

# Muscles express motor patterns of non-innervating neural networks by filtering broad-band input

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**We describe three slow muscles that responded to low-frequency modulation of a high-frequency neuronal input and, consequently, could express the motor patterns of neural networks whose neurons did not directly innervate the muscles. Two of these muscles responded to different frequency components present in the same input, and as a result each muscle expressed the motor pattern of a different, non-innervating, neural network. In an analogous manner, the distinct dynamics of the multiple intracellular processes that most cells possess may allow each process to respond to, and hence differentiate among, specific frequency ranges present in broad-band input.**

Neurons receive input across an extremely wide frequency range (from high-frequency sound at tens of kHz to circadian rhythms at  $\sim 10$   $\mu$ Hz). Muscles receive input across a narrower but still wide range (10  $\mu$ Hz to several hundred Hz, the maximum firing rate of neurons). Neurons and muscles cannot accurately follow the higher portions of these ranges. For instance, the refractory period limits neuron firing to a few hundred Hz, and relaxation kinetics prevent muscles from accurately following motor neuron bursts faster than  $\sim 1$  Hz<sup>1</sup>. Neuronal and muscle intracellular processes (for example, Ca<sup>2+</sup> and second messenger concentration, protein kinase activation, protein phosphorylation and gene expression) are also slow, with time courses ranging from tens of milliseconds (10–100 Hz) to minutes ( $\sim 0.01$  Hz) or longer<sup>2–15</sup>, and therefore would also be expected to be unable to accurately follow high-frequency input.

Restriction of responsiveness to a particular frequency range might be thought to limit function. Indeed, the inhibitory and neuromodulatory muscle innervation in several invertebrates may be present to increase the frequency range over which the muscles can accurately follow their inputs<sup>16–19</sup>. However, selectivity for low frequencies may also be advantageous because it allows receivers to demodulate, and hence respond to, low-frequency signals carried in a modulated high-frequency (carrier wave) input. The wide range of kinetics present in the responses of neurons and muscles to input suggests that these systems might have the cellular mechanisms necessary to perform such demodulation, and different patterns of temporal input can differentially alter various intracellular processes<sup>12,20–24</sup>. However, except for the exclusion of inputs with frequencies of 1 Hz or greater in vertebrate skeletal muscle<sup>1</sup>, no examples in which the signal of interest is carried in the low-frequency component of a broad-band input have been described in biological systems on the cellular level.

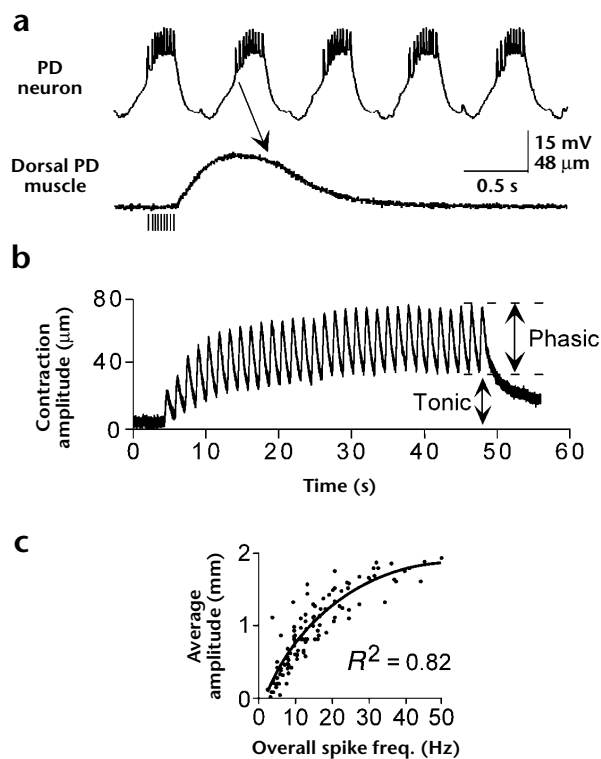
We describe here three slow muscles driven by a bursting neural network with a cycle frequency of 1 Hz. This network's

output is modulated by two other, much slower rhythmic neural networks. The muscles responded to these modulations and followed the slow network activity, even though no neuron of the slow networks innervates the muscles. These results extend the known range of functional low-frequency response some two orders of magnitude, and provide an example of a signal carried partially or exclusively in a slow modulation imposed on the target's driver from elsewhere in the nervous system. Furthermore, two of these muscles are innervated by the same motor neurons and, hence, receive identical neuronal input. However, because of differences in their contractile properties, one muscle followed both slow networks, whereas the other followed only one. These two muscles responded to different frequency domains of one signal, and thus provide a clear example of both encoding of information by the nervous system and its decoding by neuronal followers, on the basis of frequency. Preliminary accounts of this work appeared in abstract form (L.G.M. *et al.*, *Soc. Neurosci. Abstr.* 25, 1642, 1999; J.B.T. & S.L.H., *Soc. Neurosci. Abstr.* 25, 1641, 1999).

## RESULTS

We studied the pyloric neuromuscular system of the lobster (*Panulirus interruptus*), a part of the stomatogastric system that is driven by the pyloric neural network. Every 0.5 to 2 seconds, the motor neurons of this network produce 5–10 action potential bursts of 100–500 millisecond duration<sup>25</sup>. Pyloric muscles are non-spiking muscles that contract as a graded function of their neuronal input<sup>26</sup>. Based on the pyloric network's cycle period and bursting nature, it was thought that each motor neuron burst induces a single muscle contraction that fully relaxes between bursts, and that the muscles thus temporally mirror the bursting activity of their input<sup>25,27–30</sup>. Work on pyloric muscle contractions evoked by nerve stimulation in the crab, *Cancer borealis*, and the shrimp, *Palaemon serratus*, support this belief<sup>31–33</sup>.

**Fig. 1.** In some pyloric muscles, overall spike frequency codes steady-state average contraction amplitude. (a) Top trace, intracellular PD neuron recording; bottom trace, isotonic contraction of an extremely slow muscle innervated by the PD neurons (the dorsal PD muscle) induced by motor nerve stimulation mimicking the first burst of action potentials (vertical lines below muscle trace) in the PD neuron trace. If the muscle were driven by the neuron, the next contraction (arrow) would occur before the first contraction finished, resulting in intercontraction summation. Most hyperpolarized PD neuron membrane potential,  $-62$  mV. (b) Rhythmic stimulation (burst duration, 390 ms; spike frequency, 20 Hz; cycle period, 2 s) of the motor nerve innervating the dorsal PD muscle resulted in (after intercontraction summation had stabilized) a sustained tonic contraction on which rhythmic contractions rode. (c) After intercontraction summation had stabilized, overall spike frequency (spike number per burst divided by cycle period) coded average contraction amplitude (one half phasic plus tonic contraction) for this muscle. All data are from the cpv1b muscle<sup>39</sup>.

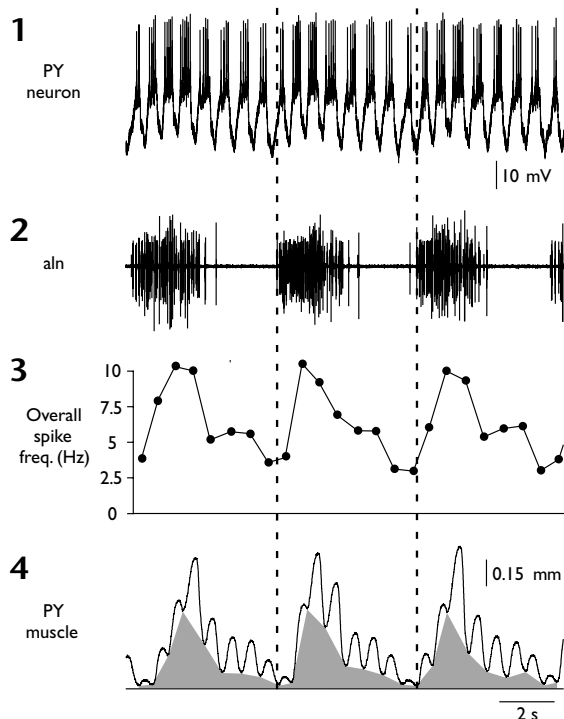


However, in *Panulirus* some pyloric muscles contract and relax much too slowly to follow their motor neuron bursts faithfully<sup>34,35</sup> (Fig. 1a). The top trace shows the characteristic rhythmic action potential bursts of a pyloric dilator (PD) neuron. The second trace shows an isotonic contraction (muscle shortening against constant load) of an extremely slow muscle innervated by the PD neurons (the dorsal PD muscle). This contraction was induced by stimulation of the motor nerve with parameters matching the first burst in the neuron trace. If the neuron were driving this muscle (in all experiments the motor nerve was cut to prevent spontaneous neuron input to the muscle), the next contraction would have occurred before the contraction induced by the previous burst had ended (arrow; angled to account for the long delay between the neuron burst and the contraction beginning).

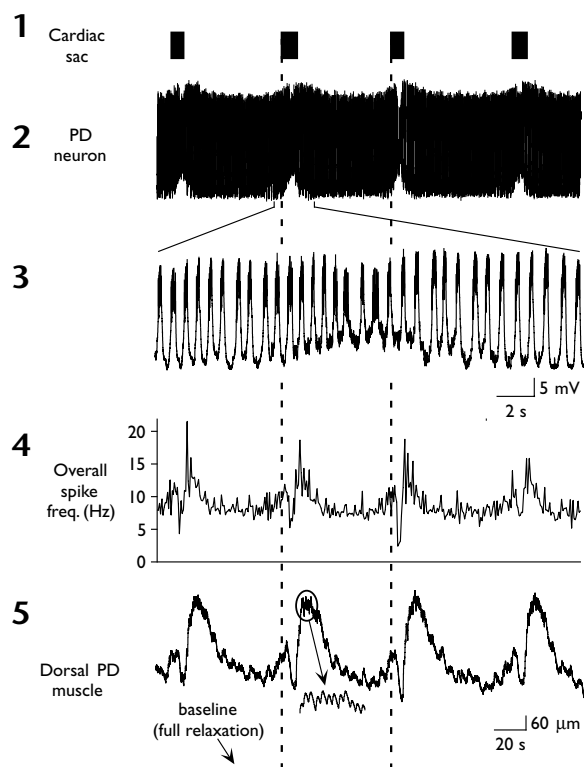
When the motor nerve was rhythmically stimulated with bursts of shocks using physiologically relevant parameters, the muscle contractions temporally summated (Fig. 1b). After the

summation had stabilized, the muscle's response therefore consisted of a sustained, tonic baseline contraction on which rode phasic contractions that matched the stimulation cycle period. Here the average contraction amplitude (one half the phasic contraction amplitude plus the tonic contraction amplitude) of the muscle is particularly important, and after muscle summation has stabilized, this average amplitude is well predicted by the overall spike frequency (spike number per burst divided by cycle period) of the motor neuron (Fig. 1c; ref. 34 and L.G.M. & S.L.H., *Soc. Neurosci. Abstr.* 24, 1891, 1998). These data suggest that if the overall spike frequencies of pyloric motor neurons were slowly modulated by extrinsic influences, average contraction amplitude of pyloric muscles might similarly vary.

The stomatogastric nervous system also contains the gastric mill and cardiac sac networks, which have much slower cycle periods (5–10 seconds and ~1 minute, respectively) than the pyloric network<sup>25</sup>. Gastric mill and cardiac sac activity modulates pyloric activity<sup>25,28,36–38</sup>, and the overall spike frequency of several pyloric neurons varies with gastric mill and cardiac sac activity (J.B.T. & S.L.H., *Soc. Neurosci. Abstr.* 25, 1641, 1999). Using stimulation parameters determined from previously measured effects of gastric mill and cardiac sac network activity on



**Fig. 2.** A muscle innervated by PY neurons expressed a gastric mill network contraction pattern even though no gastric mill neurons innervate the muscle. Trace 1, intracellular recording of the PY neuron whose activity was used to stimulate the muscle's motor nerve. Most hyperpolarized PY neuron membrane potential,  $-55$  mV. Trace 2, extracellular recording from the aln of the activity of the GM neurons of the gastric mill network. Trace 3, PY neuron overall spike frequency. Trace 4, isotonic contractions of a PY neuron-innervated muscle. Gray shading shows the tonic component of the muscle contraction; the muscle almost fully relaxed only at the end of each gastric mill cycle (dashed lines mark one cycle period). Solid horizontal line, fully relaxed muscle length. Recording is from p8 muscle<sup>39</sup>.



**Fig. 3.** Although no cardiac sac neurons innervate it, the extremely slow dorsal PD muscle primarily contracted in a pattern matching the very slow cardiac sac rhythm. Trace 1, schematic representation of cardiac sac network activity; rectangles correspond to cardiac sac bursts, dashed lines mark one cardiac sac cycle period. Trace 2, intracellular recording of the PD neuron whose activity was used to stimulate the muscle's motor nerve. Trace 3, time expansion of PD neuron activity immediately before, during and after a cardiac sac burst. Most hyperpolarized PD neuron membrane potential,  $-65$  mV. Trace 4, PD neuron overall spike frequency. Trace 5, isotonic contractions of a dorsal PD muscle (inset is a time expansion showing small contractions in pyloric time). Scale bars, 20 s (traces 1, 2, 4 and 5) or 2 s (trace 3) and 5 mV (traces 2,3) or 60  $\mu$ m (trace 5). Recording is from the *cpv1b* muscle<sup>39</sup>.

pyloric network output, we explored the effects of altering pyloric neuron activity on pyloric muscles by stimulating motor nerves innervating various pyloric muscles (Fig. 2). The first trace shows an intracellular recording from a pyloric (PY) motor neuron; the second trace is an extracellular recording from the anterior lateral nerve (aln), which carries the axons of the gastric mill (GM) neurons of the gastric mill network<sup>39</sup>. PY neuron firing waxed and waned as a function of gastric mill network activity (the dashed lines mark one gastric mill network cycle), and the third trace shows that this change in PY neuron activity resulted in corresponding changes in PY neuron overall spike frequency.

The fourth trace shows the contractions of a muscle innervated by the PY neurons induced by motor nerve stimulation exactly matching the PY neuron spiking pattern shown in the first trace. As overall spike frequency increased at the beginning of each GM neuron burst, the phasic amplitude and summation of the pyloric-timed PY muscle contractions increased and, consequently, the muscle's tonic contraction component (gray shading) increased. As overall spike frequency decreased during the GM neuron interburst interval, the muscle's phasic contraction amplitude and summation and, thus, its tonic contraction, decreased, allowing it to return nearly to rest length before the next GM neuron burst. Thus, although no gastric mill neuron innervates this muscle, it nonetheless displayed both a pyloric (the rapid rhythmic contractions) and a gastric mill (the tonic contraction component) motor pattern.

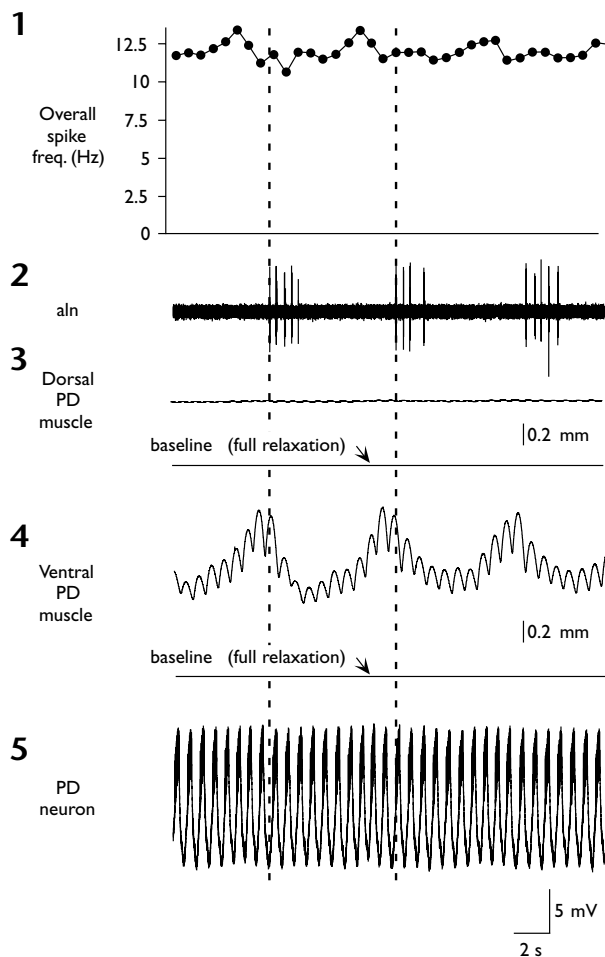
The changes in the PY muscle tonic contraction can be qualitatively understood by considering Fig. 1b and c. Contractions of the extremely slow dorsal PD muscle took 10–15 pyloric cycles to stabilize (Fig. 1b). The PY muscle is faster than the dorsal PD muscle, but it nonetheless took 2–3 cycles to stabilize in response to rhythmic stimulation and shows similar coding dependence (M. Rehn, L.G.M. & S.L.H., unpublished observations). When PY neuron overall spike frequency changes, the muscle's contraction amplitude changes toward an average contraction appro-

priate for this new spike frequency. If overall spike frequency remained at the new value, the muscle would stabilize at the average contraction amplitude predicted by the equivalent of Fig. 1c for the PY muscle. However, overall spike frequency does not stabilize; consequently, the muscle is driven toward different regions of this curve each pyloric cycle, which generates the slow variations in average contraction amplitude (Fig. 2).

For the relatively fast PY muscle, the contractions in pyloric time were a large percentage of total contraction amplitude. In contrast, the extremely slow dorsal PD muscle responded to PD neuron input modulated by the very slow,  $\sim 0.01$  Hz, cardiac sac network primarily with a slow, rhythmic variation in tonic contraction amplitude (Fig. 3). During cardiac sac network bursts (trace 1; each rectangle represents one cardiac sac burst, dashed lines mark one cardiac sac cycle), PD neuron activity was markedly altered, but the neuron continued to burst in pyloric time (trace 2; expanded over one cardiac sac burst in trace 3). PD neuron overall spike frequency (trace 4) showed a triphasic increase–decrease–increase pattern with each cardiac sac burst. When the motor nerve innervating the extremely slow dorsal PD muscle was stimulated with this activity pattern, the muscle's contractions in pyloric time (shown on expanded time scale in inset) were only  $\sim 8\%$  of the maximum contraction amplitude, whereas its contractions in cardiac sac time were  $\sim 50\%$  of maximum contraction amplitude (trace 5). Thus, even though no motor neurons of the cardiac sac network innervate it<sup>39</sup>, the muscle primarily contracted in cardiac sac time.

These data show that the PD and PY muscles can respond to slow modulations of PD and PY neuron activity. However, because the activity patterns of the PD and PY neurons are different, the PD and PY muscles receive different input; these data, therefore, do not show how different muscles respond to the same input. As well as innervating the extremely slow dorsal PD muscle, the PD neurons also innervate the faster ventral PD muscle, which allowed us to investigate whether the two muscles selectively respond to different frequency domains within an identical neural input.

In addition to showing large ( $\sim 15$  Hz) variations in overall spike frequency in time with the slow cardiac sac network ( $\sim 0.01$  Hz; Fig. 3), the PD neurons often also showed small ( $\sim 2.5$  Hz) variations in overall spike frequency (Fig. 4, trace 1) in time with the faster gastric mill network (0.1–0.2 Hz; trace 2; dashed lines mark one gastric mill cycle). The extremely slow dorsal PD muscles showed much smaller variations in gastric mill time than did the faster ventral PD muscles and, depending on gastric mill cycle period, sometimes completely excluded the gastric mill signal. In these cases (Fig. 4, trace 3), the



**Fig. 4.** Different muscles can respond to different frequency domains in the same neural signal. The PD neurons innervate both the very slow dorsal PD muscle and the faster ventral PD muscle. PD neuron overall frequency (trace 1) varied as a function of gastric mill phase (trace 2, GM neuron activity recorded from the aln; dashed lines mark a single cycle period). Muscle contraction amplitude of the extremely slow dorsal PD muscle did not vary as a function of gastric mill phase (trace 3), whereas that of the faster ventral PD muscle did (trace 4). Trace 5, intracellular PD neuron recording used to stimulate the muscles' motor nerves. Most hyperpolarized potential of PD neuron membrane,  $-65$  mV. The dorsal PD muscle is the cpv1b muscle; the ventral is the cpv2b muscle<sup>39</sup>.

input. These results have implications not only for interpreting studies of the pyloric system, but also, more generally, for understanding how and to what extent biological systems can respond to input signals carried in different frequency domains.

### Pyloric network function

In response to bath application of various neuromodulatory substances<sup>29,40–43</sup> or stimulation of modulatory inputs<sup>25,28,29,37,38,44–48</sup>, the pyloric network produces different neural outputs. It is tempting to interpret these changes as inducing changes in a pyloric-timed motor pattern. The contraction of several pyloric muscles in phase with other, slower neural networks suggests, however, that changing pyloric neural activity will have complex effects on pyloric motor output. In particular, these data imply that understanding the functional effects of changes in pyloric neuron activity may require consideration not only of pyloric activity on a pyloric cycle-by-cycle basis, but also the effects of intercontraction summation over time scales 5- to 10-fold and 60- to 100-fold longer (corresponding to gastric mill and cardiac sac cycle periods, respectively).

Pyloric cycle period varies between 0.5 and 2.0 seconds; depending on pyloric period, the relaxation times of some pyloric muscles may or may not allow intercontraction summation (T.A. Ellis, P.I. Harness, T.J. Koehnle, M. Rehn, L.G.M. & S.L.H., unpublished observations). For instance, the pyloric network shown in Fig. 2 had a period of  $\sim 0.7$  seconds. At a period of 2.0 seconds, this muscle's contractions would nearly or fully relax between neuron bursts, intercontraction summation and tonic contraction amplitude would dramatically decrease, and the variations of tonic contraction amplitude in gastric mill time would be a much smaller component of total muscle contraction. Thus one effect of changing pyloric cycle period may be to alter the expression of gastric mill activity as a variation in tonic contraction amplitude; at very slow pyloric cycle periods, these variations could be completely abolished.

### Signal extraction from different frequency domains

Considerable evidence indicates that neuronal intracellular processes are sensitive to the temporal patterning of the input the neuron receives. For instance, action potential patterning alters  $\text{Ca}^{2+}$  and second messenger concentration, protein expression and gene regulation<sup>12,20–23</sup>. The specificity of gene activation by  $\text{Ca}^{2+}$  oscillations depends on oscillation frequency<sup>24</sup>, and modeling studies suggest that multiple feedback systems sensitive to different time scales<sup>49</sup> may be necessary to explain the response of stomatogastric neurons to temporal variations in input patterns<sup>50</sup>. Although these observations are intriguing, the functional significance of these data is unclear, as the exper-

output of the dorsal PD muscle consisted of a tonic contraction, on which were superimposed very small rhythmic contractions in pyloric time but no measurable contractions in gastric mill time. In contrast, the faster ventral PD muscle (trace 4) was rhythmically active in both pyloric and gastric mill time. Both muscles also showed large contractions during cardiac sac bursts (dorsal PD muscle, Fig. 3; ventral PD muscle, data not shown). Thus, the relatively fast ventral PD muscle was rhythmically active in pyloric (1 Hz), gastric (0.15 Hz) and cardiac sac (0.01 Hz) time, whereas the slow dorsal PD muscle responded to the rapid pyloric and very slow cardiac sac signals, but failed to respond to the intermediate gastric mill signal. Note also the sensitivity of the ventral PD muscle to input in gastric mill time; the small changes in PD neuron overall spike frequency (Fig. 4, trace 1) in gastric time were almost indistinguishable in the neuron intracellular recording (trace 5), but resulted in a pronounced (50% of maximum contraction amplitude) gastric mill component in the muscle contraction.

### DISCUSSION

These data demonstrate that three slow muscles of the pyloric neuromuscular system can respond to slow modulation of the rapid pyloric pattern, and hence express, either partially (the PY and ventral PD muscles) or almost exclusively (the dorsal PD muscle), the rhythmicity of networks of neurons that do not innervate the muscles. They further demonstrate that two targets (the slow ventral and faster dorsal PD muscles) can respond to different frequency domains in the same neuronal

imental stimulation patterns used are not based on actual inputs. In contrast, our input exactly matched that produced by the system's innervating neurons.

More generally, previous experimental work on sensitivity to temporal patterns does not address the issue of whether particular second messengers selectively respond to different frequency domains. Our demonstration that two muscles responded to different frequencies in a single input (Fig. 4) directly addresses this point and demonstrates a differential response as a function of frequency domain. Presumably, this differential response largely stems from the different temporal properties of the muscles. Intracellular responses show a wide kinetic range<sup>2-15</sup>, and differential frequency response, therefore, may also be present in neuronal intracellular responses to broad-band input. Our data thus support the hypothesis that "these processes [intracellular messengers] may have...features...that limit their involvement to certain patterns of stimulation"<sup>12</sup>.

The low-frequency response shown here does not require rhythmicity of the underlying input. Slow processes driven by irregular input would smooth out high-frequency variation and would respond primarily to the input's average frequency<sup>10,14</sup>. However, if this average were slowly modulated, the system would be expected to follow the moving average and, hence, to respond to the modulation. Thus, one consequence of the distinct dynamics of various intracellular processes in a biological system may be that each process responds to specific frequency domains present in broad-band inputs. Furthermore, as expression of these processes can itself be regulated, cells may also be developmentally and functionally tuned to specific frequency domains contained in their input.

## METHODS

Stomatogastric neuromuscular systems were dissected and prepared for extracellular nerve recording and stimulation, intracellular neuron recording, and measurements of muscle contraction using standard techniques<sup>25,34,35,39</sup>. Nerve recording and stimulation were performed using suction electrodes or pin electrodes insulated with petroleum jelly, intracellular recordings were made with glass microelectrodes (filled with 0.55 M K<sub>2</sub>SO<sub>4</sub>, 20 mM KCl) and an Axoclamp 2A (Foster City, California), and contractions were measured with a Harvard Apparatus (South Natick, Massachusetts) 60-3000 isotonic muscle transducer.

Muscle rest length was maintained at approximately physiological levels. Muscle loading was determined by inducing single contractions and adjusting muscle load to achieve the maximum contraction amplitude consistent with full relaxation after the stimulation. A support bar was then placed under the transducer arm to prevent muscle overstretching. In all cases, the lateral ventricular nerve, which contains both the PD and PY neuron axons, was stimulated to induce muscle contractions. There are two PD neurons and six to eight PY neurons. To ensure activation of all axons innervating a muscle, stimulation amplitude was incrementally increased using single-burst stimulations until increases in contraction amplitude ceased. No neuromodulatory axons are known to innervate the PD and PY muscles and no pyloric neurons are known to contain modulatory co-transmitters. Furthermore, comparisons of contractions before and after long stimuli revealed no modulation by the nerve. We therefore believe that our data reflect simple classical neuromuscular innervation and muscle contraction.

Data were digitized with a Cambridge Electronic Design (CED, Cambridge, UK) 1401 plus and analyzed using CED and Kaleidagraph (Reading, Pennsylvania) software; figures were prepared in Canvas (Miami, Florida). In Fig. 1, the nerve was stimulated using a World Precision Instruments (WPI; Sarasota, Florida) stimulus isolation unit and a Grass (Quincy, Massachusetts) S48 stimulator; in Figs. 2-4, nerves were stimulated using a WPI Pulsemaster A300 stimulator driven by the CED.

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