Complexity of Neuromodulators in Aplysia Motor Neurons
Sean Lorenz – Report (December 11, 2006)

Introduction

It is well known that "the brain has a body," meaning motor function in the nervous system is intrinsically tied to an animal's body and environment not just to the central nervous system. In order to understand this relationship, extensive studies of adaptive behavior that focused on interaction of nervous system, body, and environment in the Aplysia buccal system have yielded progressive results in the past fifteen years. The overall goal by studying buccal motor neurons in Aplysia is to derive generalized principles for control mechanisms in other biological systems. Whether or not future results can prove this is yet to be seen.

The Aplysia buccal system was chosen for study of conventional and peptide cotransmission due its discrete targets that express both kinds of neurotransmitters in a simple, well-studied network - primarily in the accessory radula closer (ARC) neuromuscular system composed of two motor neurons (B15 and B16) in the buccal mass. In turn, studying the molecular neurophysiology of the ARC muscle has shown to benefit study of behavioral characteristics as well. Brezina, et al. continue by saying:

> The ARC muscle is controlled by just two motor neurons. In contrast to this central simplicity, there is great peripheral complexity, generated by a dynamically complex network of actions of numerous endogenous modulatory neurotransmitters and neuropeptides that shape the contractions of the muscle. For any central motor command, this modulatory network multiplies the range of possible peripheral outputs of the NMT (neuromuscular transform).[1]

This quote contains the culminated results of numerous experiments investigating several aspects of the interaction of central motor command and peripheral modulation.

---

[1] Vladimir Brezina, et al., "Modeling Neuromuscular Modulation in *Aplysia*. III. Interaction of Central Motor Commands and Peripheral Modulatory State for Optimal Behavior," *The Journal of Neurophysiology*, 93 (2005): 1523.

In order to make any claims concerning optimal motor behavior there are several issues to consider. First, individual motor neurons must be identified and their transmitter expression at varying membrane potentials must be understood. Second, once the neurons are identified, the types of transmitters released must be categorized and understood in terms of their excitatory, inhibitory or transient junction potentials. Then, temporal differences of transmitter release from different vesicle types must be assessed in order to accurately predict firing patterns for each neuron. The spatial differences of transmitter release is also essential for determining temporal release probabilities based on transmitter type and origin of vesicle loading.

Once this experimental data can be accurately represented, interaction between motor neurons at varied firing frequencies is needed. Consequently, simultaneous stimulation or interburst interval experiments can give results for variance in transmitter release effects – namely seeing whether an excitatory or inhibitory response is evoked. This information is important for understanding differences in regular versus irregular firing patterns at the neuromuscular junction.

At this point we must consider network interaction of motor neurons and the properties of release reactions, thus mathematical models enter the picture.[2,3] A temporal dependence model can then be represented with a more complex non-linear model that explains modulation at the neuromuscular junction and the motor neurons firing upon it. A properly working model of this sort imports *in vitro* firing inputs from prior experiments in order to output properly shaped contraction results.

---

[2] Brezina, et al., "Temporal Pattern Dependence of Neuronal Peptide Transmitter Release: Models and Experiments," *The Journal of Neuroscience*, 20(18)(Sept. 15, 2000): 6760-72.
[3] Brezina, et al., "Modeling Neuromuscular Modulation in *Aplysia*. III."
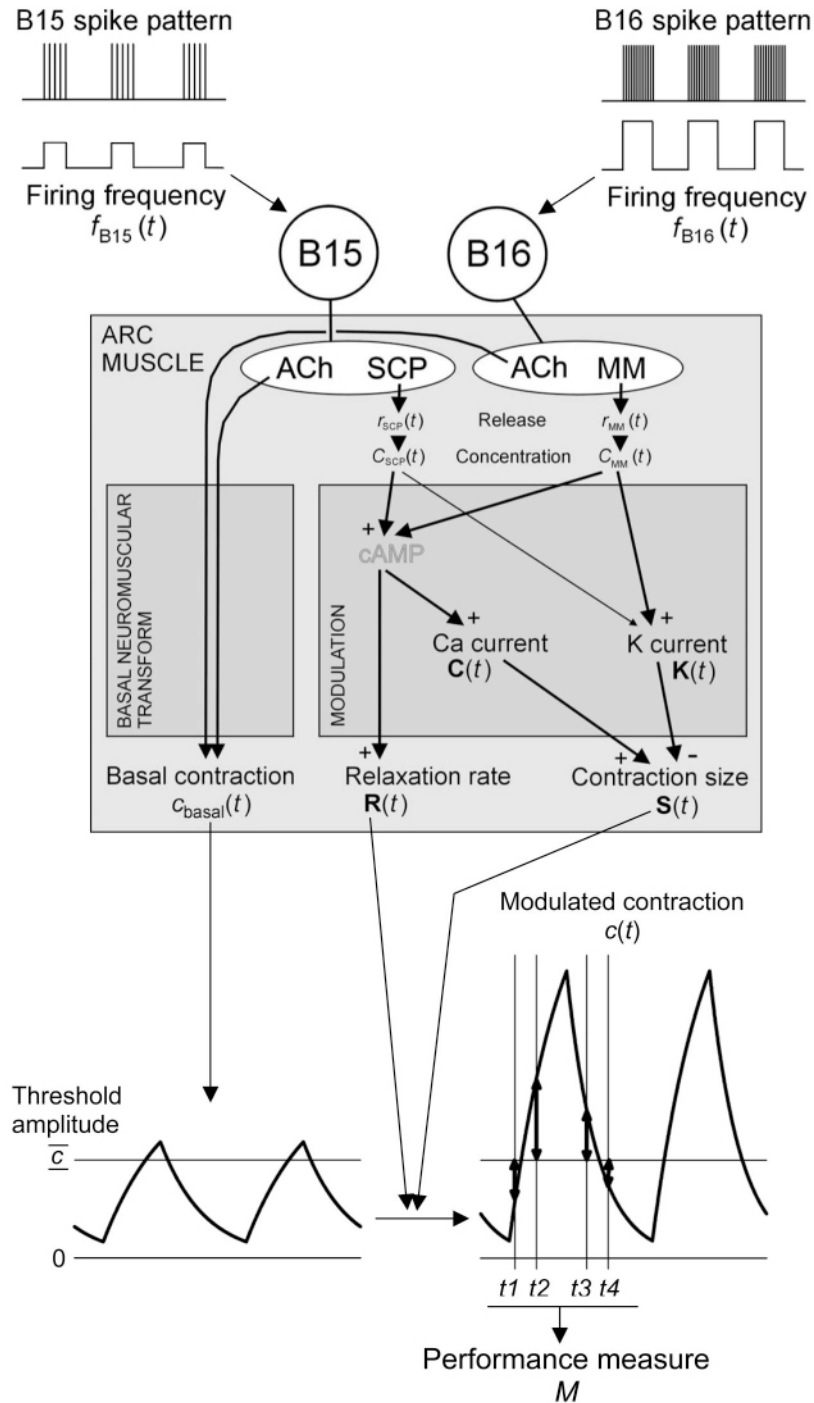
Figure 1. Model schematic of B15/B16 ARC modulation

So what are the advantages of studying such a systems in Aplysia and developing

mathematical models from it? Brezina, et al. believe that these factors explain facilitation or

constraining control of a network by another network, namely how the CNS controls behavioral

performance in the periphery. He also believes that these principles can be applied to understanding the vertebrate gut or neuromodulation in nervous systems other than the Aplysia.

Methods

I will report methods from three papers: one that explains modulation in I3a muscle, one that explains modulation in the ARC neuromuscular system, and one that details the modeling process of the ARC system. All experiments were performed with *Aplysia californica*.

PAPER ONE[4]

The functional role of neuropeptide transmitters must meet three experimental requirements: 1) peptide transmitters must be localized to individual, identified neurons, 2) release of these transmitters must be monitored, and 3) the physiological data of released transmitters need to be understood.[5] This was done by first identifying the buccal motor neurons. Animals were immobilized with injected isotonic $MgCl_2$, the buccal mass/ganglia complex removed and all nerves severed except ipsilateral buccal nerves 2 and 3. The ganglia were pinned in a chamber and superfused with high-Mg^{2+}, high-Ca^{2+} ASW to increase firing threshold and inhibit spontaneous activity. Neurons were impaled with two microelectrodes, one to inject current and one to monitor membrane potential. Neurons were identified by their position, size, nature of synaptic input, and muscle innervation patterns.

Next, an intracellular injection of ^3H-choline and synthesis of labeled ACh was performed. Thirdly, synthesis of methionine-containing peptides in the newly identified motor neuron B47 was determined. Identified neurons were stained via intracellular electrode and synthesized peptides were also labeled, then radiolabeled peptides were released from identified

[4] Paul J. Church, et al., "Modulation of Neuromuscular Transmission by Conventional and Peptide Transmitters Released from Excitatory and Inhibitory Motor Neurons in *Aplysia*," *The Journal of Neuroscience*, 13(7)(July 1993): 2790-2800.
[5] http://cmp.bsd.uchicago.edu/faculty/pLloyd.html

neurons in the primary culture. After stimulations and interburst intervals, the cell was hyperpolarized for long periods of time, perhaps to restore ionic concentrations to their prior levels. I3a muscle contractions were then measured with depolarizing current pulses. Note that high external Ca^{2+} concentrations were used here in the ganglion to allow for easy inward Ca^{2+} flow at the synapse. One question would be why this was done. Do these conditions parallel *in vivo* studies of the buccal system?

Most important to this study was measurement of I3a EJPs and IJPs recorded by an electrode pressed down firmly on the muscle. It took 10 EJPs of a burst at 15 Hz to get a muscle contraction. This is probably due to the summation effect of junction potentials in order to produce a contracting effect. Lastly, cAMP levels in I3a muscle were measured because elevation of cAMP indirectly measures release of the SCPs. This may be due to cAMP's important ability to influence neuronal signaling by binding to ligand-gated ion channels, thus being an indication of SCP binding to the channel.

The major limitation of this experiment is *in vitro* results rather than *in vivo*, however, the methods used in this experiment are thorough and obviously refined over many years of experimentation. Another concern is whether or not any other neuropeptides or conventional transmitters were present in the neuron prior to dissection. Also, no pharmacological experiments isolating MMa or SCPs has been presented in order to make sure each of these peptides were the cause for inhibition and excitation, respectively. Plotted data was shown only at the muscle-level with no data given for release probabilities of either peptide.

As for the strengths of the methods used, since the experiment was meant to show conventional transmitter interaction with peptide transmitters, the *in vitro* method performed was highly effective. The evidence shown by stimulation frequency gave well-defined results that

made it hard to doubt otherwise. To make sure results were indeed plausible, however, blocking

of ACh-Rs would have been another experiment worth showing.

PAPER TWO[6]

In this experiment (ten years after PAPER ONE), single fibers, dissociated from the ARC

muscle (also in the buccal system) were impaled with a single microelectrode and then voltage-

clamped. To study modulator-activated K^+ current, a depolarized steady voltage of -30 mV was

used to open K^+ channels and create inward current. As is common, Ba^+ current through Ca^{2+}

channels was used to study current through Ca^{2+} channels. The kinetics of ARC muscle

relaxation rate were recorded from the two-neuron system of B15 and B16 (either one or the

other at a time) impaled and stimulated with brief current injections to create certain patterns,

presumably to mimic the normal feeding pattern of the Aplysia. It is unusual that such a pattern

was used to release ACh from the neuron terminals yet the experimenters do not discuss the

process of peptide modulators. It would have been beneficial to see this data, since it is intrinsic

to the experiment.

More thoroughly laid out in the methods here was the modeling set-up. Rather than

elaborate on the details of the model I will mention the broad sections used to derive realistic

mathematical modeling from prior experimental data, then offer comments on what was covered

and what needs further consideration in the model. First and foremost, they modeled realistic

motor neuron firing patterns that cycle in three distinct types – biting, swallowing, and rejection.

A fourth null set was included to account for inactivation, i.e. the Aplysia not feeding. Bursting

patterns were derived from probabilistic firing frequency of the motor neurons over time. One

excellent feature of the model is that parameters for cycle period, interburst interval, and burst

---

[6] Vladimir Brezina, "Neuromuscular Modulation in *Aplysia*. I. Dynamic Model," *Journal of Neurophysiology* 90 (2003): 2592-2612.

duration are not *fixed* as in prior models. Instead, they vary on the cycle type over time. From the models I've seen in other papers thus far on cellular-level motor function, the non-linear aspect of neuron firing in the network is not well accounted for.

The major case for adding this mathematical feature (at least in this paper) is to account for variance in transmitter release based on vesicle type as well as variance in peptide release in either inhibitory or excitatory states. Also important was factoring in availability of modulator release alongside the probability of release. It may have been buried in the data, but I did not see figures illustrating the variance based upon the interburst pattern of motor neuron firing nor the effect of holding this pattern at different membrane potentials.

Also modeled were modulator concentrations which release relative to the firing frequencies of B15 and B16. In behavior it was found that B16 fires at higher frequency than B15 but no explicit reason is given for this. Further on in the model it mentions B16 releasing far more MM than B15, thus it may have to do with the amount of firing needed to release MM requires higher stimulation. It could be that since MM acts to inhibit motor neurons in the feeding system, higher frequency is needed for this inhibition than excitation in B15. It also seems that raising the firing frequency in B16 may be required to level release of SCPs in B15 and MM in B16.

As for modulating the three modulator effects, much progress had been made in this model compared to prior models which had to create separate static models for each factor – SCP, MM, Ca^{2+} current, K^+ current, etc. This model gives a more generalized form in order to yield dynamic effects. This effect is represented by the sum of individual effects on n modulators in the system represented in this simple time evolution equation: $X(t) = \sum_{i=1}^{n} X_i(t)$. Other key

modeling elements discussed include enhancement of Ca^{2+} current, activation of K^+ current, measurement of contraction size, and increase of relaxation rate.

Most interesting in their model is the accuracy of mimicking *in vitro* current traces as well as the dynamics of each modulator over time yielding accurate physiological results based on data from prior papers such as the Church 1993 paper mentioned earlier. There are several questions to be asked though. How can this model be applied to anything other than Aplysia buccal system? How tied is this model to Aplysia data rather than a model representing motor function in other animals? Does the model work for something more than a two-neuron system? Lastly, they propose other systems that this could function with, yet what if the modulators for those systems are different than MM or SCP? This would probably yield different time-dependent results on the muscle contraction.

Results

For discussion group I chose a paper from 1993 published in *The Journal of Neuroscience* by Paul J. Church at University of Chicago; I'll discuss the details of their findings first since it sets up the data for following experiments. The paper discussed excitatory and inhibitory motor neurons that can function in two states due to the presence of both conventional *and* peptide transmitters. This dual nature was found to be pronounced in motor neuron B47 in Aplysia buccal system, which can act selectively to either inhibit or excite the effects of a second motor neuron innervating the same muscle.[7] Church found that most buccal motor neurons are indeed cholinergic, however, when a neuron has an inhibitory or mixed response, the excitatory motor neurons to those fibers are *non*cholinergic. Further, when motor neurons B3 and B47 were

---

[7] Paul J. Church, et al., "Modulation of Neuromuscular Transmission by Conventional and Peptide Transmitters Released from Excitatory and Inhibitory Motor Neurons in *Aplysia*, *The Journal of Neuroscience*, 13(7)(July 1993) : 2790-2800.

simultaneously stimulated an inhibitory junction potential was evoked. This was believed to

occur due to the B47-evoked cholinergic IJPs decreasing the amplitude of the B3-evoked EJPs in

I3a muscle fibers. However, during a B3 *interburst interval* increase of EJPs and contractions

were due to release of myomodulins from B47 terminals. Sustained high-frequency bursts

released neuropeptides for all three motor neurons (B3, B47 and B15). Thus, I3a shows

heterosynaptic modulation involving conventional and peptide transmitters releasing

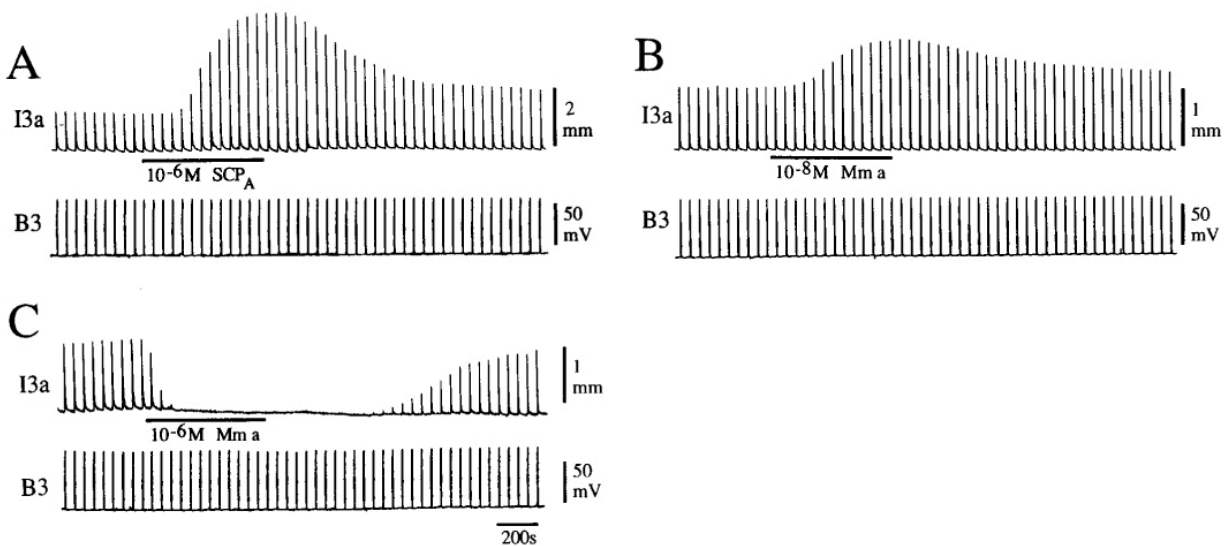conventional transmitters in short bursts and both types of transmitter in long bursts.

Figure 2. Variations on muscle response depending on peptide type and concentration

    Crucial to this study as well as for those following was the role played by a few particular

neuropeptides often acting as contransmitters with ACh[8]: small cardioactive peptides (SCPs),

myomodulin A (MMa), FMRFamide, and Buccalin A. SCPs are expressed by excitatory motor

neurons and increase the responsiveness of the feeding motor program and the endogenous

activity of specific feeding motoneurons. They also act as extremely important modulators in the

buccal muscle system. Buccalin A is colocalized with SCPs at the neuromuscular junction but

---

[8] The neuropeptide is usually released only when firing rates in the presynaptic neuron are high, probably due to higher Ca^{2+} concentrations in the terminal.

instead of increasing the size of muscle contractions, it decreases them. FMRFamide is also expressed by excitatory motor neurons and plays an important role in cardiac activity regulation. MMa, on the other hand, is expressed by inhibitory motor neurons and modulate neuromuscular signaling in the Aplysia feeding system.

SCPs and MMs were found to be of utmost importance for the results in the buccal system in particular. These two types of neuropeptides shape contractions via three primary muscle actions: 1) potentiation of contractions by enhancing depolarized Ca^{2+} current essential for contraction, 2) depression of contractions by activating K^+ current thus opposing ACh-induced depolarization and Ca^{2+} influx, and 3) increased relaxation rate of contractions by modulating elements necessary for muscle contraction.[9]

Subsequent experiments built upon the foundation shown in the 1993 Church paper, exploring the role of large dense core vesicles (LDCVs) that release the peptide NTs listed above. More specifically, differential targeting of cholinergic and peptidergic vesicles were shown to play a large role in the distinct release requirements and spatial/temporal characteristics of conventional as well as peptide NTs.[10]

---

[9] Brezina, et al. 2005, 1524.
[10] Tuula Karhunen, et al. "Targeting of Peptidergic Vesicles in Cotransmitting Terminals," *The Journal of Neuroscience*, Vol. 21 RC 127 (2001).
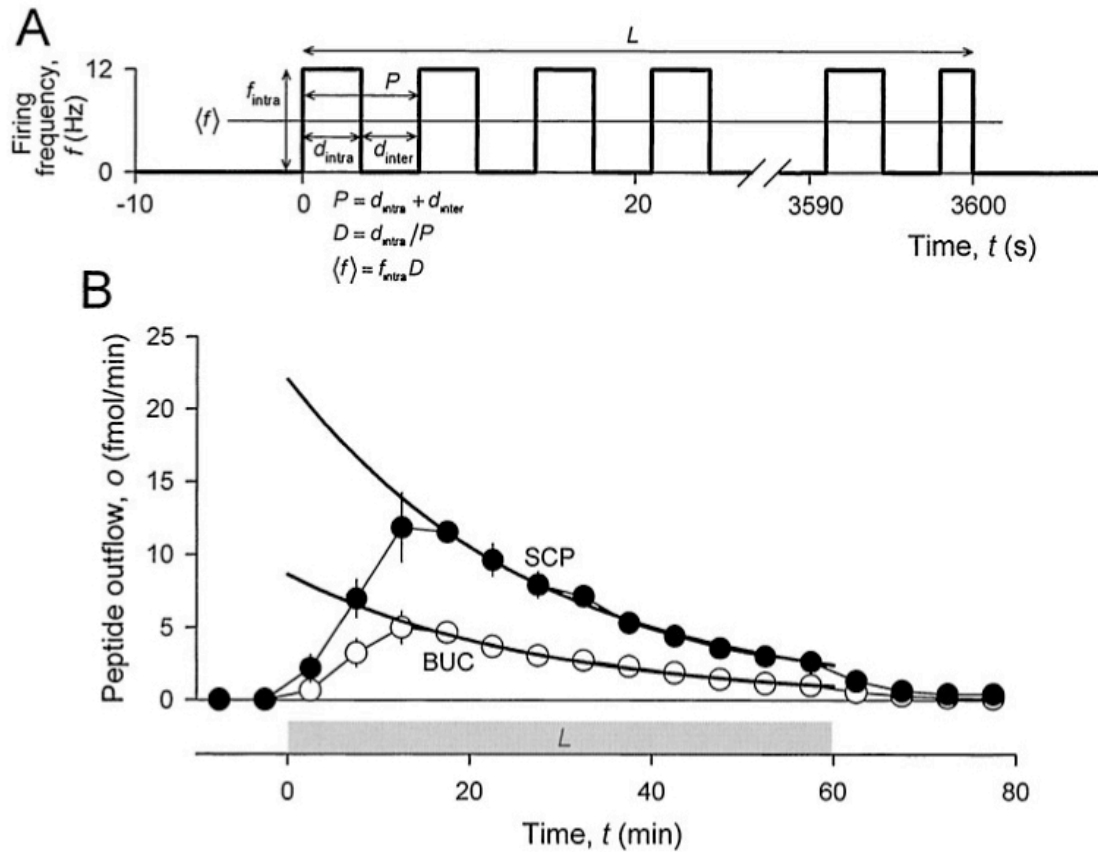
Figure 3. Typical firing pattern of motor neuron B15 and
corresponding peptide outflow from the ARC muscle

The final paper to discuss here is the Brezina 2003 paper that culminates data from prior

Church and Brezina experiments in order to build a functioning model of the ARC muscle in the

buccal system. Most important is to note the evolution of the variables in the system over time in

relation to motor neurons B15 and B16. A key finding was integration of data to solidify the idea

that modulators do different things at different times. The model showed three effects from MM

and SCP release: 1) enhanced Ca^{2+} current in muscle, 2) K^+ current activation and 3) relaxation

rate increase (if Ca^{2+} and K^+ current combine to get contract size modulation). The model also

talks extensively about the switching trajectory and change in modulatory states, but this is more

system-level analysis at this point so I will refrain from elaborating on the details.
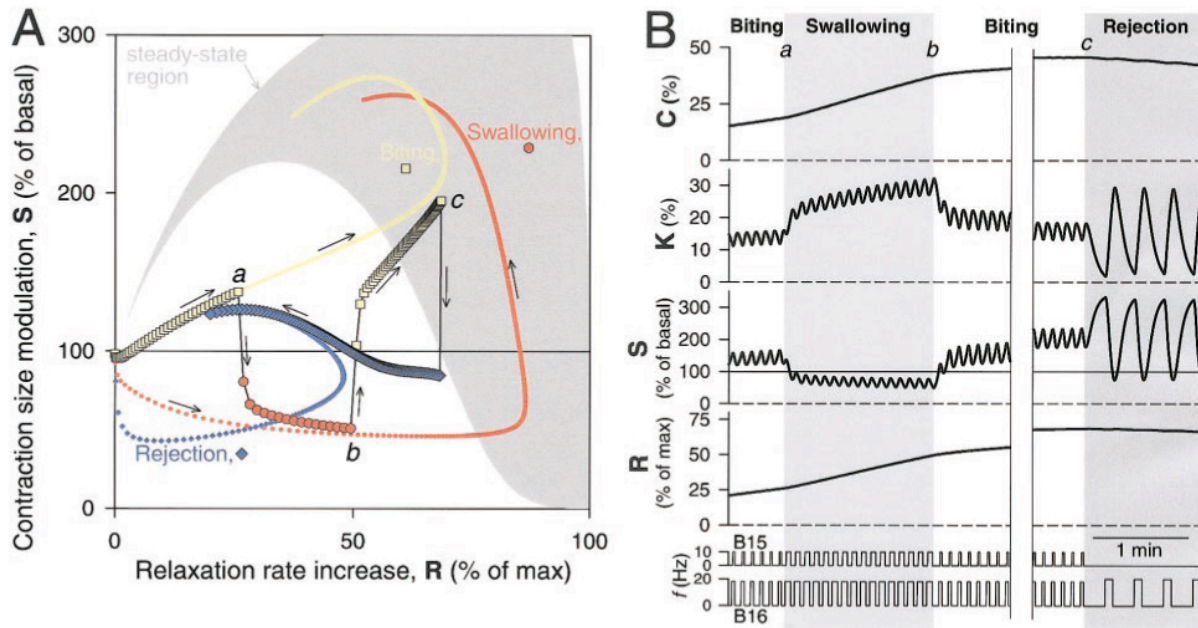
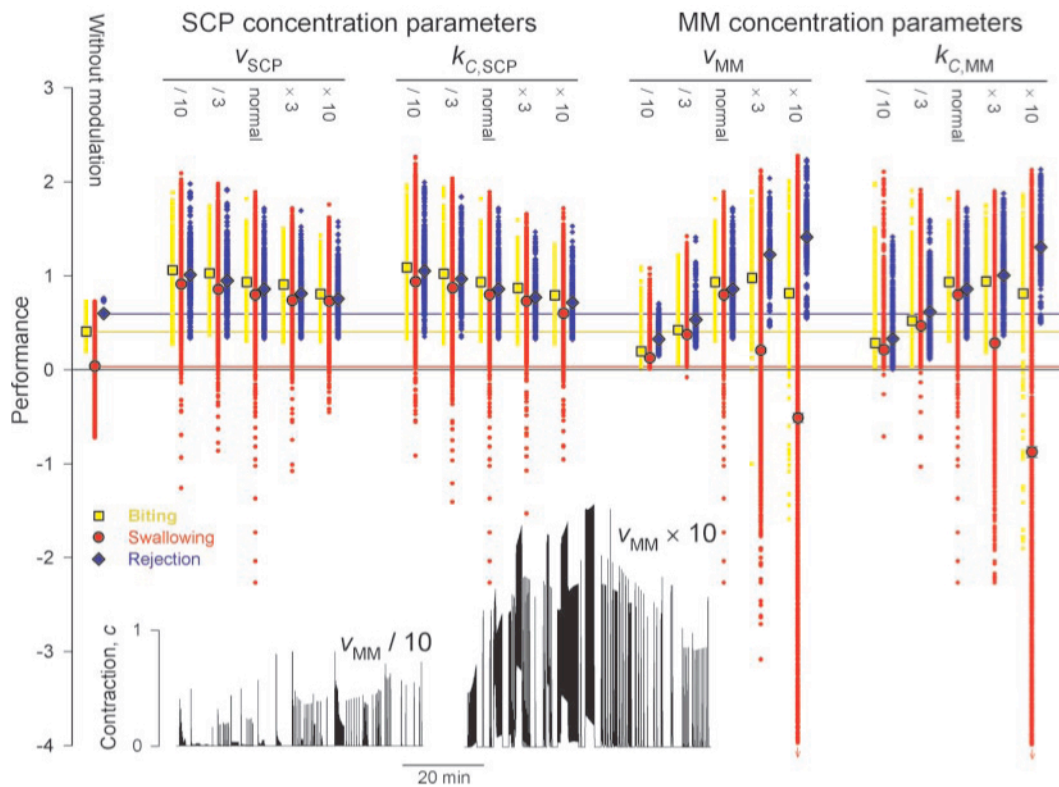*Figure 4.* Trajectory of modulation in output space during feeding.

Figure 5. Sensitivity of performance to variation of key parameters governing SCP and MM concentration

Discussion

The current hypothesis for modeling neuromuscular modulation in *Aplysia* has its strength in its simplicity. Since they are investigating a two-neuron system, honing in on the specific motor output has yielded progressive results. Perhaps of utmost importance in the findings here is the relation of peptide neurotransmitters that act as modulators when released from the presynapse onto the neuromuscular junction. Motor neurons may have highly regular firing rates, but the output of those firing neurons can exhibit very different results depending on their interaction as well as interaction of the peptides they express. Neurophysiologically speaking, this is important for understanding time-dependence of quantal release with regards to peptides in large core dense vesicles as opposed to small core vesicles that release more conventional transmitters (in this case ACh). Having a more accurate representation of the dynamic effect between the release of these two different forms of transmitters can help us better understand what occurs at the neuromuscular junction as far as EJPs and IJPs are concerned. Also intriguing is the progress in understanding the cellular and molecular elements of a system well enough to construct highly accurate mathematical models from the data. As of 2005, Brezina, et al. have been able to enter input values into the model taken from prior datasets of B15 and B16 and get accurate results.

A few things in the model, however, still need to be addressed. How does the model and experimental data account for the nonlinear nature of neurotransmitter modulation in actual Aplysia feeding? Also, how does this data correlate with other complex systems as they postulate? Lastly, there seems to be no mention of pharmacological blocking of MMs or SCPs.

Results for this sort of study would be worth seeing. Also, what is the effect of other "classical" neurotransmitters like serotonin in relation to MM or SCP?

Brezina, et al. say that other somewhat predicable, higher-order temporal structure should adapt to the results they found. Future directions should try to find correlation of this experimental data with other complex networks of modulator and neurotransmitter action. Another direction would be to better understand how motor output from the CNS effects and predicts the stochastic nature of motor output onto the muscle as noted by the nonlinear nature of varied transmitters.

In conclusion, although motor neurons may look normal and uninteresting based primarily on spike pattern analysis, there is much more going on within the cell that determines response on the muscle. In addition to spike pattern and firing frequency, it is important to test other factors such as transmitter type release, transmitter time difference for release, cAMP results, and the relation between neurons during certain types of firing intervals. Thus it seems that even "simple" neurons are not so simple.