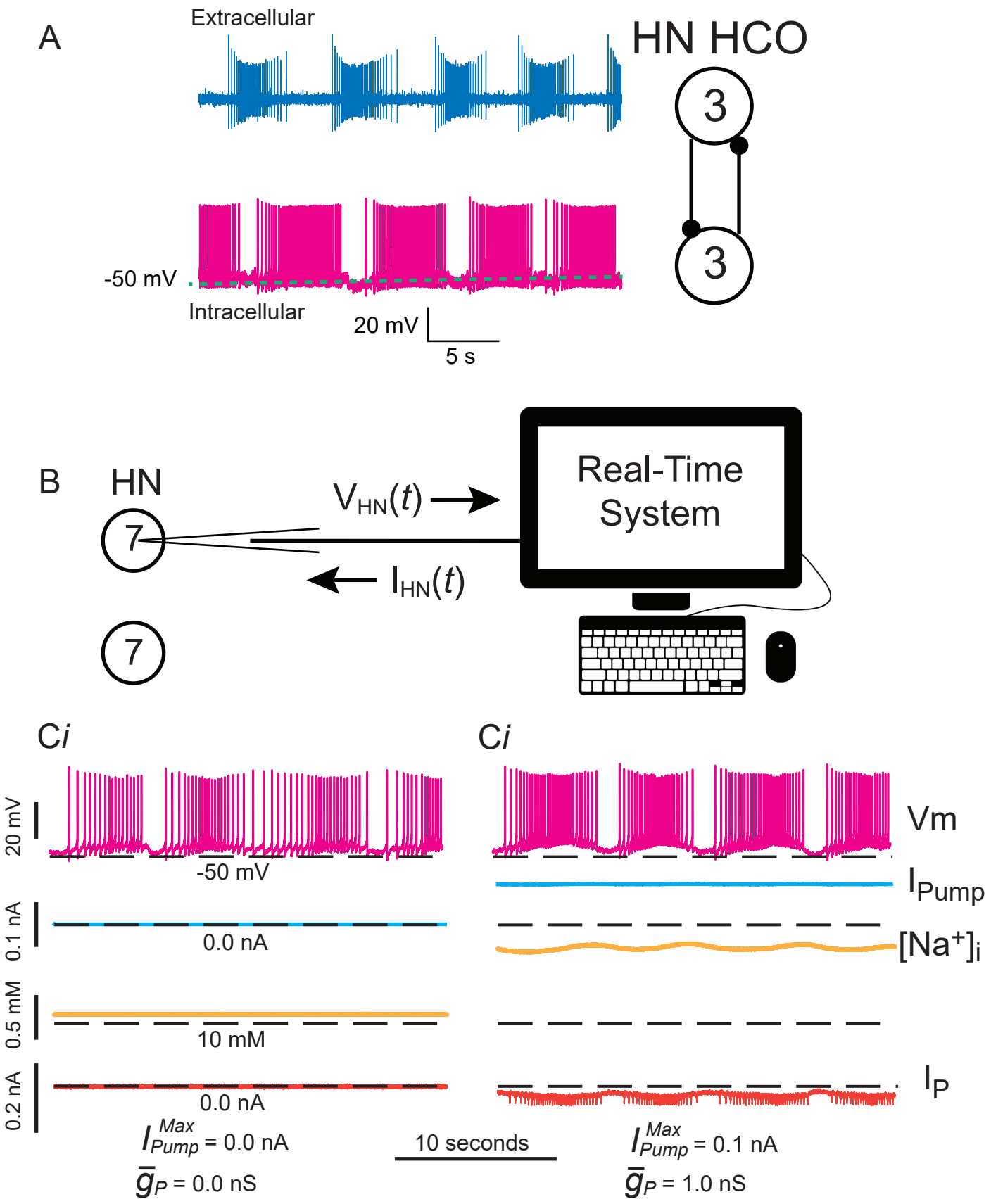

Figure 2

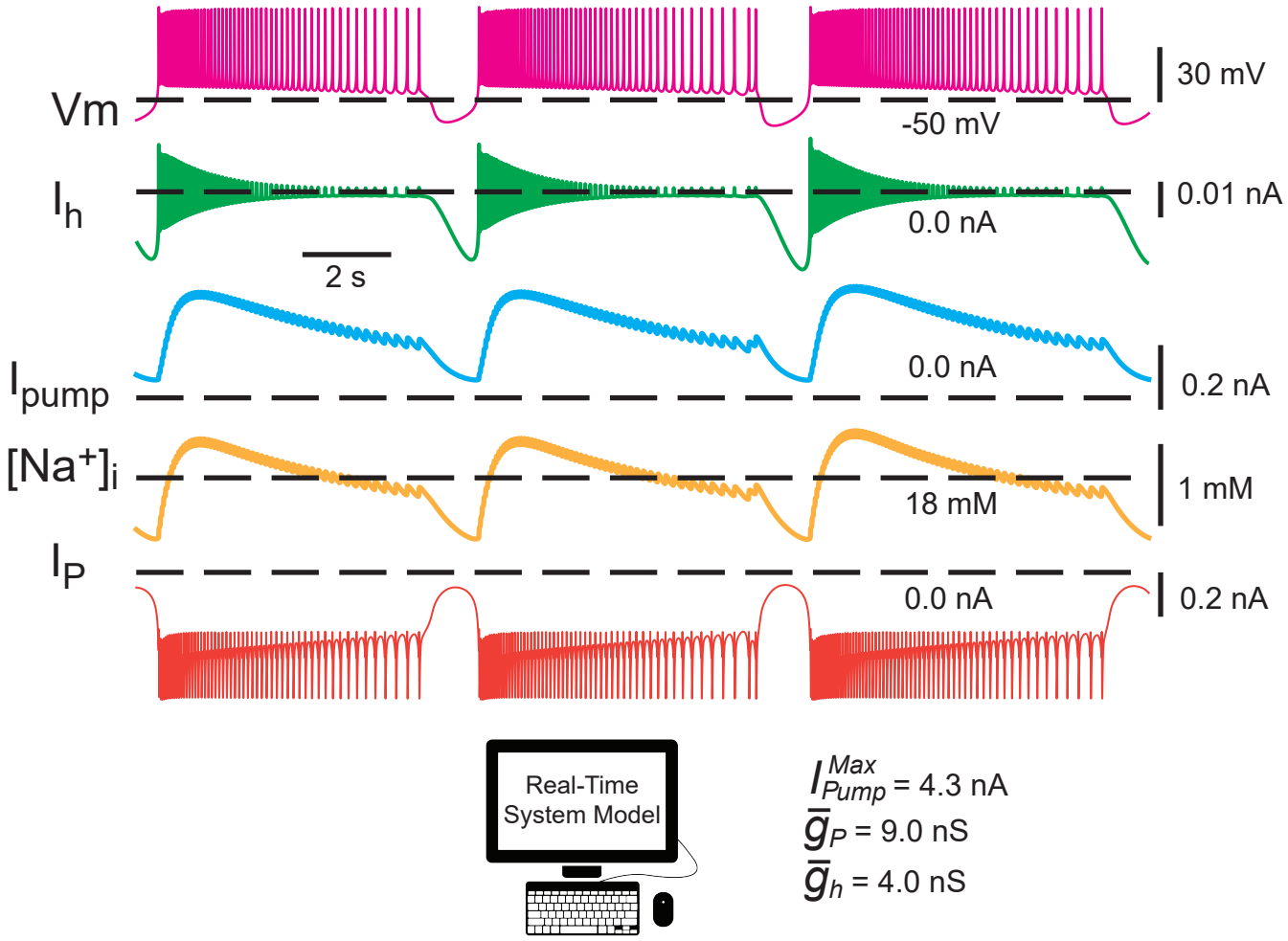

Figure 3

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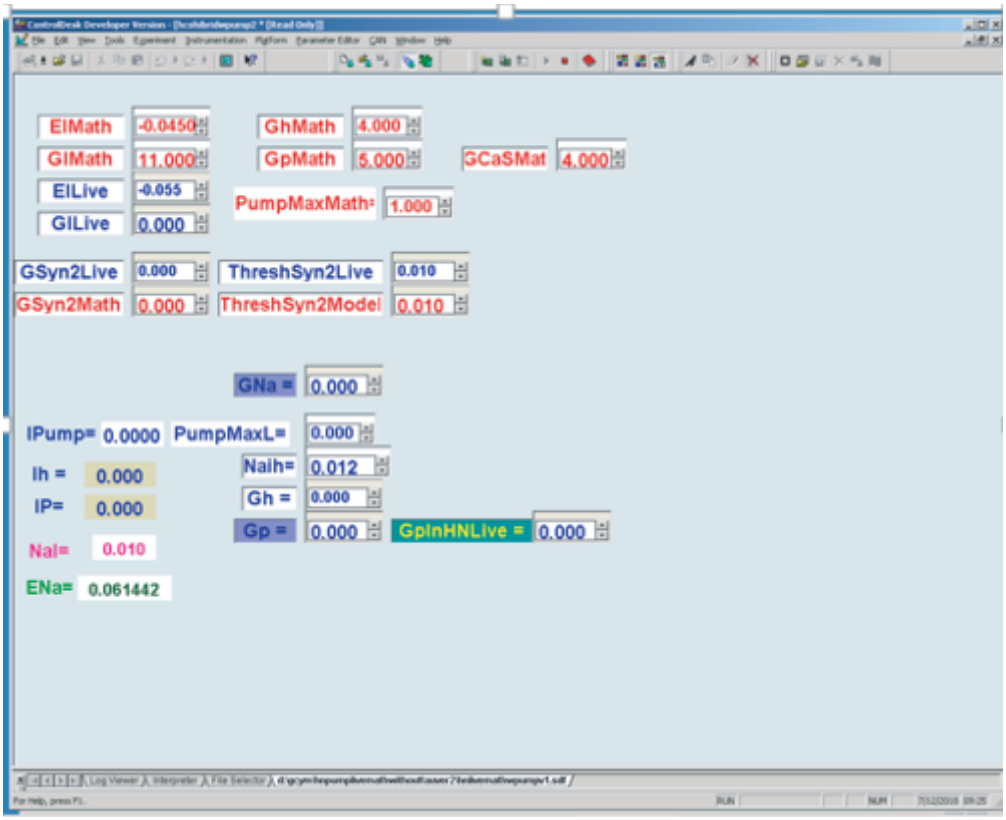


Figure 4

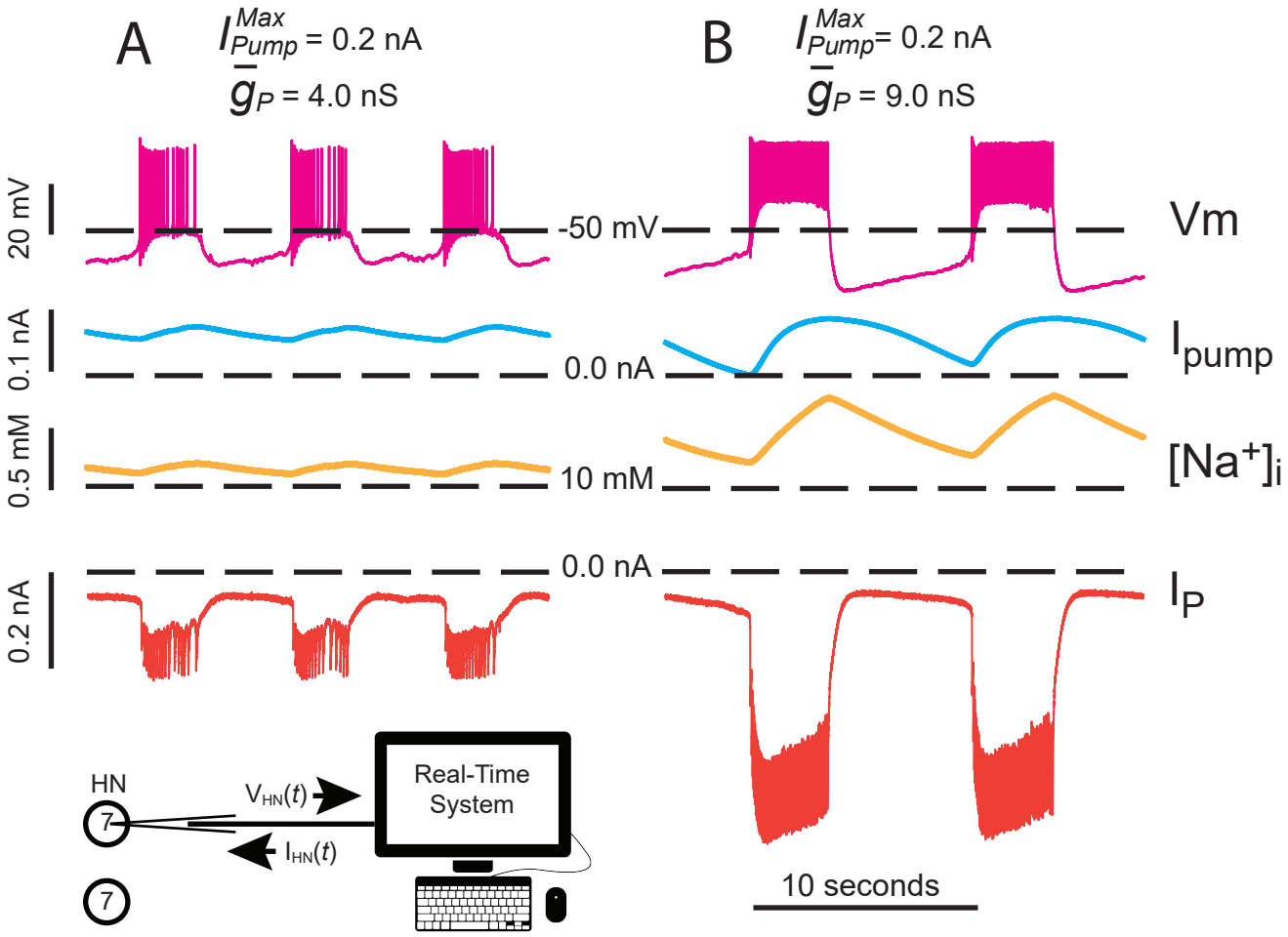
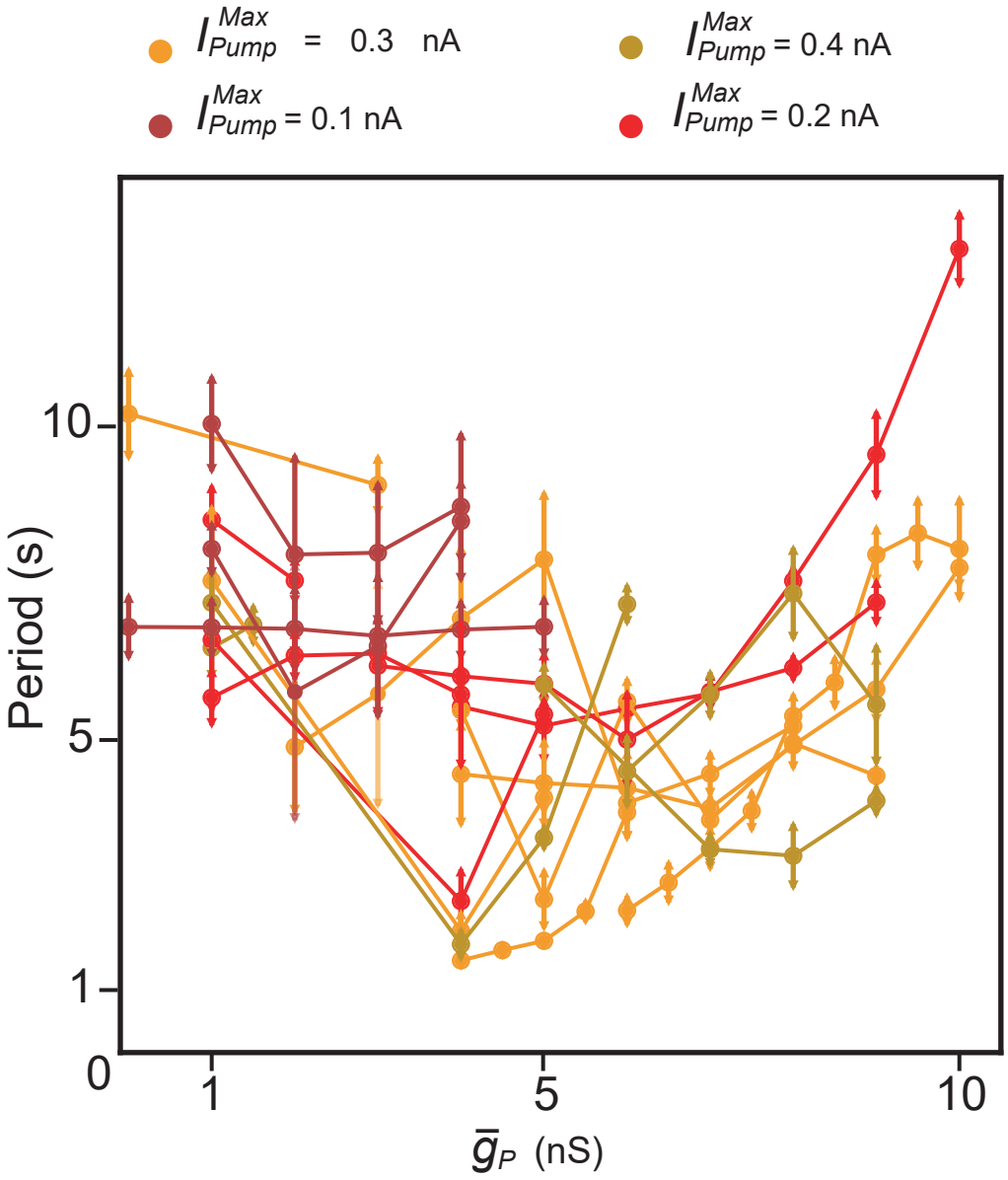


Figure 5



Name of Material/Equipment	Company	Catalog Number	Comments/Description
ANIMALS			
Hirudo verbana	Leech.com, https://www.leech.com/collections/live-leeches		live leeches 2-3 grams
CHEMICALS			
ARTIFICIAL POND WATER			
$CaCl_2$	Sigma Aldrich	C5670-100G	1.8 mM add last after adjusting pH
glucose	Sigma Aldrich	G7021-100G	10 mM
HEPES	Sigma Aldrich	H4034-100G	10 mM
Instant Ocean (sea salt)	Spectrum Brands Inc., Madison, WI		0.05% (w/v) diluted in deionized water
KCl	Sigma Aldrich	P9333-500G	4 mM
NaCl	Sigma Aldrich	S7653-250G	115 mM
NaOH 0.1 N Solution	Sigma Aldrich	2105-50ML	Adjust to pH 7.4 with NaOH
MICROELECTRODES			
K Acetate	Sigma Aldrich	P1190-100G	2 M
KCl	Sigma Aldrich	P9333-500G	20 mM
SALINE			
EQUIPMENT			
#5 Forceps	Fine Science Tools Dumont	11251-30 OR 11251-20	For general leech dissection
AxoClamp 2A/2B DCC electrometer	Axon Instruments Molecular Devices	2A/2B	For recording of neuronal membrane potential and discontinuous current clamp
Black resin	Dow Sylguard	170	Lines general dissect dish

Capilary glass 1 mm outer diameter, 0.75 mm inner diameter	A-M Systems	615000	For fabricating sharp microelectrodes
Clear resin	Dow Sylguard	184	Lines Petri dish used to mount ganglion for electrophysilogy
Dark field condenser	Nikon	Dry 0.95-0.80 MBL 1210	For illuminating the ganglion preparation during cell impalement
Digidata 1440A	Axon CNS Molecular Devices	1440A	Performs A to D and D to A for data acquisition and stimulation during electrophysiology
Digital signal processing board	dSpace	CLP1104	Our software implements all the conductances/currents in our model HN neuron on a DS1103 dSPACE PPC Controller Board in real-time at a rate of 20 kHz with a ControlDesk GUI (dSPACE, Paderborn, Germany)9.
Falming/Brown Microelectrode Puller	Sutter Instruments	P-97	For fabricating sharp microelectrodes
Fiber-Lite high intensity illuminator	Dolan Jenner Industries	170D	For illuminating the general dissection and for illuminating the ganglion preparation during cell impalement

Headstage amplifier for AxoClamp 2A	Axon Instruments	HS-2A Gain:0.1LU	Now part of Molecular Devices for recording of neuronal membrane potential and discontinuous current clamp
Light guide	Dolan Jenner Industries	Rev R 38 08 3729107	For illuminating the general dissection and for illuminating the ganglion preparation during cell impalement
Micromanipulator	Sutter Instruments	MPC-385	Micromanipulator for cell impalement with microelectrodes
Micromanipulator controller	Sutter Instruments	MPC-200	Controls micromanipulators for cell impalement with microelectrodes
Minuten pins	BioQuip	0.15 mm diameter 1208SA	Should be shortened by curtting to ~5 mm
Optical Breadboard 3' x 5' x 8"	Newport	Obsolete	With the 4 pneumatic Isolators below used to construct a vibration free workspace for electrophysiology
Oscilloscope	HAMEG Instruments	HM303-6	To monitor electrode setteling during DCC
Pascheff-Wolff spring scissors	Moria		Supplied by Fine Science Tools (Foster City, CA) catalog # 15371-92

pClamp 9 Software	Axon Instruments	9	Now part of Moleculear Devices uses the Digidata 1440 for data acquisition and stimulation during electrophysiology
Pneumatic Isolators 28"	Newport	Obsolete	With optical breadboard used to construct a vibration free workspace for electrophysiology
Simulink / MATLAB software	MathWorks	2006 (Obsolete)	Implements dynamic clamp on the digital signal processing board
Stereomicroscope	Wild	M5A	10x Eye Pieces used for dissecting the leech and removingand desheathing ganglia
Steromicroscope	Wild	M5	20x Eye Pieces used in electrophysiologcal station to visualize neuron for microelectrode penetration
Student Vannas Spring Scissors	Fine Science Tools	91500-09	For general leech dissection

TITLE

Contribution of the Na/K pump to rhythmic bursting, explored with modeling and dynamic clamp analyses.

AUTHORS AND AFFILIATIONS:

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Keywords: bursting activity, dynamic clamp, hybrid systems, leeches, modulation, Na/K pump, neuronal models

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SUMMARY

Presented here is a method for manipulating the Na/K pump and persistent Na current in leech heart interneurons using dynamic clamp.

ABSTRACT

The Na/K pump, often thought of as a background function in neuronal activity, contributes an outward current (I_{pump}) that responds to the internal concentration of Na^+ ($[Na^+]_i$). In bursting neurons, such as those found in central pattern generators (CPGs) neuronal networks that produce rhythmic movements, one can expect the $[Na^+]_i$ and thus I_{pump} to vary throughout the burst cycle. This variation with electrical activity and the independence from membrane potential endow I_{pump} with dynamical properties not available in channel-based currents (e.g. voltage- or transmitter- gated, or leak channels). Moreover, in many neurons the pump's activity is modulated by a variety of modulators further expanding the potential role of I_{pump} in rhythmic bursting activity. Here we will show how to use a combination of modeling, and dynamic clamp methods to determine how I_{pump} and its interaction with persistent Na current influence rhythmic activity in a CPG. Specifically, we will focus on a dynamic clamp protocol and computational modeling methods in heart interneurons of medicinal leeches.

Video Link

The video component of this article can be found at http://www.jove.com/video/

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INTRODUCTION

Heartbeat in leeches is driven by a central pattern generator (CPG) consisting of 9 bilateral pairs of heart interneurons (HN) distributed across as many mid-body segmental ganglia. At the core of the CPG are mutually inhibitory pairs interneurons in the 3rd and 4th segmental ganglia that form half-center oscillators (HCOs) (**Figure 1A**) but retain rhythmic bursting when synaptically isolated pharmacologically using bicuculline[1]. Others, such as the pair in the 7th segmental ganglia – the focus of this protocol are independent bursters receiving only descending input and thus easily isolated by severing the ganglion from the rest of the nerve cord. This independent bursting activity is sensitive to introduced leak current caused by penetration with sharp microelectrodes for recording but vigorously burst when recorded with loose patch methods[1].

We have previously modeled both individual HN neurons and HN HCOs (Hodgkin-Huxley based single isopotential compartment models of HN neurons containing all known voltage-gated and synaptic currents) and successfully captured all the burst characteristics of living system[2]. Myomodulin, an endogenous neuropeptide in leeches, markedly decreases period (T) of the burst rhythm of isolated HN neurons and HN HCOs. This modulator acts to increase h current (hyperpolarization-activated inward current, I_h) and to decrease I_{pump}[3]. This observation led us to explore how I_{pump} interacts with I_h, and their co-modulation contributes to rhythmic activity of HN neurons. We found that activation of the pump by increasing $[Na^+]_i$ (using the ionophore monensin) speeds the HN burst rhythm in both HN HCOs and isolated HN neurons[4]. We also found that this speed up was dependent on I_h; when I_h was blocked (2mM Cs^+) then the burst period was not altered by this method of pump activation, but the burst duration (BD) was curtailed and the interburst interval (IBI) increased in both HN HCOs and isolated HN neurons[4].

The goal of the protocol described here is to manipulate I_{pump} precisely and reversibly in real time to discover how it interacts with voltage gated currents (in the current protocol persistent Na current) to control rhythmic bursting in single heart interneurons. To accomplish this goal we use dynamic clamp, which artificially introduces, upon command, a precise amount of any current that can be calculated as the model is running. This method has advantage over pharmacological manipulation of the pump, which affects the entire tissue, can have off target effects, are often hard to reverse, and cannot be precisely manipulated in amount. Dynamic clamp[5,6] reads the voltage of a recorded neuron in real time (Figure 1B) and calculates and injects in real time the amount of any current based on model equations and the set values of any $\bar{g}_x$ or I_x^{max}. Similar methods can easily be applied to any neuron that can be recorded intracellularly but parameters will have to be rescaled to the neuron chosen and the neuron should be isolated from synaptic inputs, e.g. pharmacologically.

PROTOCOL

Invertebrate animal experimental subjects are not regulated by the NIH or Emory and Georgia State Universities. All measures are nevertheless taken to minimize suffering of the leeches used in this work.

1. Prepare isolated Ganglion 7 from the leech nerve cord

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1.1. Maintain leeches *Hirudo verbana* in artificial pond water (0.05% (w/v) sea salt) diluted in reverse osmosis water at 16° on a 12:12 light-dark cycle.

1.2. Prepare leeches for dissection, by cold-anesthetizing in a bed of crushed ice >10 minute until immobile.

1.3. Fill a black resin lined dissecting dish submerge to a depth of ~1cm with chilled saline with 115 NaCl, 4 KCl, 1.7 CaCl2, 10 D-glucose, and 10 HEPES (in mM); pH adjusted to 7.4 with 1 M with NaOH. Pin the leech dorsal side up in a black resin lined chamber at least 20 x 10 cm with a depth of at least 2 cm above the resin and the resin at least 2 cm thick.

1.4. Under a steromicroscope at 20 X magnification with oblique light guide illumination, make a longitudinal cut at least 3 cm long with 5 mm spring scissors through the body wall in the rostral 1/3 of the body.

NOTE: Any stored blood meal can be removed by suction with a fire polished Pasteur pipette.

1.5. Use pins to pull aside the body wall and expose internal organs. Isolate an individual mid-body ganglion 7 (seventh free segmental ganglion caudal to the brain).

1.6. Open the sinus in which the nerve cord resides using the 5 mm spring scissors. Be sure to split the sinus dorsally and ventrally leaving two strips of sinus. Use sharp #5 forceps to help guide the cutting and hold the sinus.

1.6.1. Keep the sinus attached to each of the two bilateral nerve roots that emerge from the ganglion (it adheres tightly to each root), because these strips of sinus will be used for pinning out the ganglion.

1.7. Remove the ganglion from the body by cutting the rostral and caudal connective nerve bundles that link the ganglia as far from the 7th ganglion as possible and the sinus strips, and then cut the roots lateral to where they emerge from the sinus.

1.8. Pin (using old blunted #5 forceps) with shortened minuten insect pins, ventral side up, in clear resin lined petri dishes. Insert pins in the strips of sinus and loose tissue adhering to the roots and the rostral and caudal connectives as far from the ganglion as possible. Make sure the ganglion is taught both longitudinally and laterally.

1.9. Increase the magnification of the steromicrosciope to 40 X or greater, and adjust the oblique illumination so the neuronal cell bodies can be easily seen on the ventral surface of the ganglion just below the perineurium.

1.10. Remove the perineurium (desheath) the ganglion with microscissors. Start the desheathing by cutting the loose sheath between the roots on one side and continue the cut laterally to the

other side making sure to keep the scissor blades superficial and not harm the neuronal cell bodies directly beneath the sheath.

1.11. Make a similar superficial cut caudally from the lateral cut along the midline. Then grab the caudolateral flap of sheath on one side with the fine #5 forceps and pull it away from the ganglion and cut it off with the microscissors.

1.12. Repeat on the other side; this exposes both HN(7) neurons for recording with microelectrodes.

1.13. Place the preparation dish in the recording setup and superfuse with saline at a flow rate of 50mL/min at room temperature.

2. Identify and record leech heart interneurons with sharp microelectrodes

2.1. For the duration of the recording of HN(7) neuron (recordings last between 30 to 60 minutes), acquire and digitize the intracellular current and voltage traces from a neurophysiological electrometer sampling at rate of 5 KHz with a digital data acquisition (A to D) and stimulation (D to A) system and display on a computer screen.

2.2. Under a steromicroscope at 50 to 100X with dark field illumination (from below), tentatively identify a HN(7) neuron of the bilateral pair by its canonical location at the posteriolateral position in midbody ganglion seven.

2.3. Now aim to penetrate the putative HN(7) neuron with a sharp microelectrode filled with 2 M K Acetate am 20 mM KCl using a micromanipulator.

2.4. Place the microelectrode very near the target cell body. Continuously observe the recorded potential with the electrometer and set this potential to zero mV before penetrating the neuron.

2.5. Penetrate the neuron with the microelectrode along a single axis of movement of the manipulator moving slowly and using the electrometer buzz function set a 100 ms until a negative shift in membrane potential and spiking is observed.

2.6. Set the electrometer in discontinuous current clamp mode (DCC) ≥ 3 KHz to simultaneously record membrane potential and pass current with the single microelectrode (capacity compensation set to just below ringing and then dialed back 10%). Inject a steady current of 0.1 nA with the electrometer steady current injector for a minute or two to stabilize the recording.

2.7. Definitively identify the HN(7) neuron is by its characteristic spike shape and weak bursting activity (Figure 1C).

2.8. Perform any data analysis offline after the experiment is completed and all data is stored to disk.

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3. Build a real time HN or other model neuron

3.1. Custom build the software using a digital signal processing board (DSB) in a desk-top computer to implement in real time the model currents described in[2,4] or different model currents for other neurons/experiments.

3.2. Use Hodgkin-Huxley style equations as these are the generally preferred method for representing model currents. See[7] for a detailed description of the implementation of the real time HN model and dynamic clamp prior to the addition of the pump current.

[**Explanatory Note:** A**ll** the currents of a living HN(7) neuron, including the pump current, I_{pump}, are incorporated in the HN model as follows:

$$C\frac{dV}{dt} = -\left(I_{Na} + I_P + I_{K1} + I_{K2} + I_{KA} + I_h + I_{CaF} + I_{CaS} + I_{Leak} + I_{pump}\right) \quad (1)$$

where C is the membrane capacitance (in nF), V is the membrane potential (in V), t is time (in s). See[2,4] for detailed ionic current descriptions and equations. The complete HN model neuron runs in real time (Figure 2). The software will be made available on GitHub upon publication suitable to run on the digital signal process board described in the Table of Materials.

Here the focus of enquiry is the Na^+/K^+ pump (I_{pump}) current and voltage-gated currents contributing significant Na^+ flux: a fast Na^+ current (I_{Na}), a persistent Na^+ current (I_P).
The Na^+/K^+ pump exchanges three intracellular Na^+ ions for two extracellular K^+ ions, thus producing a net outward current; importantly it exchanges 3 time as much Na^+ as this current indicates, which is important for calculating the intracellular Na^+ concentration. The Na^+/K^+ pump current depends on intracellular Na^+ concentrations, and is expressed with the following sigmoidal function:

$$I_{pump} = \frac{I_{pump}^{max}}{1+\exp\left(\frac{[Na]_{ih}-[Na]_i}{[Na]_{is}}\right)} \quad (2)$$

where $[Na]_i$ is the intracellular Na^+ concentration, I_{pump}^{max} is the maximum Na^+/K^+ pump current, $[Na]_{ih}$ is the intracellular Na^+ concentration for the half-activation of the Na^+/K^+ pump, and $[Na]_{is}$ the sensitivity of the Na^+/K^+ pump to $[Na]_i$. Intracellular Na^+ concentration that occur as a result of the Na^+ fluxes carried by ionic currents and the Na/K pump (The contribution of I_h and I_{Leak} to the Na^+ flux is small and not considered in the real-time model.):

$$\frac{d[Na]_i}{dt} = -\frac{I_P + I_{Na} + 3\,I_{pump}}{vF} \quad (3)$$

where, v is the volume (~6.7 pL) of the intracellular Na^+ reservoir, F is Faraday's constant, and the extracellular Na^+ concentration is kept constant.
We differentiate between voltage-gated and leak conductances and the pump current which is regulated by calculated intracellular Na^+ concentration ($[Na^+]_i$). $[Na^+]_i$ is built up through Na^+ entry via the fast Na current (I_{Na}) that produces action potentials (spikes) and the persistent Na current (I_P) that provides the depolarization to support spiking. $[Na^+]_i$ is in turn reduced by the action of the pump – extrusion of Na^+.]

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We cannot have paragraphs of text in the protocol section, please move the detail description to a supplementary file. This can also be moved to the intro/discussion as appropriate.

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4. Implement and vary dynamic clamp conductances/currents

4.1. Use the custom built dynamic clamp software for the DSB to implement and then change in real time dynamic clamp any of the GUI (Figure 3) accessible programed conductances/currents of the HN real-time model of the HN(7) neuron.

4.2. Use GUI entry boxes in the software (Figure 3) to make changes as the model is running in the I_{pump}^{max} and $\bar{g}_P$.

4.2.1. First add small amounts of I_{pump}^{max} and $\bar{g}_P$ with dynamic clamp to stabilize bursting of the HN(7) neuron (Figure 1C), which is weakened by microelectrode induced leak.

NOTE:

4.2.2. Start by adding a baseline value of I_{pump} (I_{pump}^{max} = 0.2 nA), which makes up for the microelectrode induced leak but depresses excitability, and then gradually increase $\bar{g}_P$, which increases excitability, until regular bursting ensues usually about 1-4 nS (Figure 4).

4.2.3. Now systematically co-vary these currents (0.1 nA steps I_{pump}^{max} and 1 nS steps $\bar{g}_P$) to the recorded HN(7) neuron with dynamic clamp (Figure 3) and assess their effects on burst characteristics: spike frequency (f – reciprocal of the average of the interspike interval during a burst), interburst interval (IBI – time between the last spike in one burst to the first spike in the next burst), burst duration (BD – time between the first spike in a burst and the last spike in a burst), burst period (T – time between the first spike in a burst and the first spike in the subsequent burst).

4.2.4. Change these values, to become familiar with the technique and then venture out. Specifically holding a fixed value of I_{pump}^{max} sweep through 1 nS steps of $\bar{g}_P$. Now increase the fixed value of I_{pump}^{max} by 0.1 nA and again sweep through $\bar{g}_P$). Each parameter pair must be implemented for at least 8 bursts so that reliable average measures of f, IBI, BD, and T can be made.

4.2.5. Continue with sweeps for as long as the neuron remains viable as assessed by strong spiking and a stable baseline potential of oscillation. Collect data from several neurons (from different animals) to generate a composite graph like in Figure 5.

Representative Results

Modeling with the addition of I_{pump}[4] brought the experimental findings presented in Introduction into sharper focus and began to explain the pump-assisted mechanism of bursting. The real time model demonstrated here has been tuned ($\bar{g}_x$ or I_x^{max} parameters chosen) so that it produces regular rhythmic activity falling within the bounds of normal activity as observed in experiments – spike frequency (f), IBI, BD, T) and continues to produce such activity when the myomodulin-modulated parameters I_{pump}^{max} (the maximal pump current) and $\bar{g}_h$ (maximal h conductance) are

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varied or co-varied in the model. The parameters values determined can be used as a benchmark or canonical set for modeling experiments. In these model instances I_{pump} oscillates throughout the burst cycle as $[Na^+]_i$ around a baseline level. I_{pump} contributes to burst termination during the burst phase and activates I_h during the IBI; notice its maximal level near burst initiation (Figure 2).

The real-time HN model has all implemented currents[2,4] available for dynamic clamping but here the focus was on I_{pump}^{max} and $\bar{g}_P$ which are changeable on the fly in the dynamic clamp GUI (Figure 3). Dynamic clamp allows the experimenter to add or subtract (negative $\bar{g}_x$ or I_x^{max}) any conductance or current into a neuron artificially that mimics the voltage and ionic dependence of a real conductance or current. Thus, it is possible to fully explore how a particular conductance/current interacts with the cells endogenous conductances/currents.

The real time HN model indicates that the persistent Na current (I_P) in HN neurons contributes much of the Na^+ entry determining $[Na^+]_i$ (Figure 2) and thus I_{pump}. Because I_P is active at relatively negative membrane potentials, it opposes I_{pump} even during the IBI. These observations indicate that it is instructive to explore interactions between I_{pump}^{max} and $\bar{g}_P$ in isolated HN neurons with dynamic clamp as in [8,9,10]. These experiments (currently continuing) are performed with sharp microelectrodes recordings in single synaptically isolated HN(7) neurons (seventh ganglion severed from the nerve cord). Thus far these experiments show that robust bursting is restored in tonically active HN neurons (due to microelectrode penetration introduced leak) by co-addition of I_P and I_{pump} with dynamic clamp (Figure 4). This is an important observation indicating that a bursting mechanism is available in these neurons (even when leak compromised) that results from the interaction of I_{pump} and I_P. Preliminary results indicate a strong complicated space of interaction, which can be explored in model and experiment (Figure 5).

We conclude:

- I_{pump} in response to periodic increases in $[Na^+]_i$ during bursting activity contributes to the burst rhythm through burst termination (decreasing BD).
- Interaction of I_P and I_{pump} constitutes a mechanism which is sufficient to support endogenous bursting activity; this mechanism can reinstate robust bursting in HN interneurons recorded intracellularly in ganglion 7.
- The interaction between I_P and I_{pump} through $[Na^+]_i$ affects HN burst period non-monotonically and ensures robustness of autonomous bursting.
- These conclusions are in line with experiments and modeling in vertebrate systems[11,12].

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Discussion

Modeling, dynamic clamp, and the resulting analyses that it enables are useful techniques for exploring how individual and groups of ionic conductance/currents contribute to the electrical activity of neurons. The herein demonstrated use of these techniques shows how the Na/K pump's current (I_{pump}) interacts with voltage-gated currents, particularly the persistent Na current (I_P) to promote robust bursting in the leech heartbeat pattern generator's core heart interneurons. By adding dynamic clamp experiments to modeling it is possible to test models in way that are not possible with ordinary voltage recording and current clamp techniques. The

results gathered in the dynamic clamp experiments (Figure 5) will be used to further refine the HN model. The basic method of dynamic clamping demonstrated here can be customized to reflect the properties of any neuron under study as long and a mathematic model of neuronal currents can be determined with voltage clamp experiments.

To be successful in implementing experiments of the type shown here require carful impalement of a HN or other neuron when using a sharp microelectrode, because strong bursting is curtailed by the electrode penetration[1]. (Whole cell patch recording techniques, which minimize introduce leak, are also applicable to other neurons but do not work well on leech neurons.) It is critical that the impalement of the HN neuron cause minimal damage to the neuron (added leak) and input resistance should be monitored and must be in the range of 60 - 100 MOhms for successful experiments[4].

Dynamic clamp is a powerful technique, but it has limitations imposed by neuronal geometry because the artificial conductances are implemented at the site of the recording electrode – usually the cell body – not at the site where rhythm-generating currents are usually localized[5,6,10]. In leech HN neurons, the cell body is electrically close to integration zone (main neurite) of the neuron where most active currents are localized, and spikes are initiated.

Disclosures

None

Acknowledgements

We thank Christian Erxleben for preliminary dynamic clamp experiments on HN(7) neurons that demonstrated their bursting capabilities. Angela Wenning aided the experiments with expert advice.

References

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363 Figure 1

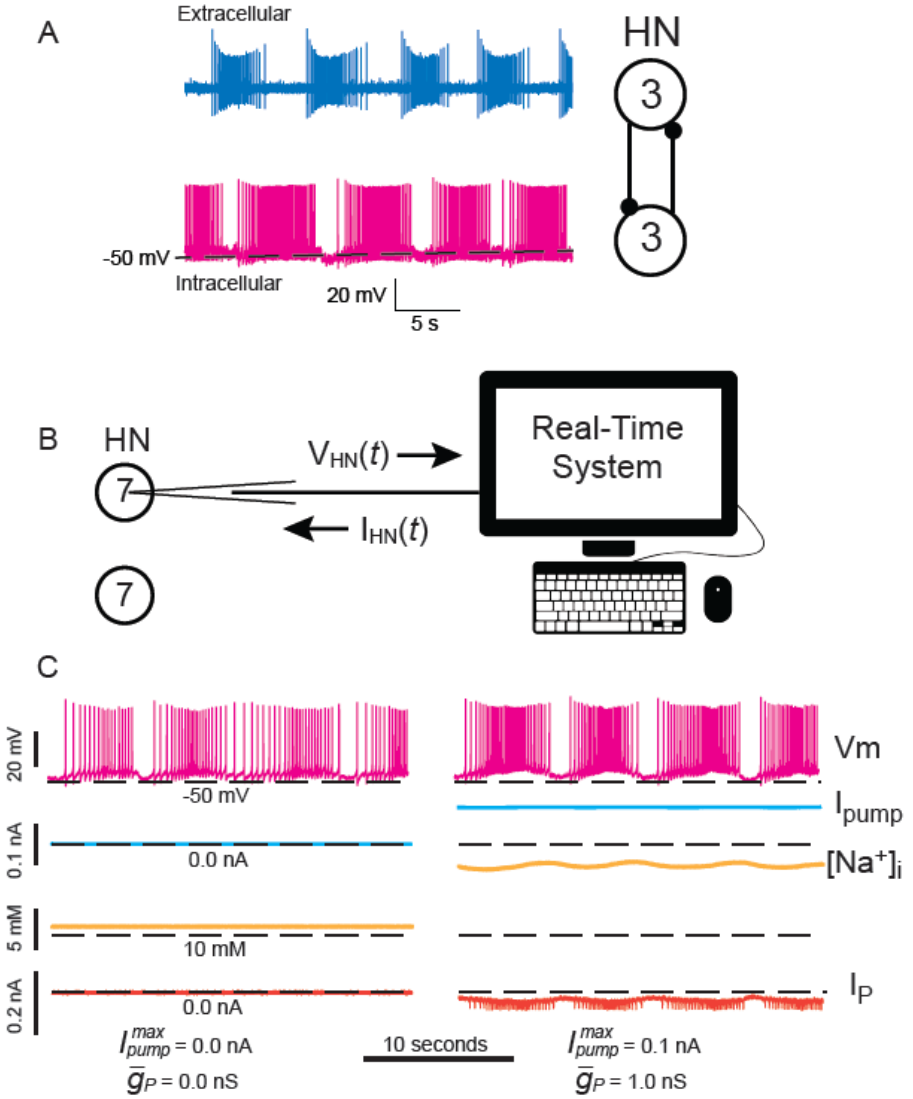

Figure 1. Leech heart interneuron electrical activity and implementation of I_{pump} and $\bar{g}_P$ with dynamic clamp.

(A) Normal bursting activity recorded extracellularly and intracellulary in a leech heartbeat HCO from a third ganglion; schematic of the recorded neuron and their mutually inhibitory synaptic connections at right. (B) Dynamic clamp schematic when recording a HN(7) interneuron in an

isolated ganglion 7; note there is no synaptic interaction between the two HN(7) interneurons. (C) Bursting in a leak compromise HN(7) interneurons recommenced by adding a baseline value of I_{pump} (I_{pump}^{max} = 0.2 nA), which makes up for the microelectrode induced leak but depresses excitability, and $\bar{g}_P$, which increases excitability, until regular bursting ensues (4 nS). Black dashed lines indicate baseline values.

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366 Figure 2

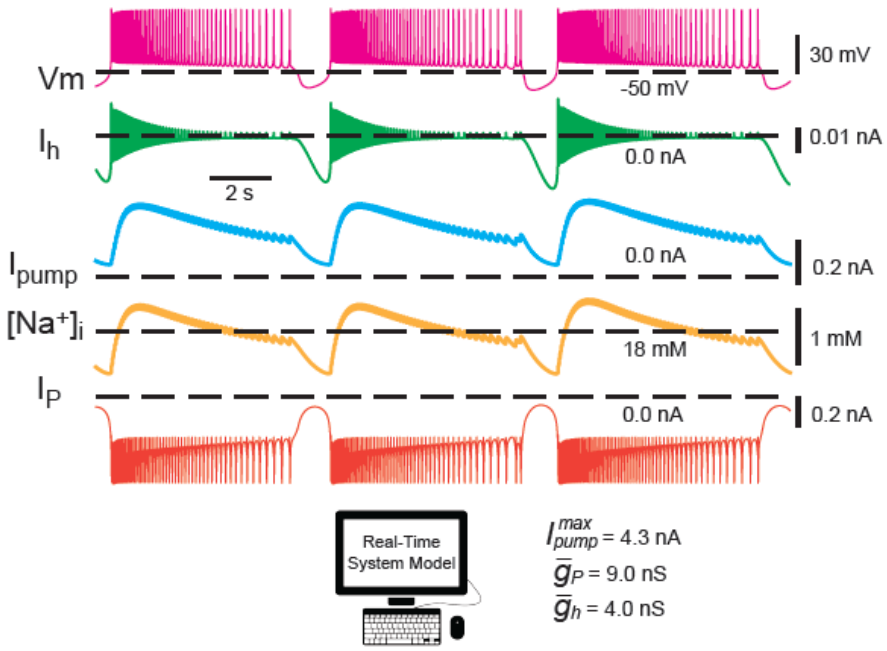

Figure 2. Single cell HN interneuron model showing traces for membrane potential (Vm), I_h, I_{pump}, $[Na^+]_i$, and I_P.
Outward hyperpolarizing currents are negative and inward depolarizing currents are positive. Black dashed lines indicate baseline values.

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Figure 3

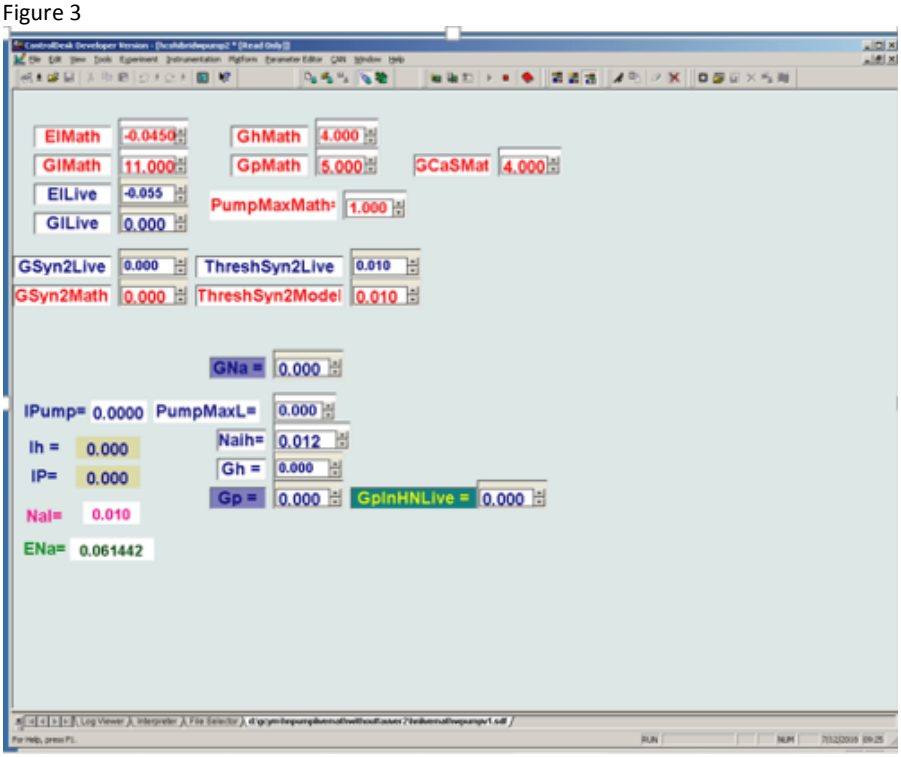

Figure 3. GUI of real time HN model and dynamic clamp implemented on a digital signal processing board.

Upper Left: Red Math are user-determined parameter boxes are for the real time model whereas Blue Live are user-determined parameter boxes are for the dynamic clamp. El, reversal potential of the leak current; Gl, leak conductance; Gh, h current maximal conductance; Gp, P current maximal conductance; GCaS, slow calcium current maximal conductance; PumpMax, pump maximal current; [GSyn2 maximal synaptic conductance to the respective neuron; ThreshSyn2 spike crossing threshold for mediating a synaptic potential – these used to make a hybrid half center oscillator and not illustrated here.]. Lower Left for Dynamic Clamp. At the very left are 5 computed values of dynamic clamp variables IPump, pump current injected; Ih, h current injected (not used here); IP, P current injected; NaI, calculated internal Na concentration; ENa, calculated sodium reversal potential. Lower Left for Dynamic Clamp. To the right of the computed variables are 6 user determined parameter boxes; GNa, assumed endogenous fast sodium maximal conductance use to calculate Na^+ flux associated with action potentials; PumMaxL, maximal pump current to be injected by dynamic clamp; Naih see equation (2); Gh, maximal conductance to determine h current to be injected by dynamic clamp; Gp, assumed endogenous p maximal

conductance use to calculate Na^+ flux associated with endogenous P current; GpinHNLive, maximal conductance to determine P current to be injected by dynamic clamp.

394 Figure 4

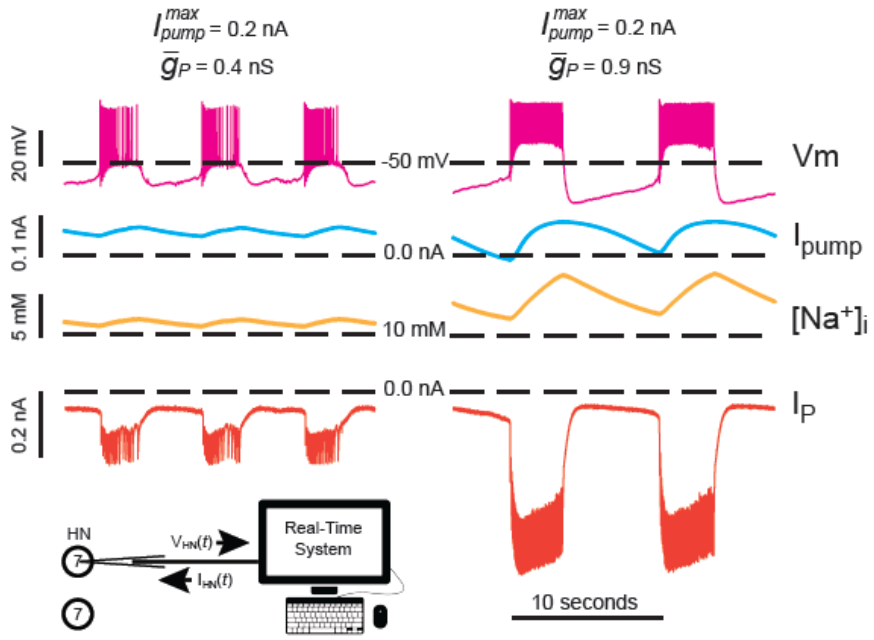

Figure 4. Dynamic clamp analysis of independent HN(7) bursting.
Upregulation of $\bar{g}_P$ from 4.0 to 9.0 nS slows down the independent HN burst rhythm. Experimental traces show rhythmic bursting in isolated HN(7) neuron with dynamic clamp. The ranges of oscillation of $[Na^+]_i$ and Vm increase with upregulated $\bar{g}_P$. Traces top to bottom: recorded Vm, injected I_{pump}, calculated $[Na^+]_i$, and injected I_P. Black dashed lines indicate baseline values.

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Figure 5

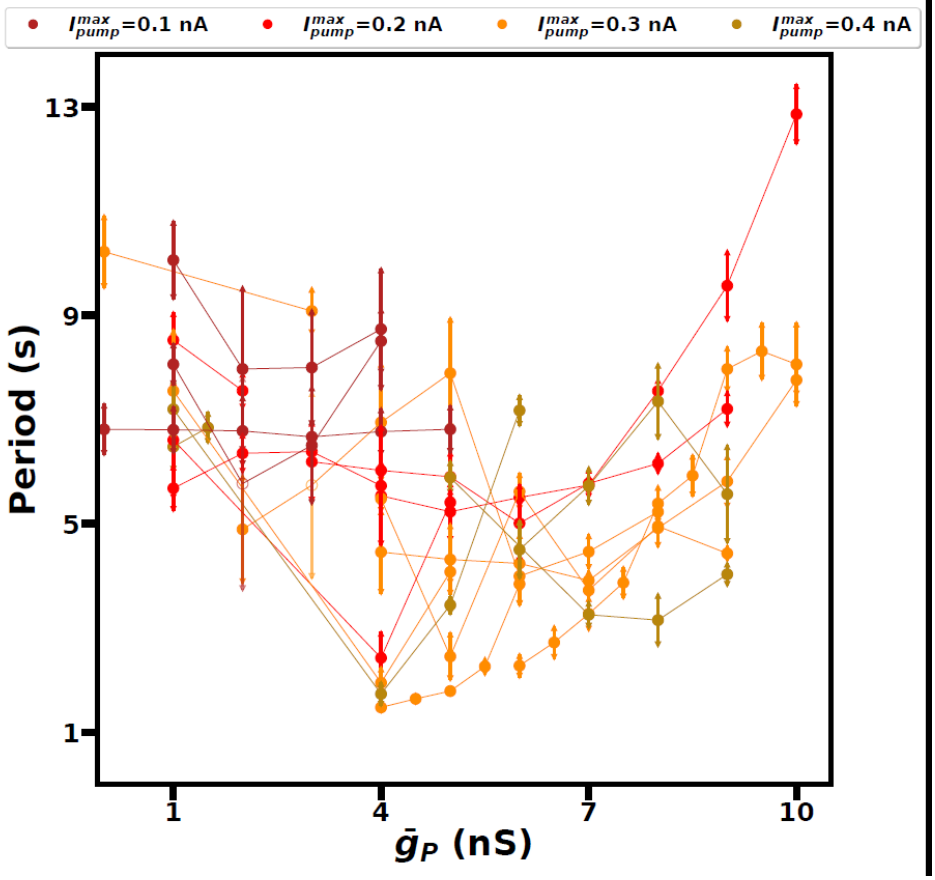

Figure 5. Dynamic clamp analysis of independent HNHN model bursting.
Upregulation of $\bar{g}_P$ tends to decrease, and then increase HN burst period. Colors represent levels of across I_{pump}^{max}.

Figure Legends

Figure 1. Leech heart interneuron electrical activity and implementation of I_{pump} and $\bar{g}_P$ with dynamic clamp.

(A) Normal bursting activity recorded extracellularly and intracellulary in a leech heartbeat HCO from a third ganglion; schematic of the recorded neuron and their mutually inhibitory synaptic connections at right. (B) Dynamic clamp schematic when recording a HN(7) interneuron in a isolated ganglion 7; note there is no synaptic interaction between the two HN(7) interneurons. (C) Bursting in a leak compromise HN(7) interneurons recommenced by adding a baseline value of I_{pump} (I_{pump}^{max} = 0.2 nA), which makes up for the microelectrode induced leak but depresses excitability, and $\bar{g}_P$, which increases excitability, until regular bursting ensues (4 nS). Black dashed lines indicate baseline values.

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Upregulation of $\bar{g}_P$ tends to decrease, and then increase HN burst period. Colors represent levels of across I_{pump}^{max}.

From: em.jove.145d9.6e1156.38eb9243@editorialmanager.com on behalf of Vineeta Bajaj
To: Calabrese, Ronald
Subject: [External] Revisions required for your JoVE submission JoVE61473R1 - [EMID:f9a9f97dc24d9fce]
Date: Friday, September 18, 2020 4:47:48 PM

CC: "Ricardo Javier Erazo Toscano" rerazotoscano@student.gsu.edu, "Parker J. Ellingson" pellingson3@gmail.com, "Gennady S. Cymbalyuk" gcymbalyuk@gmail.com

Dear Dr. Calabrese,

Your manuscript, JoVE61473R1 "Contribution of the Na/K pump to rhythmic bursting, explored with modeling and hybrid systems analyses.," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually.

Your revision is due by **Oct 02, 2020**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Vineeta Bajaj, Ph.D.
Review Editor
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About JoVE

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Tried.
2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Done. Please include a single line space between each step, substep and note in the protocol section. Not sure what you want here but I think I have it.. Please use Calibri 12 points Done.
3. Please provide an email address for each author. Done.
4. Please include a Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Presented here is a protocol …” Done.

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol. Done.
6. Please do not include in text citation in the abstract section. Fixed.
7. Please ensure the Introduction include all of the following with citation:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application Tried.
8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. Done.
For example: Spectrum Brands Inc., Madison, WI, Dow Sylgard® 170, Corning, NY, Moria® 84 Pascheff-Wolff spring scissors, Axoclamp® 2A amplifier, Simulink® (MathWorks, Natick, MA), Didgidata® 1440A (Molecular Devices, Sunnyvale, CA), etc.
9. Please revise lines 207-209 as it matches with previously published literature. Done.
10. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Done.
11. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.). Done.
12. The Protocol should contain only action items that direct the reader to do something. Done.
13. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step. We tried to do it in a numbered way. There are many many substeps.
14. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? For this please include all button clicks in the software, knob turns, mechanical actions, command lines if any, etc. If using large scripts, please include it as supplemental file. Tried.
15. 1.2: How do you confirm the depth of anesthesia in leeches? Fixed.
16. 1.3: What is used to make the incision? How big is the incision? Please specify all surgical instruments used. Done.
17. 2.1: Please specify the volume used. Electrode volume unknown and immaterial; just fill it something less that 100micorliters for a typical microelectrode, but I really never measure it..
18. 2.2: How many recordings are done? How do you perform the recordings, please include button clicks, and knob turns, etc. Tried.
1. 3, 4: Please make all the substeps as action steps. Please clearly describe the actions being performed. Tried.
2. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Done.
3. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted. I don't understand this.

4. Please note that much of steps 3 and 4 are more of a discussion that a filmable protocol. If these steps are to be filmed, please provide explicit stepwise details for each step. Calculations cannot be filmed. Made an explanatory note.
5. Please ensure Representative Results is written in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included. Please do not make points and use paragraph style with complete sentences. Tried.
6. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." All figures unplublished.
7. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Tried.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The text provides the relevant background information to accompany a video. All the critical steps in the process are explained with the help of figures. All Material/Equipment are fully listed. The authors are appropriate to produce this video with one of the authors (Professor Calabrese) an internationally recognised authority on leech neurophysiology.

Major Concerns:

none

Minor Concerns:

either in Introduction, line 36, or Abstract, line 31, give Central Pattern Generator (CPG) in full the first time the abbreviation is used. Done in Abstract. line 105: helpful to provide information on modern equipment equivalent board/software. Not sure how to do this without mentioning brand names. Figure 1: give scale for extracellular recordings. Voltage scale meaningless for entracellular loose patch recordings. Figure 1C: label left hand column Ci and right hand column Cii and describe as such in text and legend. Done. Similarly in Figure 4, label left hand column A and right hand column B and describe in text and legend. Done. Figure 5 legend: should be expanded to make figure clearer to the reader. Done.

Reviewer #2:

The Abstract is good and interesting but I have a major concern that needs addressing. Bursting depends on a number of variables particularly the membrane voltage and the ion gradients. The aim of the paper is to control bursting by adding pump current. However it is not clear how pump current can be added (lines 156-158). To state that the pump current controls bursting is confusing. It seems like putting the cart before the horse. Bursting depends on [Na+]i which determines pump activity. The greater [Na+]i the stronger the pump activity.

If increasing pump current is actually simply injecting charge then [Na+]i is not affected by that change, but the membrane voltage is which has a major impact on bursting. Bursting will occur if the voltage gated channels are opened by the rise in membrane voltage.

There does not seem provision for dynamic clamp of Na+ in the set-up. So, what I infer from the presentation, is that this paper is not about adding Ipump current but injecting electric charge. If that is the case, the work has to be presented in that light. Injecting charge changes the membrane potential and hence impacts bursting and excitability in general. But it is a very different problem than modeling a system with actual increases in pump current that changes the ion concentrations.

The principal innovation of our method is the implementation of a pump model in dynamic clamp. As covered in the now **Explanatory Note**, our pump model depends on calculated intracellular Na^+ concentration (equations 2 and 3). We have added to the **Explanatory Note** for clarity "We assume baseline living HN values of $\bar{g}_P$ (5 nS) and $\bar{g}_{Na}$ (150 nS) and take account of any added dynamic clamp $\bar{g}_P$." The pump current is thus a reflection of intracellular Na^+ concentration, and it is integral to the bursting mechanism, so it is reasonable to say it controls bursting.

Reviewer #3:

Manuscript Summary:

This manuscript will generate a masterful JoVE project: the experiments are interesting on their own and they provide an excellent tutorial on how to use dynamic clamp as well as a clear discussion of what kinds of questions can and cannot be addressed with this technique.

Major Concerns:

I have no problems with or suggestions about the data presented, the experiments described, or the interpretations of results.

Minor Concerns:

The numbers refer to line numbers in the manuscript.

32: it would be useful to have a short description of what is meant by a "hybrid system" in this study. We have deleted all reference to hybrid systems except in the legend of Fig. 3 where the term is used and explained so that the GUI interface as it exists is explained; "…these used to make a hybrid (living/model) half-center oscillator and not illustrated here."

32: here and elsewhere, the use of dashes to set off parenthetical clauses is a bit too informal. It ends up being shorthand, and it is not always clear what has been left out. We have endeavored to remove such use of such dashes and make the text plainer.

33: the use of "but" is jarring; I thought I had missed something. "and also include" would be better. Reworded for clarity.

40: "(pharmacologically bicuculline)" is a bit telegraphic; inserting "using" would make it less ambiguous, and smoother, if that is indeed what was meant. Done.

41: the "bursters" are "independent" of both intersegmental and segmental connections; they should point out here that there are no segmental connections to worry about. Reworded for clarity.

46: "H-H" presumably (as in line 107) means "Hodgkin and Huxley"; it should be written out here, at its first use. Done.

69: "reverse osmosis water" is confusing; presumably they mean "distilled water using reverse osmosis". The important part is that the water was distilled, not the process of distillation. RO water is not distilled. We substituted in de-ionized.

96: here and elsewhere, the authors lapse into lab manual speak (second person), telling the reader how to do the experiment. Mostly, they use first person ("we did this"), which is

preferable. Whichever they choose, they should not use both. Moved to the imperative as instructed by the editor.
123-4: presumably the reason for the "3 times [not "time"] as much" is that two of the Na ions are replaced by K ions. Whatever the reason for this 3X difference, it should be stated. We corrected times and have endeavored to make the reason for the 3X in Equation 3 clearer. We corrected the times and have endeavored to clarify the 3X in equation 3.
137: use "conductances and currents".
Done.
139: "built" not "build" Done.
139-141: this is not a sentence.
Fixed.
148, 158: second person again.
Fixed.
154; 159-61: more shorthand dashes. Fixed.
163-165: it would be extremely useful to have a table defining these abbreviations, as well as all the abbreviations for the currents and conductances. Editor asked that these be removed from the original text because these definitions had been previously published.
178: this dash should be a semicolon.
Fixed.
182: add "we" after "here". Fixed.
182: "on the fly" is a bit informal; "as the model is running" would be better. Fixed.
184: what is in the parentheses should either be explained (are these examples?) or dropped; I vote for the latter. Fixed.
185: another second person. Fixed.
205-6: this is a very awkward (and confusing) statement; it needs to be stated more clearly.
Fixed.
Fig 1 legend: (A), (B), and (C) should be at the beginnings of the sentences describing them, not at the ends. Fixed.
Fig 2 legend: the list of currents, concentrations, and voltages should be in the same order as in the figure, as an aid to the reader. Done.
Fig 3 legend: in this legend, the same current is listed as "P current" (235), "P-current" (241, 249) and "p current" (248). They should pick one convention and stick with it, here and in the text. Fixed.
236-239: this discussion of synaptic conductances that are not used in this study is very hard to follow, partly because it breaks some rules of grammar. It needs to be revised. Deleted.

Reviewer #4:
Manuscript Summary:
This manuscript will illustrate the use of the dynamic clamp to explore the role of specific conductances in neuronal and network function. I can imagine that it will have some use in the specific instance as a teaching tool, but otherwise it is a "proof of concept", as I can't imagine it will otherwise have a large audience.

Major Concerns:
Manuscript is sloppy and somewhat idiosyncratic.

1. What data gave rise to the kinetics of the pump removal of the intracellular Na+? Model fitting to normal HN function and in the presence of the Na+ ionophore Monensin (Kueh et al. 2016).
2. In all legends, please indicate is parts of these figures are from previously published papers? All original data.
3. Where are the values of Na+ intracellular coming from in Figures with dynamic clamp?

Calculated using Eq 3.

Minor Concerns:
1. Sentence on lines 25-27 is grammatically and syntactically challenged. Please repair. Fixed.
2. Sentence on lines 43-46 is grammatically and semantically challenged. Please repair. Fixed.
3. Line 53 needs a "how" after and Fixed.
4. Line 91 "am" should be "and" Fixed.
5. Line 175 should be "parameter" not "parameters" Fixed.
6. Line 182, should be "here we focus" No.
7. Line 263, should be "that they enable" Fixed.
8. Line 269 syntax problem models in ways not possible Fixed.
9. Line 273 "mathematical" Fixed.
10. Line 276, "careful" Fixed.

Reviewer #5:
The protocol has been written nicely and it would be very useful for modelling not only in leech but also in other systems. My comments are below:

1. Introduction line 4 "…that form half-center oscillators (HCOs) (Figure 1)…": Figure 1 is cited here, but it does not explain the HCOs. A more appropriate figure would be better. Fixed. Legend now explain the shema of the HCO

2. I understood that this is a Methods collection, but it would be better to introduce some information about other rhythmic neurons/system in the Introduction, so this proposed method would be applied to or adapted to other studies. Introduction is focused on the Method and necessary background for the demonstration of the experimental system.

3. In Protocol, part 1: The second step "Prepare leeches…" is not clear regarding how to cold- anesthetize the animal, in the dissecting dish or not? Fixed.

4. In Protocol, part 1: As the third step "Pin the leech dorsal side up…" is not illustrated by a figure, it would be helpful to cite a paper with such figures and methods explained. Many leech papers are cited.

5. In Protocol, part 2: As no figures provided, citations seem necessary for this part of the Protocol. Citations seem unnecessary but can be added. Many leech papers are cited..

6. Representative Results: Figure 1 is hardly explained in the text. Either change it or add more content in the text. Figure 1 is now cited in the text appropriately and legend edited.

7. The Discussion is more like a conclusion. Extend the discussion or maybe change the heading to "Conclusion"? Discussion now focuses on strengths and limitations of the technique.

8. There are quite some typos and mistakes, for example:

In Protocol, part 1: "Prepare isolated Ganglion 7 from the leech nerve cord": "G" should not be capitalised. Fixed.

In Protocol, part 1: First paragraph "…12:12 light-dark cycle)." ")" is missing. Fixed.

"CaCl2" should be "CaCl2" Fixed.

"pH adjusted to 7.4 with 1 M with NaOH." Delete the second "with". Fixed.

"Remove the perineurium (desheath) the ganglion": "of" is missing before "the ganglion" Fixed.

Na+/K+ pump or Na/K pump? Should be consistent throughout the paper. Fixed.