Cycle-to-Cycle Variability of Neuromuscular Activity in Aplysia Feeding Behavior

Charles C. Horn, Yuriy Zhurov, Irina V. Orekhova, Alex Proekt, Irving Kupfermann, Klaudiusz R. Weiss, and Vladimir Brezina

Monell Chemical Senses Center, Philadelphia, Pennsylvania 19104; Center for Neurobiology and Behavior, Columbia University, New York 10032; and Department of Physiology and Biophysics and Fishberg Research Center for Neurobiology, Mount Sinai School of Medicine, New York, New York 10029

INTRODUCTION

Most studies of biological parameters focus on the average values of these parameters, treating their dispersion or variability as error relevant only to establishing the location of the “true” average. Yet this variability often has real existence and functional significance. This becomes especially clear as one rises up the levels of organization toward behavior. In a cyclical, rhythmic behavior, for example, each cycle represents a real investment of time and energy by the animal. Each cycle is shaped by a particular set of parameter values—amplitude, speed, and so on—that, when the cycle is measured against the goal of the behavior, determine the functional success or failure of that cycle. Then the degree to which the parameter values may differ from cycle to cycle becomes very significant, both to the animal itself and to us when we analyze its behavior.

Here we quantify and analyze the variability in the cyclical consummatory feeding behavior of Aplysia. This behavior comprises subtypes such as biting, swallowing, and rejection of unsuitable food, all driven by the same central pattern generator (CPG) and performed with the same neuromuscular plant. Since its first description by Kupfermann (1974), extensive studies have revealed much about the behavior and the neuromuscular mechanisms that produce it (reviewed by Chase 2002; Elliott and Susswein 2002; Hooper et al. 1999; Kupfermann et al. 1997). However, its variability has not been systematically studied.

The previous work in the system, indeed, leads to two differing expectations as to the variability. On the one hand, the neuromuscular system contains mechanisms—in particular, numerous endogenous neuromodulators—that tune its operating parameters for optimal functional performance. Furthermore, the tuning differs in the different subtypes of feeding behavior (Brezina and Weiss 2000; Brezina et al. 1996, 2000a,b; Hooper et al. 1999). This might suggest that the operating parameters are constrained to a narrow range of values in each subtype, so that if we examine successive cycles of a particular subtype, performed under the same circumstances, we will find them all to be very similar. On the other hand, many of the modulatory tuning processes, as well as basal processes in the neuromuscular transform—the transform of motor neuron spike patterns to muscle contractions—and in the CPG, have slow dynamics (Brezina et al. 2000a, 2003a). The history dependency of these processes might then be expected to produce cycles which, with different histories, are different from one another. Indeed this is predicted by mathematical models of the modulation in the system (Brezina et al. 2003a, b).

If there is variability, it might not be random, but might have a distinct spatiotemporal structure in the multidimensional space of the various parameters. This structure, in turn, might help us identify something that is at present unknown in this or...
indeed most behaviors, that is, the higher-order variables that the nervous system is attempting to control to make the behavior successful (see, e.g., Scholz and Schöner 1999; Todorov and Jordan 2002).

In this paper we therefore study the variability of the *Aplysia* feeding behavior. We begin with reduced preparations that, although lacking the full mechanical and sensory integrity of the intact animal, allow access to several internal levels of the neuromuscular system. We are able to record feeding-related neuromuscular activity simultaneously at three levels—the overall cycling of the CPG, motor neuron firing, and muscle contraction. We then use chronically implanted electrodes to extend our findings to intact animals fed in a semicontrolled manner or engaged in spontaneous feeding. Finally we return to the reduced preparations where we can perturb aspects of the neuromuscular function for a more analytical examination.

In connection with this order of approach, we emphasize another aspect of our strategy in this paper. In the intact animal, behavior emerges from an interaction between the nervous system, body, and environment (Chiel and Beer 1997). In such a coupled system, there are likely to be multiple layers of variability (Beer et al. 1999) that will be very difficult to separate. In particular, in a behavior that, in its several subtypes and parametric adaptability, is as responsive to environmental demands as *Aplysia* feeding behavior, it is difficult to be sure that the observed variability is not being driven simply by variations in the environment. For this reason, we focus here on experimental paradigms that attempt to keep the external stimuli to the system as constant as possible. This is of course easier to accomplish in the reduced preparations without sensory feedback. For the same reason, we pay relatively little attention to conventional global measures of variability that lump together many, possibly heterogeneous, cycles. Rather, we concentrate on a local measure, the pairwise differences between the successive cycles in a sequence, which provide, in essence, their own internal control. Remarkably, we find that, even with a constant stimulus in the reduced preparations that reveal in an unmediated manner the operation of the CPG, successive cycles are very different. Under these controlled conditions, this fact of large central variability can be established relatively unambiguously. The central variability can then be followed as it emerges in the overall neuromuscular variability in the intact, freely behaving animal.

**METHODS**

*Esophageal motor programs in a reduced buccal–accessory radula closer neuromuscular preparation*

**EXPERIMENTS.** The preparation was the standard preparation used for recording motor neuron-elicited contractions of the ARC (accessory radula closer, or IS) muscle (e.g., Cohen et al. 1978; Orekhova et al. 2003; Weiss et al. 1979), here combined with extracellular nerve recording and stimulation. Briefly, the preparation consisted of the bilateral buccal ganglia, the ARC muscles, and the connecting buccal nerves 3 through which the buccal motor neurons B15 and B16 innervate the ARC muscle. The cerebral ganglion, connected to the buccal ganglia by the cerebral–buccal connectives, was also retained. The buccal ganglia (but not the cerebral ganglion) were desheathed. One ARC muscle was pinned out in a separate subchamber and connected to an isotonic transducer (Model 60-3000, Harvard Apparatus, Holliston, MA) to measure the length of the muscle with a light counterbalancing load. The ipsilateral motor neurons B15 and B16 were impaled with standard intracellular microelectrodes and their membrane voltage was monitored with an intracellular amplifier (Axoclamp 2A/B, Axon Instruments, Union City, CA). Electrical activity in three buccal nerves, i.e., the I2 nerve, the ipsilateral buccal nerve 2, and the radula nerve (for nomenclature see Cohen et al. 1978; Hurwitz et al. 1994), was recorded differentially through suction electrodes connected to an extracellular amplifier (Differential AC Amplifier Model 1700, A-M Systems, Carlsborg, WA). (The radula nerve records are not used in this paper.) The extracellular amplifier, under the control of a separate stimulator (Grass S48/S88, Astro-Med, West Warwick, RI), was also used to stimulate the ipsilateral esophageal nerve. All signals were sampled and recorded simultaneously by a computer using Digidata 1322A data-acquisition hardware and pCLAMP 8.0 software (Axon Instruments). Most experiments were done at 15–16°C, although some were done at room temperature with no obvious difference.

Two protocols of esophageal nerve stimulation were used. In the “continuous” protocol (e.g., Fig. 1), the stimulation consisted of a long train of regular voltage pulses with parameters adjusted so as to elicit identifiable motor programs (see following text) at moderately frequent intervals while the stimulation continued. The individual voltage pulses were typically 7–10 V in amplitude, 7–15 ms in duration, delivered at 2–3 Hz. Blocks of stimulation lasting 3 min (with parameters constant within each block), separated by 7-min rest periods, were repeated as long as the motor programs continued to be elicited. In the “discontinuous” protocol (e.g., Fig. 14), the regular voltage pulses were delivered to the esophageal nerve in more intense but short bursts, at 4–8 Hz for 3.5–8.5 s repeated every 60–100 s. These parameters, and those of the individual voltage pulses, were adjusted for each preparation so that each burst of stimulation was reliably followed by one, and only one, complete motor program. When the programs began to fail, the block of stimulation was terminated. However, after readjustment of the stimulation parameters and a prolonged (>10 min) rest, another block of reliable motor programs could often be recorded in the same preparation.

**DATA ANALYSIS.** Motor programs were identified by the presence of a characteristic coordinated pattern of electrical activity in the I2 nerve and buccal nerve 2, that is, first a burst of at least moderately intense activity in the I2 nerve, then, beginning very soon after the end of the I2 nerve burst, a characteristically shaped burst of intense activity in buccal nerve 2 (see Figs. 1 and 14; for previous use of these criteria see, e.g., Hurwitz et al. 1996; Jing and Weiss 2001; Morgan et al. 2002; Morton and Chiel 1993a). The protraction phase of the program was defined to be coincident with the I2 nerve burst; the retraction phase was defined as lasting from the end of the I2 nerve burst to the end of the buccal nerve 2 burst. Only those blocks were analyzed that had 4 or more identified motor programs, and at least some ARC muscle contraction. Altogether, 1,076 programs in 166 blocks from 28 preparations in the continuous stimulation protocol, and 856 programs in 37 blocks from 11 preparations in the discontinuous protocol, were accepted for further analysis.

Initial processing of the raw records was done in Clampfit (Axon Instruments). The programs were identified and the beginning and end times of their protraction and retraction phases were marked by eye. This appeared to be sufficiently reliable for the purposes of this paper, given that the bursts of I2 and buccal nerve 2 activity usually began and ended quite abruptly (e.g., Fig. 14). Motor neuron B15 and B16 spike times were automatically tabulated using the threshold event detection module of Clampfit 9.

Subsequent processing of the data, its collation across multiple blocks and preparations, and most statistical analysis was done in Mathematica (Wolfram Research, Champaign, IL). (The Kolmogorov–Smirnov test was run in Clampfit 9.) For some analyses, the motor neuron B15 and B16 spike times were converted to instantaneous firing frequency functions, assigning to each time point in an interspike interval the reciprocal of the duration of that interspike interval. The instantaneous ARC muscle contraction amplitude during...
any program was defined as \( \frac{\text{instantaneous muscle length}}{\text{muscle length at the beginning of the program}} \); thus contraction of the muscle is positive in sign. Because different preparations, with different muscle sizes and somewhat different loads, could differ significantly in their contraction amplitudes, all contraction amplitudes were normalized by the maximal amplitude reached in that particular preparation. Other measured variables were initially not normalized, although for some analyses all variables were secondarily normalized by the mean in each stimulation block. Further details of the analysis are given in RESULTS and in the figure legends.

Data analysis. The same methods and criteria were used as with the buccal-ARC data, except that, because the interprogram interval preceding the program in each cycle (see Fig. 1) was also analyzed, the first program in each block, for which this interval is not well defined, was excluded from the analysis. Altogether, 741 programs in 94 blocks from 12 preparations were accepted for further analysis. The instantaneous opener muscle contraction amplitude during any cycle was defined as \( \frac{\text{instantaneous muscle length}}{\text{muscle length at the beginning of the interprogram interval}} \).

In vivo nerve activity during ingestion of seaweed strips

Experiments. Chronic electrodes were implanted en passant on the radula nerve and buccal nerve 2 as described by Horn et al. (1999) and

**Figure 1.** Typical 3-min block of motor programs elicited in the reduced buccal-accessory radula closer (ARC) neuromuscular preparation by the “continuous” protocol of esophageal nerve stimulation. Simultaneous recording of (top to bottom) ARC muscle length, membrane voltage of the ARC motor neurons B15 and B16 (recorded intracellularly), and electrical activity in the I2 nerve and buccal nerve 2 (recorded extracellularly). The 3-min period of esophageal nerve stimulation is indicated at the bottom. Bursts of activity in the I2 nerve and buccal nerve 2, respectively, were taken to define the protraction and retraction phases of each program (gray rectangles; see METHODS).
and the neuronal frequency functions. Because both nerves contain multiple units merely of the aggregate activity of each nerve. cellular record, these frequencies must be regarded as measures of the minimal threshold were automatically tabulated using the threshold detection module of Clampfit (only the radula nerve was analyzed in this data set) were identified as continuous sequences of ≥5 events with interevent intervals of <1 s. After identification of the bursts, the aggregate activity within them was computed from the times of events of all sizes as above. Altogether, 1,449 bursts were identified from one animal.

**In vivo ARC muscle activity during spontaneous feeding**

**EXPERIMENTS.** A chronic electrode was implanted in the ARC muscle and recordings were made as above (see also Cropper et al. 1990; Evans et al. 1996). Continuous recordings were made as the food-deprived animals fed spontaneously on seaweed (*Gracilaria*) introduced into the tank.

**DATA ANALYSIS.** Similar methods were used as above. Bursts of electrical activity in the muscle were identified automatically; bursts were defined as continuous sequences of ≥10 large events with interevent intervals of <1 s. Altogether, 5,791 bursts were identified from 4 animals.

**Motor neuron-elicited ARC muscle contractions in the reduced buccal-ARC preparation**

**EXPERIMENTS.** These experiments used the standard buccal-ARC neuromuscular preparation without extracellular nerve recording or stimulation. Some of the experiments were new, but a large part of the data set was obtained by realanalysis of control runs from previous studies that used this preparation (e.g., Brezina et al. 1996, 2000a,b; Orekhova et al. 2003). Either motor neuron B15 or B16 was intracellularly stimulated with repetitive brief current injections to fire bursts of spikes such that each burst elicited an ARC muscle contraction of moderate amplitude. B15 was typically fired at 10–13 Hz, B16 at 15–25 Hz, for 1 or 1.5 s every 20 or 30 s; within each experiment, the pattern of spikes was completely regular and invariant from burst to burst. The results of interest were indistinguishable for the two motor neurons and have been pooled.

**DATA ANALYSIS.** Similar methods were used as above. Because the muscle contraction lagged considerably behind and outlasted the motor neuron burst, the mean contraction amplitude was evaluated over a standard 5-s interval starting with the first spike of the burst. This interval included segments of the contraction baseline, but this did not matter because we focused primarily on the cycle-to-cycle variability. The mean contraction amplitude was normalized by the maximal amplitude reached in that particular preparation. In total, 2,762 contractions were analyzed from 28 preparations.

**VARIABILITY IN APLYSIA FEEDING BEHAVIOR**

**DATA ANALYSIS.** Similar methods were used as above, except that the beginning and end times of the bursts of radula nerve activity (only the radula nerve was analyzed in this data set) were identified automatically. The identification used the multimodal distribution of the interevent intervals of the largest units in the nerve, presumably the neurons B8. Based on this distribution, bursts were defined as continuous sequences of ≥5 events with interevent intervals of <1 s. After identification of the bursts, the aggregate activity within them was computed from the times of events of all sizes as above. Altogether, 1,449 bursts were identified from one animal.

**In vivo nerve activity during spontaneous feeding**

**EXPERIMENTS.** Chronic electrodes were implanted on the radula nerve and buccal nerve 2 and recordings were made as above. In these experiments continuous recordings were made as the food-deprived animals fed spontaneously on seaweed (*Gracilaria*) introduced into the tank.

**DATA ANALYSIS.** Similar methods were used as above. Because the muscle contraction lagged considerably behind and outlasted the motor neuron burst, the mean contraction amplitude was evaluated over a standard 5-s interval starting with the first spike of the burst. This interval included segments of the contraction baseline, but this did not matter because we focused primarily on the cycle-to-cycle variability. The mean contraction amplitude was normalized by the maximal amplitude reached in that particular preparation. In total, 2,762 contractions were analyzed from 28 preparations.

**FIG. 2.** Variability in the esophageal motor programs: temporal profiles of motor neuron B15 and B16 firing (A, B) and ARC muscle contraction (C). Waveforms of instantaneous firing frequency of the motor neurons B15 and B16 and normalized ARC muscle contraction amplitude were computed (see METHODS) for all programs in the data set (n = 1,076) and aligned at their protraction–retraction boundaries (vertical line at time = 0 in the left column of plots); the 10th, 25th, 50th, 75th, and 90th percentiles of the resulting ensemble distribution were then determined at each time point. These percentiles, plotted over all of the time points, are shown in the left column. Before the start of protraction and after the end of retraction of each individual program, its waveforms were assigned a nominal negative value. As a result, each ensemble percentile waveform becomes negative, and so appears to end in these plots, at the corresponding percentile of the distribution of protraction or retraction durations, explicitly shown by the gray bar at the bottom. Right column of plots compares the areas (for the firing frequencies, equivalent to numbers of spikes) under the 10th, 25th, 50th, 75th, and 90th percentile waveforms of the ensemble with the percentile distributions of the areas under the 1,076 individual program waveforms.

**J Neurophysiol • VOL 92 • JULY 2004 • www.jn.org**
RESULTS

Esophageal motor programs in a reduced buccal-ARC neuromuscular preparation

The reduced preparations used in this work consisted of the buccal ganglia, which contain the feeding CPG, still connected to parts of the buccal mass, the complex muscular organ that moves the food-grasping structure, the radula (see Elliott and Susswein 2002; Kupfermann 1974). The CPG generates cyclical, rhythmic motor programs that can be recorded intracellularly in the firing of many individual CPG interneurons and buccal motor neurons as well as extracellularly in the activity of nerves that project to the buccal-mass musculature (Fig. 1). In this work, following established practice (e.g., Hurwitz et al. 1996; Jing and Weiss 2001; Morgan et al. 2002; Morton and Chiel 1993a), we used the activity of two nerves, the I2 nerve and buccal nerve 2, respectively as markers of the two major phases of each program that, in the intact animal, produce protraction and then immediately retraction of the radula. We define a “motor program” as one unit of these two phases, and a complete “cycle” as a motor program together with the preceding interprogram interval (see Fig. 1). Simultaneously, in the buccal-ARC preparation, we recorded the firing of two buccal motor neurons, B15 and B16, and contractions of the muscle that these neurons innervate, the accessory radula closer (ARC, or I5) muscle (Cohen et al. 1978). The ARC is routinely studied as a representative buccal-mass muscle (Brezina et al. 2003a; Hooper et al. 1999).

To elicit the programs, again following previous work (e.g., Chiel et al. 1986; Morgan et al. 2002), we electrically stimulated another of the buccal nerves, the esophageal nerve. In our standard “continuous” protocol (see METHODS), we stimulated the nerve with brief shocks delivered at a moderate frequency (2–3 Hz) continuously for 3 min. Figure 1 shows one such “block” of stimulation. As can be seen, multiple motor programs were typically elicited. After a rest period of 7 min,

![FIG. 3. Variability in the esophageal motor programs: distributions of absolute values of principal program (A) and neuromuscular activity (B) parameters. All programs in the data set (n = 1,076) were measured for protraction duration, retraction duration, program duration (= protraction duration + retraction duration), interprogram interval (the interval before a program; thus the first program in each 3-min block did not yield an interprogram interval, or a cycle period), cycle period (= program duration + interprogram interval), the mean firing frequencies of the motor neurons B15 and B16 in protraction and retraction, and the mean normalized ARC muscle contraction amplitude in protraction and retraction. Plotted here are the distributions of these 11 parameters, scaled so as to approximate probability density functions. Thin vertical lines indicate the 10th, 25th, 50th, 75th, and 90th percentiles of each distribution (where the lower percentiles appear to be missing, they are compressed against the left side of the plot). In most of the plots, the last bar contains pooled larger values (small right-pointing arrow); in some cases the first bar, containing zero values, extends off the top of the plot (small upward arrow).]
another block was recorded. Altogether, we collected 1,076 programs in 166 blocks (~6.5 programs/block on average), satisfying our criteria (see METHODS) for further analysis.

Variability of the esophageal motor programs

Although the esophageal nerve stimulation was perfectly regular and continuous throughout the 3-min block, the elicited motor programs were not at all regular. As Fig. 1 shows, they varied in all respects—in their timing, their intensities of nerve activity, their motor neuron firing frequencies and patterns, and the sizes and shapes of their muscle contractions. We used several complementary approaches to quantify and analyze this variability.

The downstream levels of the neuromuscular system, the muscle contractions and, ultimately, functional movement, integrate in a complex way multiple upstream parameters (Brezina et al. 1997, 2000a). The contractions of the ARC muscle, for example, depend on the overall firing frequencies of both motor neurons B15 and B16, on the detailed pattern of the firing of each neuron and the mutual relationship of the two patterns, and on the duration of the firing, determined by the duration of the protraction and retraction phases generated by the CPG. To simultaneously visualize the variability in all these parameters, we examined the variability of entire waveforms. We collected together the waveforms of the instantaneous firing frequencies of B15 and B16 and of the ARC contraction amplitude over the duration of the protraction and retraction phases of all 1,076 programs in the data set, aligned at their protraction–retraction boundaries. In Fig. 2, left column, we have then plotted, at each time point relative to this boundary (vertical line), the 10th, 25th, 50th (median), 75th, and 90th percentile values across the entire ensemble of waveforms. The corresponding values at successive time points constitute the 10th, 25th, 50th, 75th, and 90th percentile values seen. The time at which each percentile waveform first rises above zero marks the corresponding percentile of the distribution of protraction durations; where it eventually falls.

**FIG. 4.** Variability in the esophageal motor programs: distributions of cycle-to-cycle differences of the principal program (A) and neuromuscular activity (B) parameters. This figure is constructed like Fig. 3 and the same data set was used, except that here the parameters were further processed to show the distributions of differences between successive programs in each 3-min block of esophageal nerve stimulation. Differences in each block were “block-normalized” (see Variability of the esophageal motor programs in RESULTS), so that −1 and 1 on the horizontal scale indicate decreases and increases, respectively, equal in magnitude to the mean of the parameter. All distributions have been scaled so as to approximate probability density functions and are plotted on the same vertical, as well as horizontal, scale. Thin vertical lines indicate the 10th, 25th, 50th, 75th, and 90th percentiles of each distribution (where some percentiles are missing, they are located off the edges of the plot). SD of each distribution, σ, is given. In all plots, the first bar contains pooled smaller values (small left-pointing arrow) and the last bar contains pooled larger values (small right-pointing arrow); in some cases, bars extend off the top of the plot (small upward arrows).
below zero, of retraction durations (the phase duration percentiles are explicitly indicated by the gray bar at the bottom). Clearly, there is a many-fold difference in the motor neuron firing frequencies and muscle contraction amplitude, at any particular time and overall, as well as in the protraction and retraction durations, between the smallest and the largest programs in the data set.

These plots somewhat exaggerate the variability, however, because the percentiles were computed independently at each time point. Only with perfect correlation between adjacent time points and among all program parameters would the data set actually contain, for instance, a 50th-percentile—median—individual program waveform identical to the median waveform of the ensemble seen in Fig. 2, left. The programs certainly did not have such perfect correlation (see following text). Without such correlation, the waveforms of individual small or large programs would be expected, on statistical grounds, to be less extreme than the corresponding waveforms of the ensemble. To examine this, in Fig. 2, right column, we have compared the cumulative percentile distribution of the ensemble with that of the individual programs, plotting on the vertical axis in each case a single overall parameter, the total area under the waveform (for the firing frequencies, equivalent to the total number of B15 or B16 spikes fired during the program). Although over the middle range of percentiles the individual programs indeed vary less steeply than does the ensemble, there is still a several-fold difference between, say, the 10th- and 90th-percentile individual programs.
The plots in Fig. 2 show well the simultaneous multidimensional variability of entire waveforms, but they do not lend themselves easily to further statistical treatment. A different quantification of the variability in the data set is therefore presented in Fig. 3. Here we have simply plotted, one by one, the distributions of values of 11 principal parameters of the overall cycle-to-cycles and phasing of the programs (Fig. 3a) and the firing of the motor neurons B15 and B16 and contractions of the ARC muscle (Fig. 3b) measured from all 1,076 programs in the data set. We will refer to such values measured from individual programs as “absolute” values, in contrast to the relative cycle-to-cycle differences between programs introduced below. The 10th, 25th, 50th (median), 75th, and 90th percentiles of each distribution are marked by the thin vertical lines. Again, the bulk of each distribution is very broad, and there is furthermore in most cases a long right-hand-tail, extending even offscale, in which the values are many times those at the left-hand-end of the distribution.

These absolute-value distributions give a good idea of the overall extent of the variability across all preparations, under all conditions. However, these distributions are so broad partly because they mix together at least four different kinds of variability: 1) variability between preparations; 2) variability between the blocks of programs, in particular systematic trends over successive blocks in the same preparation; 3) systematic trends over successive programs in the same block; and 4) “random” variability between individual programs. Variability of types 1 and 2 was least interesting to us here: variability between preparations could simply reflect, for instance, their different sizes, and trends over successive blocks conditions such as cumulative fatigue. To eliminate variability of these two types, we normalized the absolute parameter values by the mean of the values in each block, producing “block-normalized” distributions that were somewhat less broad than those in Fig. 3. We do not show these distributions because they represented merely an intermediate stage in our analysis—they still combined the two most interesting types of variability, types 3 and 4.

To quantify the “random” variability of type 4 alone, we examined pairwise cycle-to-cycle differences between successive programs in the same block. In Fig. 4 we have plotted the distributions of these differences for the same 11 parameters as in Fig. 3. The differences were computed from the block-normalized parameter values, so that −1 and 1 on the horizontal scale indicate decreases and increases, respectively, from one program to the next that are equal in magnitude to the mean of the parameter. The distributions are roughly symmetrical around zero, showing that any systematic trends of type 3 have been for the most part eliminated. Each distribution has a distinct central peak, but this peak is still quite broad, and there are still long tails indicating some very large differences between successive programs. The plots in Fig. 4 also give the SD (σ) of each distribution. With the block-normalized values used, σ is a dimensionless measure similar to the coefficient of variation that is often used to quantify variability (see, e.g., Shadlen and Newsome 1998; Stevens and Zador 1998; Zoccolan et al. 2002). As can be seen, σ is of the order of 0.3–0.6 for many of the parameters, but 1 or more for some parameters, in particular those of the ARC muscle contraction.

**Systematic trends in the esophageal motor programs**

Having thus isolated the “random” variability of type 4, we could return to the variability of type 3—systematic trends over successive programs in the same block. One indication that such trends existed was that the cycle-to-cycle differences in
Fig. 4 were distributed differently than expected if they had originated by purely random assortment of the absolute parameter values in Fig. 3. Figure 5A shows this formally for two representative parameters: the interprogram interval and the mean firing frequency of motor neuron B16 in protraction. We performed, essentially, a Monte Carlo simulation to construct the distributions of cycle-to-cycle differences expected from pairs of values resampled at random from the absolute-value distributions in Fig. 3 (thin continuous curves in Fig. 5A) or, more important, from the block-normalized versions of those distributions (thick continuous curves). The actual distributions of cycle-to-cycle differences, reproduced from Fig. 4, are shown by the gray histograms. The actual distributions clearly have a narrower central peak than the random expected distributions, reflected in a generally smaller value of $\sigma$. Yet in some cases—for instance, that of the interprogram interval—the actual distribution also had longer tails, leaving $\sigma$ unchanged. The single-dimensional parameter $\sigma$ was thus inadequate to capture the more complex differences in the shapes of the distributions.

**FIG. 6.** Correlations between parameters of the esophageal motor programs: three examples. A: correlation between absolute values, as well as cycle-to-cycle differences, of the interprogram interval and the cycle period. B: correlation between absolute values, but not cycle-to-cycle differences, of the interprogram interval and the mean firing frequency of B16 in protraction. C: no correlation between either absolute values or cycle-to-cycle differences of the protraction duration and the retraction duration. All values were block-normalized. Nonlinear least-squares Marquardt–Levenberg regression was used to fit a cubic polynomial; where any correlation was found, the fit is shown. Coefficient of determination, $R^2$, is given.
To demonstrate statistically significant differences between the distributions, we used two complementary approaches. First, we compared the entire distributions using the Kolmogorov–Smirnov test, a test that is very sensitive to cumulative differences in the shapes of distributions (e.g., Hoel 1971; Press et al. 2002). The Kolmogorov–Smirnov test found a significant difference ($P < 10^{-10}$) between the actual distribution and either random expected distribution for each of the two parameters. Then, to determine which parts of the distribution were different, we constructed and compared binwise 95% confidence intervals around each distribution (thin vertical lines in Fig. 5A; see legend for details). The asterisks (*) at the top of each plot in Fig. 5A mark where the actual distribution is significantly greater than the block-normalized random expected distribution, the plus signs (+) the converse.

The generally narrower shape of the actual distributions indicated that successive programs were more similar than if they had been completely independent of one another. Although other explanations are possible, this could be if on average—under the overlay of the large “random” variability—the programs progressively evolved throughout the block. To visualize these trends directly, for each of the 11 parameters, we examined the location of the distribution of the subset of all first, second, third, and so forth, programs from each block, within the entire distribution of the block-normalized absolute values from all programs. Plotted in Fig. 5B are the medians of the program subdistributions expressed as percentiles of the entire distribution. If the subdistributions were randomly sampled from the entire distribution, the program medians should be centered around the median of the entire distribution, the line marked “Random sampling.” Instead, the medians of most of the parameters, including the two studied in Fig. 5A (“4—interprogram interval” and “8—B16 in protrac- tion”), clearly trend systematically from smaller to larger values as the programs proceed. Others, conversely, trend from larger to smaller values (“9—B16 in retraction”).

**Structure of the variability of the esophageal motor programs**

Figure 5B reveals coherent structure in the parameter space of the motor programs. Yet this structure is not informative in the way we would wish, because there are reasons to believe that the trends that give rise to the structure represent evolution of the type of program, from a relatively ingestive (biting- or swallowing-like) to a more egestive (rejection-like) type (see DISCUSSION). In the intact animal, such trends would be thought of as reflecting a progressive change in functional goal, rather than different ways of reaching the same goal. Can we find structure even when the goal remains the same—when the progressive trends are eliminated, in the “random” variability of the cycle-to-cycle differences?

We examined, as a first attempt at the problem, simply pairwise correlations between different parameters. These correlations fell into three classes, examples of which are shown in Fig. 6, A, B, and C, respectively. As expected, there were strong correlations, both in the absolute values and in the cycle-to-cycle differences, where one parameter was an intrinsic part of the other, as for example the interprogram interval was part of the cycle period (Fig. 6A). More interestingly, there were correlations, albeit considerably weaker ones, between the absolute values of some parameters that were not intrinsi-
cally linked, such as the two studied in Fig. 5, the interprogram interval and the firing of B16 in protraction (Fig. 6B, left). These correlations were a reflection of the trends found in Fig. 5B. When the trends were eliminated by focusing on the cycle-to-cycle differences, the correlations disappeared (Fig. 6B, right). Finally, there were no correlations at all, either in the absolute values or in the cycle-to-cycle differences, between still other parameters, such as the protraction duration and the retraction duration (Fig. 6C).

More sophisticated methods that consider the relationships between all parameters simultaneously, such as principal-components analysis or multidimensional scaling (see, e.g., Hand et al. 2001), might yet find some structure in the cycle-to-cycle variability. However, the strong suggestion of these results is that, when the next program is to be generated by the system, the “choice” of any one of its parameters does not depend on the choice of any other parameter. In this sense the variability is truly random.

**Variability of esophageal motor programs in a reduced buccal-opener preparation**

The ARC muscle closes the radula (Cohen et al. 1978; Orekhova et al. 2001), but it is not the only muscle that does this. The radula is also closed by the firing of motor neurons B8, probably through contraction of the I4 muscles (Morton and Chiel 1993b; Orekhova et al. 2001). There is therefore a degree of degeneracy in the mapping of muscle contractions to movements (see, e.g., Beer et al. 1999; and DISCUSSION): potentially similar closing movements of the radula can be accomplished by contractions of the ARC muscle, the I4 muscle, or combinations of both. The constraint on the ARC muscle to contract in the same way in each cycle may consequently be reduced. Could this account for the particularly large variability seen in the ARC contractions in Figs. 3 and 4?

To help address this question, we performed another set of experiments, essentially identical to those described so far but, instead of B15 and B16 and the ARC muscle, recording the firing of motor neuron B48 and the contractions of the radula opener muscle complex I7–I10 (Evans et al. 1996). The I7–I10 complex is a key muscle that opens the radula and thus would definitely be expected to participate in each functional cycle. The results are shown in Fig. 7. Figure 7A shows part of a typical 3-min block of esophageal motor programs, and Fig. 7B shows the distributions of the cycle-to-cycle variability of the firing frequency of B48 and the contractions of the opener muscle, drawn from the 741 cycles analyzed from these experiments and presented as in Fig. 4. Because B48 fired and the opener muscle contracted strongly in the interprogram interval and in protraction, but not in retraction (Fig. 7A), the cycle-to-cycle distributions are shown for the interprogram interval and protraction. As can be seen, the variability in Fig. 7B is large, essentially no different from what it was in Fig. 4B for the firing of B15 and B16 and contractions of the ARC muscle.

**Variability of neuromuscular activity during feeding in intact animals**

The large variability described so far was found in reduced preparations lacking some control mechanisms, such as sen-

![FIG. 8. Typical in vivo recording of buccal nerve 2 and radula nerve activity during ingestion of a 10-cm strip of seaweed, which was presented to the animal just before the beginning of the segment shown, and was entirely ingested by the end. Small black rectangles mark the approximate times of observed swallows. Expanded records at the bottom show one of the motor programs, defined by coordinated bursts of activity in the two nerves (gray rectangles; see METHODS).](http://jn.physiology.org/)

---

168 HORN ET AL.

*J Neurophysiol* • VOL 92 • JULY 2004 • www.jn.org
sory feedback loops, that might increase variability, but might also conceivably reduce variability. Is there large variability also in intact animals, during normal feeding behavior? To answer this question, we performed three sets of in vivo experiments in which we used chronically implanted electrodes to record the activity of buccal nerve 2 and of the radula nerve (another of the buccal nerves), or the electrical activity of the ARC muscle, while the animals were fed in a semicontrolled manner or engaged in spontaneous feeding.

First, we fed the animals with standard strips of seaweed, each 10 cm long, long enough that the animal, having grasped one end, required multiple swallowing cycles to ingest the entire strip. Figure 8 shows a typical recording from buccal nerve 2 and the radula nerve during the ingestion of one complete strip. The small black rectangles at the top mark the times when actual functional swallowing—inward movement of the strip—was observed. For analysis, we collected complete recordings from 153 strips, comprising 1,388 cycles; thus on average, the animals required approximately 9 cycles to swallow each 10-cm strip.

It can plausibly be argued that, after the initial bite-swallow (which was excluded from the analysis; see METHODS), each swallow during the ingestion of a strip was performed under identical conditions; as in the case of the esophageal nerve stimulation, the half-swallowed strip represented a continuous, regular stimulus. Yet, as Fig. 8 shows, the cycles were again very variable. In Fig. 9 we have quantified this variability in similar ways as for the esophageal motor programs. Figure 9A shows the 10th, 25th, 50th (median), 75th, and 90th percentile waveforms of the ensemble of all program waveforms—in this case, of the bursts of buccal nerve 2 and radula nerve activity—in the data set. Figure 9B shows the absolute-value distribution of the cycle periods, and Fig. 9C the distributions of the cycle-to-cycle differences of the cycle period as well as of the total activity during the burst in each nerve. Overall, the variability is about as large as it was for the esophageal motor programs.

Interestingly, the variability appeared to progressively decrease over successive cycles that were used to ingest each strip. Because this phenomenon was itself variable, it was not at all obvious with each individual strip (e.g., in Fig. 8). However, averaging over all strips clearly revealed the phenomenon. Figure 10 shows it for two representative parameters. In Fig. 10A we have partitioned the distributions of cycle-to-cycle differences of the total buccal nerve 2 and radula nerve activity shown in Fig. 9C into the subdistributions during programs 1–3, 4–6, 7–9, and so forth, during the ingestion of

![Diagram](http://jn.physiology.org/)

**FIG. 9.** Variability in the buccal nerve 2 and radula nerve activity during ingestion of seaweed strips. A: temporal profiles of the aggregate nerve activity (see METHODS), computed from all programs in the data set (n = 1,388) as in Fig. 2. Waveforms were aligned at the beginning of the burst of buccal nerve 2 activity. B: distribution of absolute values of the cycle period, as in Fig. 3. C: distributions of cycle-to-cycle differences of the cycle period and the total activity during the burst in each nerve (the area under the profile of aggregate activity through the entire burst), as in Fig. 4.
each seaweed strip. Figure 10B plots the SDs (σ) of these subdistributions as a function of program number. The subdistributions become noticeably narrower with increasing program number: their central peak grows while their tails diminish; the vertical lines marking the 10th, 25th, 50th, 75th, and 90th percentiles come closer together, and σ progressively decreases. For each of the two parameters, the final subdistribution of programs 16–18 differs significantly (P < 10^-9 by the Kolmogorov–Smirnov test) from the starting subdistribution of programs 1–3 (the black histogram outlines repeat the latter at the bottom of Fig. 10A for comparison). Note that, because we have here analyzed cycle-to-cycle differences rather than absolute values, the trend seen here reflects not a progressive migration of the location of the average parameter value as in Fig. 5B, but rather a progressive tightening of the spread of its variability.

In another set of experiments, we similarly recorded nerve activity but allowed the animals to feed spontaneously on seaweed introduced into the tank. Figure 11A shows a typical recording, illustrating well the “fractal” temporal structure of free feeding, with bursts of activity grouped on multiple time scales. Figure 13 presents the variability of the 5,791 cycles in this data set. The variability is again large. The variability of the cycle period, in particular, is even larger than in the previous data sets. Because in these experiments the animals were free to feed, as they naturally do, in bursts—bursts of cycles—interrupted by periods of inactivity (see Fig. 12), the distribution of cycle periods has a very long right-hand tail, corresponding to the long interbout intervals (Fig. 11C). The distribution of cycle-to-cycle differences of the cycle period likewise has very long tails, for a very large total SD (σ_total) of 1.92 (Fig. 11D, left). Removing just the largest 10% and the smallest 10% of the distribution—presumably removing selectively the interbout intervals—leaves a SD (σ_central) of 0.33, similar to the values in the previous data sets.

In a third set of experiments we recorded the electrical activity of the ARC muscle during spontaneous feeding. Figure 12 shows a typical recording, illustrating well the “fractal” temporal structure of free feeding, with bursts of activity grouped on multiple time scales. Figure 13 presents the variability of the 5,791 cycles in this data set. The variability closely resembles that found in Fig. 11 for the nerve activity during free feeding and is, again, very large.

Regularization of the timing of esophageal motor programs reduces their variability

Broadly, two kinds of mechanisms might explain the large cycle-to-cycle variability found in the system. The variability appears to be random across the parameters of a particular cycle (Fig. 6), and it might be random—after the systematic trends in Fig. 5B have been eliminated—in any one parameter across successive cycles as well. It would then amount to truly random, spontaneous, uncontrollable noise in the system. Alternatively, like the systematic trends, the cycle-to-cycle variability might depend, although perhaps in a much more complex way, on the previous history of activity in the system.

To test for the second mechanism, we should alter the history of the system. In intact animals we cannot do so because we have no control over the production of the motor programs. In a reduced preparation, however, we can exert a considerable degree of control over their timing. We carried out a second series of experiments in the reduced buccal-ARC preparation, identical to the first series except using “discontinuous” esophageal nerve stimulation (see METHODS), essentially more intense but brief bursts of stimulation, each of which reliably elicited one, and only one, motor program. By repeating the bursts of stimulation at regular intervals we were able to elicit the programs at quite regular intervals (not completely regular because the latency from the stimulation to the beginning of the program, as well as the duration of the program, still varied somewhat). Figure 14 shows 4 typical programs from such an experiment; altogether, we collected 856 programs. In Fig. 15 we have quantified the cycle-to-cycle variability of the 11 principal parameters of these regular programs [gray histograms, black percentile lines, SDs (σ) in black], compared to the variability of the irregular programs reproduced from Fig. 4 (red histogram outlines, σ in red).

Clearly, the variability is substantially reduced in the regular programs (compare the shapes of the distributions, the values of σ, and note the symbols at the top of each plot, indicating statistically significant differences between the distributions; furthermore P < 10^-10 in each case by the Kolmogorov–Smirnov test). The regular programs are, of course, much less variable in parameters such as the cycle period, which we most directly control. However, they are also considerably less variable in timing parameters that we do not directly control, such as the protraction and retraction phase durations, and even, remarkably, in parameters that intrinsically have little to do with timing, such as the firing frequencies of the motor neurons B15 and B16. Perhaps least regularized, interestingly, are the contractions of the ARC muscle, but even here P < 10^-10 by the Kolmogorov–Smirnov test.
The neuromuscular transform has little intrinsic cycle-to-cycle variability

One possible explanation for the persistent large variability of the muscle contractions is that the neuromuscular transform—the sum total of the peripheral processes that transform the motor neuron spike patterns into contractions (Brezina et al. 2000a)—itself has large intrinsic variability. That is, even completely identical, regular spike patterns might produce irregular, variable contractions, as has been found in other systems (e.g., Zoccolan et al. 2002). To examine this possibility, we regularized the firing of the motor neurons B15 and B16, stimulating either one by DC injection to fire in completely regular, repetitive bursts, each of which produced a contraction of the ARC muscle. Figure 16A shows a typical segment from such an experiment. In Fig. 16B we have quantified the cycle-to-cycle variability of the mean amplitude of 2,762 such contractions [gray histogram, black percentile lines, SD ($\sigma$) in black], contrasting it with the variability of the mean contraction amplitude in retraction during the irregular (red) and regular (blue) esophageal motor programs from Figs. 4 and 15. As
can be seen, the directly elicited contractions have much less variability—indeed, essentially no variability: completely regular firing of the motor neurons produces completely regular contractions of the muscle. The ARC neuromuscular transform thus appears to have very little intrinsic variability.

**DISCUSSION**

*Neuromuscular activity in Aplysia feeding behavior is highly variable.*

*Aplysia* feeding behavior is often said to be stereotyped. Certainly, it is qualitatively stereotyped, in that we can recognize the same characteristic pattern of events in the neuromuscular system during each cycle (e.g., Figs. 1 and 14), and different patterns in different types of feeding behavior such as biting, swallowing, and rejection (Church and Lloyd 1994; Cropper et al. 1990; Hurwitz et al. 1996; Jing and Weiss 2001; Morgan et al. 2002; Morton and Chiel 1993a). Quantitatively, however, one cycle of the behavior differs from another. This has long been anecdotally recognized, and it is apparent in various published records of motor programs in reduced preparations as well as in intact animals (e.g., Chiel et al. 1986; Church and Lloyd 1994; Kabotyanski et al. 2000; Morton and Chiel 1993a,b). However, until now the variability has not been the direct object of study.

Our basic finding here is that the variability is large. We have quantified it at several levels in the neuromuscular system—in the overall cycling and phasing of the motor programs by the CPG, in motor neuron firing, and in muscle contraction—in reduced preparations and in intact, normally feeding animals. Across all cycles in all preparations, neuromuscular activity varies many-fold (Figs. 2, 3, 9, A and B, 11, B and C, and 13, A and B). Even in the same preparation, from one cycle to the next, parameters of the activity often differ by half of their mean value (Figs. 4, 7B, 9C, 11D, and 13C), and in some...
cases substantially more. In particular, the muscle contractions, the final executors of behavior, are not less variable than the upstream neural activity, but even more variable (Figs. 4B, 7B, and 15B). In our experiments here with feeding animals, we recorded only electrical activity, not muscle contractions or the movements of the behavior. However, parameters such as the cycle period apply to all levels of the system. A similar degree of variability is moreover apparent in published records of the mechanics of buccal-mass movement (Drushel et al. 1997, 1998; Neustadter et al. 2002a,b; Weiss et al. 1986) and observations, where available, of the behavior itself (Hurwitz and Susswein 1992; Susswein et al. 1976, 1986).

From published records, we expect that substantial variability is present in other “stereotyped” behaviors of invertebrates and vertebrates (see Stein et al. 1997 for numerous examples). Variability of vertebrate limb movement, in particular, is well known and in some respects can serve as a model for us here.

Structure of the variability

Dissecting the variability, we have focused particularly on two components: 1) systematic trends over successive cycles and 2) apparently random cycle-to-cycle variability.

One reason for using the reduced preparations was that we could stimulate the esophageal nerve in a constant, regular manner, hoping to elicit each motor program under precisely the same conditions. However, the conditions could not be the same in one respect: the previous history of the stimulation and neuromuscular activity was different for each successive program. Recent studies of the Aplysia feeding CPG (Proekt and Weiss 2002, 2003; Proekt, Brezina, and Weiss, unpublished observations) have shown that the character of the motor programs generated by the CPG depends not just on the immediate eliciting stimulus, but also on the internal network state of the CPG, which changes slowly with the history of the stimulation. These changes in network state correspond to changes in the type of feeding behavior, from ingestive (biting-or swallowing-like) through intermediate to egestive (rejection-like). Thus repetitive esophageal nerve stimulation, after a period of rest as in each of our blocks here, elicits programs that change progressively from intermediate to egestive. Preliminary examination (Zhurov et al. 2003) suggests that this largely explains the trends in Fig. 5B. For instance, the firing of motor neuron B16 and the contractions of the ARC muscle shift progressively from retraction to protraction, as expected if

![Graph A: ARC muscle electrical activity](image1)

**A** ARC muscle electrical activity

- Aggregate firing frequency (Hz)
- Burst duration
- Time (s)

**B** Cycle period

- Probability density
- Cycle period (s)
- Total ARC muscle electrical activity in burst

- Normalized cycle-to-cycle difference

Given the nature of the stimulus and the variability observed in the data, it is reasonable to expect that the ARC muscle activity will exhibit both systematic trends and apparent randomness. The graphs illustrate this variability, with **A** showing the aggregate activity and **B** highlighting the cycle period and total activity in a burst. The **C** graph provides further insights into cycle-to-cycle differences and normalized values, emphasizing the central tendency and spread of the data.

**Fig. 13.** Variability in the ARC muscle activity during spontaneous feeding. A: temporal profiles of the aggregate activity computed from all bursts in the data set (n = 5,791). Because the activity often increased during the burst before ending relatively abruptly (e.g., Fig. 12, bottom; Cropper et al. 1990), the waveforms were aligned at the end of each burst. B: distribution of absolute values of the cycle period. C: distributions of cycle-to-cycle differences of the cycle period and the total activity in the burst. $\sigma_{\text{total}}$ is the SD of the entire distribution, $\sigma_{\text{central}}$ just of the central 90%, from the 10th to the 90th percentile.
the muscle is to close the radula around material during protraction so as to expel it from the mouth. Another radula closer motor neuron, B8, whose firing we recorded in the radula nerve during these programs but for simplicity have not included in this paper, exhibits a similar shift (cf. Morgan et al. 2002; Morton and Chiel 1993a,b; Proekt and Weiss 2002, 2003). A full analysis of the changing character of the esophageal motor programs in this data set will be presented elsewhere.

Superimposed on these average trends, indeed masking them in many individual blocks of stimulation (e.g., Fig. 1), is a large cycle-to-cycle variability. Despite the constant stimulus, and despite the fact that on average the network state of the CPG changes too slowly to differ much from one program to the next, successive programs can be very different. Unlike the trends which produce a coherent structure and correlations in the parameter space (Figs. 5B and 6, left), the cycle-to-cycle variability is not obviously correlated across parameters (Fig. 6, right). The choice of any one parameter of a program is not constrained by the choice of any other parameter. In this sense the variability is random.

**Mechanisms of the variability**

Because it appears random, the cycle-to-cycle variability might be thought to represent simply the aggregate noise in the various components of the system, for instance its synaptic connections, that inevitably appear whenever, and however, these components are activated. However, this is not so: the cycle-to-cycle variability, like the systematic trends, does not originate locally within each program, but depends on the previous history of the system. When the history is regularized (Fig. 14), the variability is substantially reduced (Fig. 15). The system contains many processes—in the CPG, within the neuromuscular transform (Brezina et al. 2000a), and activated by the system’s numerous endogenous modulators (Brezina et al. 2003a,b)—that are slow enough to span multiple programs. We cannot even completely rule out the possibility of such processes at the input stage, if, for instance, our constant esophageal nerve stimulation activated a changing mix of axons with different refractory periods. Most likely mutual feedback and reverberation of multiple such processes, once set in motion, maintain the cycle-to-cycle variability. The cycle-to-cycle variability, while random across parameters, should then in principle not be random in any one parameter across successive programs, although such dependencies may be so complex as to also appear effectively random. Whatever the precise mechanisms, the larger functional conclusion is that the system is apparently so constituted that it does not dampen and eliminate the variability, but rather, even with constant input, amplifies and maintains the variability in the output.

**FIG. 14.** Typical appearance of regular motor programs elicited in the reduced buccal-ARC neuromuscular preparation by the “discontinuous” protocol of esophageal nerve stimulation, i.e., short, intense bursts of stimulation delivered at regular intervals as indicated at the bottom; otherwise as in Fig. 1.
An intriguing alternative is that the “random” variability is in fact deterministically chaotic (see, e.g., Rabinovich and Abarbanel 1998; Selverston et al. 2000). We have not applied the mathematical tests for chaos to our data, and with limited data deterministic chaos may be difficult to distinguish from true randomness. A chaotic system, too, may be regularized by periodic forcing input (e.g., Elson et al. 1999).

Variability is usually seen as a problem in the task of performing “reliable computation with unreliable components” (von Neumann 1956), to be solved by averaging, redundance, and, more generally, degeneracy in the mapping from one level of the system to the next higher level (Beer et al. 1999). Thus, the intrinsic variability of neuronal firing patterns (e.g., de Ruyter van Steveninck et al. 1997; Kara et al. 2000; Shadlen and Newsome 1998; Stevens and Zador 1998; Warzecha and Egelhaaf 1999; Wu et al. 1994) and muscle contractions (Hoover et al. 2002; Zoccolan et al. 2002) is reduced through synchronization, averaging, and population coding (Hoover et al. 2002; Selverston et al. 2000; Sparks 1997; Zoccolan et al. 2002) to achieve a reproducible final output of the system. In our system here, however, it appears that the variability at the upstream levels is carried down unreduced all the way to muscle contractions and behavior. Indeed, the muscle contractions are the most variable (Figs. 4B and 7B) and perhaps most resistant to regularization (Fig. 15). This is probably because they represent the point of synergistic convergence of many upstream parameters; the neuromuscular transform itself is not intrinsically variable (Fig. 16). Like the systematic trends, the cycle-to-cycle variability that emerges in the behavior thus appears to be largely a central product, very likely driven by variability of the operation of the CPG. Hence, as we have found, regularizing merely the high-level timing of the CPG regularizes, to at least some extent, all of the downstream levels too. Altogether it appears that, although there certainly is a degree of degeneracy in the Aplysia neuromuscular system (Brezina and Weiss 2000; Brezina et al. 2000; Neustadter et al. 2002a,b), this degeneracy is not sufficiently large, or the neural input is not sufficiently constrained to the degenerate region in the neuromuscular mapping (see following text), as to prevent the emergence of the CPG variability in muscle contractions, movements, and behavior.

**Analytical utility of the variability**

In analyzing vertebrate limb movement, constraints manifest in the structure of parameter variability have been exploited in a very creative way. The aim is to discover which higher-order variables, perhaps complex combinations of the lower-level
parameters, the system is attempting to control. In this view, there is a well-defined control goal, and variability arises, in part, from degeneracy in the mapping from the lower levels to that goal—from the fact that there are many degrees of freedom in the system, many ways of reaching the desired goal (Bernstein 1967). All the ways of reaching a particular goal constitute an “uncontrolled manifold” in the parameter space, so called because the system will not be motivated to reduce the variability within this manifold. It will, however, attempt to reduce the variability that is orthogonal to the manifold (see Scholz and Schöner 1999; Todorov and Jordan 2002, and references therein; for further illustration in theoretical models of walking CPGs, see Beer et al. 1999). Examination of the structure of the variability can thus reveal the control goal or functional task of the system.

In our studies of Aplysia feeding behavior, likewise, ignorance of the precise functional tasks of the neuromuscular system represents a major problem (Brezina and Weiss 2000; Brezina et al. 2003b). The systematic trends in the esophageal motor programs, if analyzed as just outlined, may help us identify the higher-order variables controlled in egestive behavior. Unfortunately, the cycle-to-cycle variability, which we would prefer to analyze because it is relatively stationary, will not be as useful. The structureless cloud of the programs in the multidimensional parameter space (of which the plots in Fig. 6, B and C, right, are 2-dimensional projections) will have equal variability along whatever manifold we choose as orthogonal to it. One possible interpretation of this is that the functional task of the system is not especially constraining (see following text).

Finally, the absolute parameter values presented in this paper (Figs. 2, 3, 9, A and B, 11, B and C, and 13, A and B) give us an idea of the physiological range of upstream activity that the downstream levels in the neuromuscular system are exposed to and must cope with during behavior, as well as of the significance that we should attribute to any experimentally induced changes, for instance after exogenous application of neuromodulators. Given that the physiological range is large, small changes, even if statistically significant, may not be functionally meaningful.

**Functional implications of the variability**

Because the functional tasks of the system remain unknown, so do quantitative measures to evaluate performance in these tasks as a function of the neuromuscular parameter values. Nevertheless, the large variability already has clear qualitative implications. We illustrate these in a hypothetical example in Fig. 17. In each of the two plots, the bottom x–y plane represents the space of neuromuscular parameters—the phase durations, motor neuron firing frequencies, muscle contraction amplitudes—of which only 2 dimensions are shown for clarity. The black dots represent individual cycles, scattered with large, apparently random variability in the parameter space as we saw, for example, in Fig. 6. Plotted in the vertical, z-dimension is then a hypothetical surface of functional performance—ultimately, for example, the amount of seaweed ingested—that can be evaluated for each point in the parameter space. Performance is higher for some parameter combinations than for others. Say that acceptable performance occurs above the red contour on the performance surface, and, correspondingly, within the red circle in the parameter plane. It is likely that performance will be acceptably high over at least the center of the scatter of cycles in the parameter plane.

We can then envisage two possibilities. First, it may be that the variability is permitted to be so large because the performance surface is broad—there are broad tolerances in the operation of the neuromuscular system, so that even very different cycles produce acceptable performance (Fig. 17, left). In other words, there is a highly degenerate mapping to functional performance in each cycle (Beer et al. 1999). Alternatively, the neuromuscular tolerances may not be broad. With the large variability, there will then be some cycles of behavior that are dysfunctional (Fig. 17, right). These may be tolerated by the animal in the absence of evolutionary pressure, or, more interestingly, because they are outweighed by higher-order advantages of the arrangement that permits them (see following text).

Building on the foundation of our work here, it should now be possible to perform behavioral experiments to distinguish...
between these two hypotheses in this system. The functional task of the system is perhaps most intuitively obvious on the behavioral level, where feeding performance can be quantified by measuring, for example, the length of seaweed strip (e.g., Hurwitz and Susswein 1992; Morton and Chiel 1993a; Weiss et al. 1986) or string (Kabotyanski et al. 2000) swallowed in each cycle. The first hypothesis predicts that, despite the large variability of the neuromuscular parameters, their values should always complement so as to produce reliable movement of food into the mouth in each cycle—the control goal on the ultimate, behavioral level. The second hypothesis predicts that, on the contrary, there will be cycles with robust electrical activity, yet little movement of food. Anecdotally, such cycles have indeed been observed (Fig. 8; see also Morton and Chiel 1993a), and “neutral” rhythms have been described in the feeding behavior of another mollusc, Pleurobranchaea (Croll et al. 1984). Large variability in the movements of ingested string can be seen in the records of Kabotyanski et al. (2000), although, as in all such studies published to date, the variability was never explicitly quantified cycle by cycle.

Such experiments will also help specify an absolute scale for the neuromuscular variability that we have found. Here we have argued that the variability is “large” using an internal standard: the variability in each parameter is comparable in magnitude to the mean value of the parameter. However, if, as we expect, variability of similar magnitude is found also in functional performance, this will provide an unambiguous external standard of comparison.

As mentioned in the INTRODUCTION, the neuromuscular system incorporates modulatory mechanisms that tune the system for optimal performance, which superficially might lead one to expect low variability. In fact, however, the present work agrees well with the conclusion of our modeling studies (Brezina et al. 2003a,b) that, although the modulatory mechanisms may improve performance on average, they do not do so in each individual cycle. Indeed, by increasing the variability, they may even degrade performance in some cycles.

Why is the CPG variable?

In view of the functional penalties that the variability probably incurs in some cycles, why is the CPG variable? We suggest that the variability reflects or even actively promotes the overall operation of a multitasking CPG in an uncertain environment.

The variability may simply reflect the fact that the network state of the feeding CPG is located in the dynamical space near the boundaries between its various behaviors, where however the ease of switching must be paid for by reduced stability, manifest in large variability, of each behavior (see Schöner and Kelso 1988). In this passive view, the variability is simply the unavoidable cost of the ability to respond rapidly to unforeseen changes in the environment.

More interestingly, the variability may be an active, integral part of the strategy for dealing with such an environment. At any point in its meal, the feeding Aplysia may be confronted with any of a wide range of seaweed types and qualities that are best ingested with somewhat different feeding movements (see, e.g., Hurwitz and Susswein 1992). Furthermore, it is likely that external tactile and chemical cues cannot fully distinguish, for example, the toughness of a piece of seaweed. How the seaweed can best be eaten, indeed whether it can be eaten at all, can be determined only by internal sensory feedback from the buccal mass and esophagus, once the attempt has actually been made. In such circumstances, a trial-and-error feeding strategy may be optimal. The feeding CPG generates randomly (or quasi-randomly by a chaotic mechanism) a wide range of movements that efficiently sample the entire space of possibilities (see, e.g., Mpitsos 2000; Rabinovich and Abarbanel 1998). Some of these movements will fail, but at least some will succeed. The successful movements can then be reinforced and stabilized—essentially, in a short-term analog of operant conditioning—so that in the next cycle the CPG is more likely to produce a successful movement and less likely to produce an unsuccessful movement. Note, however, two fundamental underpinnings of this strategy. First, considerable variability must actually be expressed in the behavior. Second,
there is a cost–benefit balance between too much and too little variability. While stabilizing and decreasing the variability pays off if conditions remain constant, it reduces the ability of the system to search for a new optimum when conditions change.

Several lines of evidence in the Aplysia feeding system already support such a scenario. There is evidence that Aplysia do not attempt to or often cannot predict whether they will be able to ingest a given strip of seaweed. Rather, they attempt to ingest it and, if unsuccessful, they cut the strip or abandon it entirely by egesting again the entire length they have already ingested (Hurwitz and Susswein 1992). Also very relevant is the work of Nargeot et al. (1997, 1999a,b). These authors found that constant, regular stimulation of a branch of buccal nerve 2 elicited, like our esophageal nerve stimulation here, a wide range of motor programs from the feeding CPG. Nargeot et al. did not quantify the cycle-to-cycle variability as we did, but they classified the programs as ingestive, intermediate, or egestive, and showed that these very different programs were produced probabilistically in “random” order. They then found, in an in vitro analog of operant conditioning, that contingent stimulation of a branch of the esophageal nerve selectively reinforced the fraction of those programs, ingestive or egestive, with which it was associated. The systematic trends that we began to analyze here in Fig. 5 can be seen as a form of such stabilization, occurring, apparently, in the CPG itself. In intact animals we have also found stabilization, manifested in progressively decreasing cycle-to-cycle variability, over successive cycles used to swallow each strip of seaweed (Fig. 10).

A trial-and-error strategy of this kind may be important in the operation of CPGs generally (Rabinovich and Abarbanel 1998). However, it may assume a particularly prominent role in the feeding of a herbivore such as Aplysia, where the variety of tasks posed by the environment is large and unpredictable but the penalty for failure in any one cycle is low. Thus, based on our initial work here, the Aplysia feeding system may be particularly suitable for the study of such questions.

GRANTS

This work was funded by National Institute of Neurological Disorders and Stroke Grant NS-41497 to V. Brezina.

REFERENCES


Kine-