Motor neuronal activity varies least among individuals when it matters most for behavior

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Motor neuronal activity varies least among individuals when it matters most for behavior. J Neurophysiol 113: 981–1000, 2015. First published November 19, 2014; doi:10.1152/jn.00729.2014.—How does motor neuronal variability affect behavior? To explore this question, we quantified activity of multiple individual identified motor neurons mediating biting and swallowing in intact, behaving Aplysia californica by recording from the protractor muscle and the three nerves containing the majority of motor neurons controlling the feeding musculature. We measured multiple motor components: duration of the activity of identified motor neurons as well as their relative timing. At the same time, we measured behavioral efficacy: amplitude of grasping movement during biting and amplitude of net inward food movement during swallowing. We observed that the total duration of the behaviors varied: Within animals, biting duration shortened from the first to the second and third bites; between animals, biting and swallowing durations varied. To study other sources of variation, motor components were divided by behavior duration (i.e., normalized). Even after normalization, distributions of motor component durations could distinguish animals as unique individuals. However, the degree to which a motor component varied among individuals depended on the role of that motor component in a behavior. Motor neuronal activity that was essential for the expression of biting or swallowing was similar among animals, whereas motor neuronal activity that was not essential for that behavior varied more from individual to individual. These results suggest that motor neuronal activity that matters most for the expression of a particular behavior may vary least from individual to individual. Shaping individual variability to ensure behavioral efficacy may be a general principle for the operation of motor systems.

Aplysia californica; individuality; motor control; pattern generator; variability

Behavior varies. Practiced motor acts can vary from performance to performance, and from performer to performer (Furuya and Altenmüller 2013). Glenn Gould’s 1955 and 1981 recordings of Bach’s Goldberg Variations differ radically, but they are more similar to each other than Murray Perahia’s recording. Furthermore, movement kinematics and kinetics may vary and yet have the same adaptive fitness. If a squirrel monkey succeeds in plucking a breadfruit, different hand trajectories or force profiles are adaptively equivalent. Variances in responses and in individuals have been found in animals ranging from insects (Hooper et al. 2006) to humans (Borzelli et al. 1999; Nussbaum and Chaffin 1997; Wu et al. 2014).

Variability has many sources. Ionic channel gating and vesicle release vary stochastically (Sakmann and Neher 2009; Yang and Xu-Friedman 2013), channel number and synaptic strengths vary (Roffman et al. 2012; Schulz et al. 2006), neuron properties may vary (Benjamin 1976), motor neuronal output varies (Williams et al. 2013), neuromodulation alters responses (Marder 2012), body mechanics alters responses to neural output (Chiel et al. 2009), and environmental loads may vary unexpectedly (Johansson and Westling 1988).

Variability has differential penetrance: Small variations may have large effects, whereas large variations may have small effects. Variation at one level may affect another level because of amplifying or filtering nonlinearities. Large increases in motor neuronal output can have a negligible effect on a muscle with little mechanical advantage; in other contexts, a single action potential can have large behavioral effects (Hooper and Weaver 2000). Variations in ionic conductances can generate similar neural patterns (Prinz et al. 2004). If a limb has more than the minimum number of degrees of freedom (DOF) necessary to move it through its workspace, there are multiple ways to position its end point at the identical location in space while resisting perturbations. DOF that generate identical endpoint positions and stiffnesses may vary more (i.e., act as the uncontrolled manifold, UCM) than DOF that do alter position and stiffness (Latash et al. 2002).

Variability can obscure connections between motor patterns and behavior (Horn et al. 2004; Lum et al. 2005). Averaging parameters from many animals may not solve this problem. A study of model oscillators demonstrated that averaged parameters did not generate oscillations (Beer et al. 1999), a study of a conductance-based model neuron demonstrated that averaged parameters did not generate physiologically relevant behavior (Golowasch et al. 2002), and using parameter sets measured from individual animals improved muscle models compared with using averaged values (Blümel et al. 2012).

To understand how motor neuronal variability affects behavior, we studied motor neuronal activity during feeding in the marine mollusk Aplysia californica. Motor patterns can be recorded in intact, behaving animals (Cullins and Chiel 2010), in which motor neurons can be identified (Lu et al. 2013). Biting (an attempt to grasp) and swallowing (food ingestion) are readily quantified (Kupfermann 1974). Biting and swallowing involve similar motor components that primarily vary in their relative amplitude (biting has a larger-amplitude protrac- tion phase, whereas swallowing has a stronger retraction phase; Neustadter et al. 2007). Thus variation in similar motor components may underlie these behaviors. Furthermore, some investigators have argued that variability may serve as a form of trial and error that could enhance Aplysia feeding (Horn et al. 2004).
Previous studies of Aplysia feeding suggested that the duration of biting behavior shortens as animals engage in biting (Rosen et al. 1989), and a previous study of variability of swallowing used real-time behavioral durations to characterize variations over time (Lum et al. 2005). We hypothesized that behavior duration alone might provide a way of distinguishing responses in a sequence, or one animal from another, and found evidence to support this hypothesis.

To study other sources of variation, we divided each motor component by the duration of the behavior in which it occurred. The central hypothesis that we wished to test was that motor components that were most important for the expression of a particular behavior would vary least from one individual to the next, whereas motor components that were less important would vary more among individuals. To determine the relative importance of motor neuronal components for behavioral output, we correlated them with measures of behavior at the same time that we characterized the ability of the same motor components to distinguish one animal from another animal, or to distinguish biting from swallowing. The data support the hypothesis that whenever a motor component is most important for behavioral expression, it varies least among animals.

MATERIALS AND METHODS

Recording in Vivo Feeding Motor Patterns

Animals. Aplysia californica weighing 350–450 g (Marinus, Long Beach, CA) were kept in aerated tanks of circulating artificial seawater (Instant Ocean, Mentor, OH; each tank had a capacity of 189 liters) at a temperature of 16°C in a room with a controlled 12:12-h light-dark cycle. Animals were fed seaweed every other day; feeding was stopped 1–2 days before surgery to increase the response to food. Animals were presented with small pieces of dried laver (2 × 2 mm); animals were selected on the basis of previously described criteria for determining that they were healthy and capable of feeding (Kupfermann 1974).

Recording in vivo identified motor neuronal activity during feeding motor patterns. Previous studies have demonstrated that all critical motor neuronal elements for controlling the feeding apparatus (the buccal mass) can be monitored by recording directly from 1) the protractor muscle (I2), whose activation is an obligate first step for effective feeding movements and whose EMG patterns represent the activity of interneurons/motor neurons B31/B32 and motor neurons B61/B62 (Hurwitz et al. 1996); 2) the radial nerve (RN), which contains axons of the motor neurons B8a/B8b controlling closure of the grasper (Morton and Chiel 1993a, 1993b); 3) buccal nerve 2 (BN2), which contains axons of the motor neurons controlling the jaw musculature, B10, B6, B9, B3, B38, and B43 (Church and Lloyd 1994; Lu et al. 2013; Morton and Chiel 1993b); and 4) buccal nerve 3 (BN3), which contains axons of motor neurons controlling some of the intrinsic muscles of the grasper (e.g., B15/B16; Church and Lloyd 1994; Cohen et al. 1978) and a multiaccion neuron, B4/B5 (Church and Lloyd 1994, Warman and Chiel 1995; Ye et al. 2006b). Nerve recordings RN, BN2, and BN3 are referred to as n1, n5, and n4 in Scott et al. (1991).

To address the problem of variability from response to response, and from animal to animal, it is critical to measure all key motor elements simultaneously. Otherwise, it is not possible to establish clear relationships among the timings of the different units on the different nerves. For this purpose, we developed a novel technique to simultaneously record from the I2 muscle and all three nerves (described in detail and validated in Cullins and Chiel 2010). In vivo feeding motor patterns were recorded from 7 Aplysia with this technique, and a total of 173 swallows and 206 bites were analyzed.

Behavioral Measurements

Biting jaw width. Biting is an attempt to grasp that does not succeed in ingesting food (Kupfermann 1974). Biting strength was quantified based on jaw width (Susswein et al. 1976; Ye et al. 2006b). Peak jaw width opening was measured from video stills and was normalized by the height of the jaws after closure for each bite to compensate for differences in the size of animals and the distance of the camera from the animal. Only bites with a clear view of peak protraction were analyzed. The total number of bites whose recordings and behavior could be analyzed simultaneously were n = 49 bites from 7 animals.

Swallowing. Seaweed strips that were ingested by animals were marked at 1-cm intervals. Both outward movement (during protrac- tion) and inward movement (during retraction) of the seaweed strip were measured. Net inward movement of the seaweed was computed by subtracting total outward movement during protraction from total inward movement during retraction for each swallow. Behaviors in which the seaweed did not move were not included in the analysis. The total number of swallows whose recordings and behavior could be analyzed simultaneously were n = 53 swallows from 7 animals.

Recording Analysis

Behavior selection. Only motor patterns that corresponded to a visible and identifiable behavior on the video were used. Transitional and blended behaviors were excluded, such as bite/swallow behaviors (Kupfermann 1974).

Measurements of identified motor neuronal activity in intact, behaving animals. Previous studies in this system have demonstrated that the durations of spike trains from identified motor neurons (Morton and Chiel 1993a, 1993b), and durations of EMG from the I2 muscle (reflecting the activity of the B31/B32 and B61/B62 motor neurons; Hurwitz et al. 1996), can be related to behavior. The times that were measured are indicated in Fig. 1, and the criteria used to define the starts and ends of bursts are listed in Table 1. To characterize the key motor features, we determined the start and stop times of the I2 EMG during the protraciton phase by measuring both the time of the first spike and the time at which the muscle EMG reached
Behavior duration was defined as the time between the start of 10-Hz I2 activity and the end of the B43 burst. RN, radular nerve; N/A, not applicable.

Two features [the start of B43 and of the 3rd largest unit on buccal nerve 2 (BN2)] were considered the start of the burst when their FF rose above firing frequency cutoffs were used to define the beginnings and ends of bursts. Activity durations of units with low-frequency activity were measured using the times of the first and last spikes rather than using a frequency cutoff. Two features [the start of B43 and of the 3rd largest unit on buccal nerve 2 (BN2)] were considered the start of the burst when their FF rose above average, as low-frequency activity often continued throughout the pattern. Behavior duration was defined as the time between the start of 10-Hz I2 activity and the end of the B43 burst. RN, radular nerve; N/A, not applicable.

Table 1. Criteria for beginning and ending of unit bursts

<table>
<thead>
<tr>
<th>Unit</th>
<th>Start</th>
<th>End</th>
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<tbody>
<tr>
<td>I2 first spike</td>
<td>First spike</td>
<td>N/A</td>
</tr>
<tr>
<td>I2</td>
<td>10 Hz</td>
<td>5 Hz</td>
</tr>
<tr>
<td>RN</td>
<td>3 Hz</td>
<td>3 Hz</td>
</tr>
<tr>
<td>BN2 3rd largest units (retraction)</td>
<td>≥Avg FF</td>
<td>N/A</td>
</tr>
<tr>
<td>B6/B9</td>
<td>First spike</td>
<td>Last spike</td>
</tr>
<tr>
<td>B43</td>
<td>≥Avg FF</td>
<td>5 Hz</td>
</tr>
<tr>
<td>B43/B5</td>
<td>3 Hz</td>
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To exclude occasional spikes that occurred outside of bursts, unit-specific firing frequency (FF) cutoffs were used to define the beginnings and ends of bursts. Activity durations of units with low-frequency activity were measured using the times of the first and last spikes rather than using a frequency cutoff. Two features [the start of B43 and of the 3rd largest unit on buccal nerve 2 (BN2)] were considered the start of the burst when their FF rose above average, as low-frequency activity often continued throughout the pattern. Behavior duration was defined as the time between the start of 10-Hz I2 activity and the end of the B43 burst. RN, radular nerve; N/A, not applicable.

was based on techniques that we have previously described (Lu et al. 2013).

Custom Mathematica software was used to identify spikes, calculate and interpolate the instantaneous firing frequencies, create a firing frequency envelope, and determine the start and end times of the main burst for each unit. Unique criteria were used to try to best capture the activity of each unit (Table 1). Developing criteria and an algorithm for accurately measuring a burst that is uniform across all behaviors and all animals is challenging. After automated measurements were generated, they were verified manually and corrections were made by two investigators working independently as in Morton and Chiel (1993a).

Differences and normalization. Previous work has shown that the absolute durations of single measures may not be as useful for defining behavior as looking at relative timings and overlaps. Thus, for example, ingestive versus egestive patterns were previously distinguished by defining the fractional overlap of the RN activity after the onset of B2 activity divided by the total length of the RN activity for that pattern (Morton and Chiel 1993a). We can compute the duration of activity of a unit, the relative timing of the start or stop of two different units, or the overlaps in activity of two units by taking the difference in timing between two of our measured features. Since we have no a priori reason to assume that any particular difference is significant, we chose to analyze the pairwise differences of all of our measured motor features. The 12 start and end times described above then generated (12 × 11)/2 = 66 timing differences. As discussed in RESULTS, we chose to normalize these times by dividing by the behavior duration, which we defined as the time between the start time of 10-Hz I2 muscle and the stop time of B43 activity. As a consequence, we had 65 normalized measures for each behavioral response (i.e., 65 measures for each of the bites and swallows generated by each of the animals).

Discrimination

Receiver operating characteristic analyses. These 65 motor pattern measures were analyzed to determine which measures could effectively classify motor patterns as bites or swallows and which measures could effectively classify individual animals based on the bite patterns or swallow patterns they generated. A well-established method for evaluating the effectiveness of a given measure for classifying groups is receiver operating characteristic (ROC) curves (Fig. 2; Fawcett 2006; Krzanowski and Hand 2009).

Fig. 1. Unit identification and duration measurements from in vivo motor programs. To characterize motor programs as completely as possible, the activity of many of the key identified motor neurons for feeding behavior were quantified from in vivo bites (A) and swallows (B), in which all of the essential motor outputs of the feeding system were simultaneously recorded. In each recording, the top channel shows EMG recorded from I2, the protractor muscle (Hurwitz et al. 1996). The 2nd channel shows ENG recorded from the radular nerve (RN; large units: activity in neurons B5a/b, grasper closers). The 3rd channel shows ENG recorded from buccal nerve 2 (BN2), which innervates the I1/I3/jaw muscle complex (Church and Lloyd 1994; Scott et al. 1991). The 4th channel shows ENG recorded from buccal nerve 3 (BN3), whose largest unit is B4/B5, a multiaction neuron (Gardner 1993; Warman and Chiel 1995; Ye et al. 2006b). Identified units are highlighted in an example bite (A) and swallow (B) recorded in vivo from the same animal. The swallow includes the same units as the bite plus B38 activity at the start and end of the swallow pattern; B38 activity often did not appear in bites. See text for further details.
We chose to use ROC curves for three reasons: First, from the ROC curve one can readily compute the area under the curve (AUC), which is equivalent to the probability that, given a randomly chosen member of one category, a discriminator will rank it more highly as a member of that category than a randomly chosen member from an alternative category (Fawcett 2006). The AUC is directly related to the Mann-Whitney U statistic (Bamber 1975) and thus serves as a nonparametric measure of the tendency of one data group to have larger values than another that is robust to class skew and to error costs (Fawcett 2006). Thus using the AUC makes no a priori assumptions about the distributions of the motor measures. Second, the AUC can be averaged over multiple comparisons, which is critical for analyzing data from multiple individual animals. Hand and Till (2001) showed mathematically that one can compute each of the pairwise AUC values from $c(c-1)/2$ different classes, sum them, and then divide by the total number of pairwise comparisons [i.e., by $c(c-1)/2$] to obtain a measure of the unweighted pairwise ability to discriminate among these different classes. In particular, for analyzing the ability of motor measures to discriminate among the 7 animals in our study, we computed the AUCs for the $7 \times 6/2 = 21$ pairwise comparisons and
then computed Hand and Till’s measure from these AUC values. Third, AUCs of different features can be ranked by their ability to classify and discriminate, as was done by Mamitsuka (2006) to sort through microarray data to determine which features were the best classifiers. An excellent review of the extensive literature on ROC curves for medical diagnostics, bioscience, and other applications can be found in the recent book by Krzanowski and Hand (2009). ROC curves have previously been used in neuroscience to analyze cortical neuronal activity for sensory discrimination and behavioral choice (Bradley et al. 1987; Kim and Shadlen 1999).

As an introduction to ROC curves, consider a scalar measure (e.g., the normalized duration of I2 activity) for which all data in one category (e.g., all bites) are above some value (e.g., 60% of behavior duration) and all data in a second category (e.g., all swallows) are below that value. One can then use that value as a threshold for accurately discriminating between unknown samples belonging to one category or the other (e.g., an unclassified ingestive motor pattern; see Fig. 2A, right). When such a discriminator is used with this threshold to predict membership in the first category, it will have a true positive classification rate of 100% and a false positive classification rate of 0% (Fig. 2A, right). If the threshold is increased, some data in the first category will be incorrectly classified as belonging to the second category or the other (e.g., an unclassified ingestive motor pattern; see Fig. 2A, right). When such a discriminator is used with this threshold to predict membership in the first category, it will have a true positive classification rate of 100% and a false positive classification rate of 0% (Fig. 2A, right). If the threshold is increased, some data in the first category will be incorrectly classified as belonging to the second and the true positive rate will decrease. If the threshold is decreased, data in the second category will be misclassified and the false positive rate will increase. The ROC curve in Fig. 2A, right, plots the true positive rate against the false positive rate for such a discriminator as the threshold is varied from high to low (the curve originates in the bottom left corner, follows the y-axis to the top left corner, and then moves directly to the top right corner).

The usefulness of the ROC curve becomes apparent when one considers what happens when the values of the discriminator do not fall neatly into two groups on either side of a threshold (e.g., some swallows have longer normalized I2 durations than some bites). For such discriminators, false positive classifications (type I errors) and false negative classifications (type II errors) will be unavoidable, regardless of what threshold is chosen (Fig. 2A, center). In the worst case, if a discriminator correctly classifies data with probability no better than chance, the true positive and false positive classification rates will increase at equal rates as the threshold is decreased and the ROC curve will lie along the 45° diagonal (Fig. 2A, left). The area under an ROC curve (AUC) is used as a measure of how well a discriminator performs in general (Fawcett 2006). In the best case (Fig. 2A, right) the AUC will be 1, which corresponds to an ideal discriminator; in the worst (Fig. 2A, left) it will be 0.5, which indicates that the discriminator provides no information about category membership (discriminators that have an AUC <0.5 can be dramatically improved by switching the category to which high values are assigned). Usually, the AUC will be intermediate (Fig. 2A, center).

The construction of an ROC curve and the calculation of the resulting AUC are illustrated schematically in Fig. 2B. Normalized I2 durations from a bite and a swallow from a single animal are highlighted (Fig. 2B, top); beneath these are shown raster plots and probability density functions of this motor measure for all bites and swallows from all seven animals (Fig. 2B, middle); and beneath this is shown the ROC curve constructed from these data as the threshold discriminating bites from swallows is raised from 0 to 1 (fraction of behavior duration) (Fig. 2B, bottom). The AUC for the curve is relatively large, which indicates that normalized I2 duration can serve as a good discriminator of bites versus swallows...

By combining discriminators, it is possible to develop a classification method that performs better on average than any single discriminator. Thus we examined whether combining multiple timing differences might create more effective discriminators. Combined discriminators can be evaluated by combining ROC curves and computing their convex hull (Krzanowski and Hand 2009). The AUC for the best single discriminator for biting versus swallowing (0.901) was not much worse than the AUC values for the best combined discriminators composed of two (0.912) or three (0.913) timing differences. As single discriminators have the most straightforward interpretation, we chose to focus on them.

For our analyses, we also determined how well motor measures discriminated among our seven experimental animals. To do this, we computed the AUC for all 21 pairwise animal discrimination tasks and averaged them to construct a mean AUC for each discriminator, i.e., we computed Hand and Till’s (2001) measure for classifying more than two data groups. To refer to individual animals, we arbitrarily numbered them from 1 to 7, and this numbering scheme is used throughout the figures.

Statistical significance: approximate randomization tests. By computing AUCs for 65 measures, we risk obtaining results that appear to be significant simply because we made a very large number of comparisons. Thus it is essential for each comparison (i.e., biting vs. swallowing or distinguishing individual animals during biting or during swallowing) to determine objectively a value for the AUC that constitutes a statistically significant result. We therefore determined critical values for statistical significance for the AUCs with approximate randomization tests (Edgington and Onghena 2007; Manly 2007).

ROC analysis depends only on the relative ranking of values from two data sets; the ROC curve and AUC do not depend directly on the actual values. Under the null hypothesis that the performance of a classifier is no better than chance (i.e., AUC not significantly greater than 0.5), the rankings of the data from two classes are randomly distributed. This means that the distribution of AUCs for any discriminator under the null hypothesis depends only on the number of data points in each class. To obtain this distribution, we repeatedly permuted the value rankings between two data sets and computed the corresponding AUC. For example, in discriminating behaviors, we permuted the ranks 1,…, n m for our data set containing n = 206 bites and m = 173 swallows and assigned the first n ranks to bites and the remaining ranks to swallows. We repeated these permutations 2,000 times and determined the critical values for the AUC from the largest α/65th quantile of this distribution, where α = 0.05 is the significance level and α/65 applies the Bonferroni correction for multiple comparisons with 65 normalized discriminators. These approximate randomization tests are equivalent to standard Monte Carlo procedures for determining the significance of the related Mann-Whitney U statistic (Sheskin 2011). ROC analyses and approximate randomization tests were performed in Mathematica using custom software.

The procedure defined critical values for the AUCs. When discriminating bites and swallows, AUC > 0.598 is significant; when discriminating animals during biting, mean AUC > 0.602 is significant; when discriminating animals during swallowing, mean AUC > 0.62 is significant.

RESULTS

Behavioral Variability

How do motor programs vary? Figure 3 shows data recorded during two bites and two swallows in two different animals. Note that the overall duration of the two behavioral responses varies both within and between animals and many components of the motor responses vary significantly. For example, note the variation in the duration of the activity on the I2 protractor muscle (Fig. 3, top trace in each recording) or in the activity of the B4/B5 neuron (Fig. 3, largest extracellular units in the 4th trace in each recording). It is also clear that there are some changes in the motor patterns as the animals switch from biting to swallowing. Distinguishing the different animals and the different behaviors from one another is very challenging given the variability in the motor patterns.
We hypothesized that it might be possible to identify subcomponents of variability due to 1) response-to-response variability, 2) animal-to-animal variability, and 3) variability due to switching from one behavior to another. Furthermore, it might be possible to transform the data so as to compensate for some of these sources of variability. In addition, we hypothesized that it would be possible to relate changes in components of the motor patterns observed in different behaviors to measures of the efficacy of that particular behavior. Finally, we hypothesized that variations from animal to animal might be reduced in motor components that played essential roles in behavior.

Although both the frequency and duration of the motor units vary (as illustrated in Fig. 3), we have focused on the durations of bite strengths for each of the 65 motor program measures was calculated for both real-time and normalized behavior duration, and these values were plotted against one another. Dashed line indicates identical values for $R^2$. The majority (70%) of the measures were on or above the line, indicating that the ability of most motor components to predict behavioral output was unchanged or improved by normalization. Circled point corresponds to $R^2$ values for plots in E1 and E2.
of the motor components (as illustrated in Fig. 1), because our preliminary analyses of the data showed that duration was a better predictor of behavior than instantaneous firing frequency, consistent with the slow time course of response of the muscles in *Aplysia* (Yu et al. 1999).

**Normalization by Behavior Duration Reduces Variability from Animal to Animal and from Response to Response**

How do the durations of behaviors, or components of motor programs, vary from animal to animal and from response to response? Since bites and.swallows may vary in their relative durations, we focused first on biting behavior. We grouped bite durations by individual animals and computed the distributions of the bite durations for each animal (as an example, 2 such distributions are highlighted in Fig. 4A). To determine the overall variability in all 7 animals, we computed the average of the AUCs of the 21 pairwise animal-to-animal comparisons (see MATERIALS AND METHODS), and this was significant (see Fig. 4 legend for statistical results).

Variations in behavior duration could affect the duration of motor program components. Normalization of motor pattern elements by behavior duration may clarify some relationships while obscuring others (Herzog et al. 1994). For example, if a component of a motor program scales linearly with the length of the motor components (as illustrated in Fig. 1), because our preliminary analyses of the data showed that duration was a better predictor of behavior than instantaneous firing frequency, consistent with the slow time course of response of the muscles in *Aplysia* (Yu et al. 1999).
of the behavior, then not normalizing by behavior duration is likely to introduce variability that is not functionally relevant. In contrast, if a component of the motor program has a fixed duration in real time, then normalizing by behavior duration may introduce spurious variability.

We focused next on the duration of activation of the I2 muscle, since I2 protracts the grasper and strong protractions are a key feature of biting (Neustadter et al. 2007). The duration of activity in the I2 muscle was significantly correlated with biting duration (Fig. 4B). The durations of the I2 motor components in each animal paralleled the distribution of overall behavioral durations (Fig. 4C1). Normalizing the I2 durations by dividing them by the duration of the behavior in which they occurred reduced the variation among all animals (Fig. 4C2). The decrease in the AUC (from 0.728 to 0.65) after normalization represents a significant reduction in individual variation in I2 duration (Wilcoxon signed-rank test performed on paired AUC differences obtained from bootstrapped data, \( P < 0.001 \)).

Swallow durations were also analyzed, and high variability was observed in durations among individual animals (average AUC = 0.71). Similarly, I2 durations varied among animals during swallowing (average AUC = 0.71). Dividing by the duration of the swallow reduced the variation (average AUC = 0.67). Thus normalization can reduce animal-to-animal variation due to differences in behavior duration in both biting and swallowing.

We also analyzed variations from response to response. Sequences of three successive swallows or three successive bites were analyzed. We took the differences between the first and second behavior and between the second and third behavior and tested for changes in duration. No significant changes were seen between the durations of the first and second swallows or the second and third swallows in a sequence (\( P = 0.126, P = 0.17 \), respectively, Wilcoxon signed-rank test). In bites, however, overall bite duration significantly decreased from the first to the second bite but not from the second to the third bite (Fig. 4D1). These observations are consistent with previous studies showing that serotonergic modulation due to the metacerebral cell (MCC) can significantly reduce biting duration while increasing biting intensity in a sequence of bites in \( Aplysia \) (Rosen et al. 1989). In addition to changes in overall duration, we also observed a significant decrease in a motor component of bites, I2 duration, from the first to the second bite (Fig. 4D2). After I2 durations were normalized by bite durations, the significant difference from the first to the second bite disappeared (Fig. 4D3).

Although these results clearly demonstrate that normalization can effectively reduce variability from animal to animal, and thus make comparisons among animals much easier, a natural objection that one could raise at this point is that behavior unfolds in real time, not in normalized behavior percentage. As a consequence, it is possible that normalizing motor components would reduce rather than enhance the ability to predict behavioral efficacy.

To address this very reasonable objection, we determined the coefficient of determination (\( R^2 \) value) for I2 duration with a measure of biting efficacy, the magnitude of protraction, which can be measured by determining the peak jaw width for each bite (Ye et al. 2006b), and compared the resulting values before (Fig. 4E1) and after (Fig. 4E2) normalization. We observed that the coefficient of determination improved after normalization. Moreover, we noted that the values of first bites became indistinguishable from values of subsequent bites after normalization (Fig. 4, E1 and E2).

How general is this result? We determined the coefficient of determination for every one of the 65 motor components that we identified in each biting response (see MATERIALS AND METHODS). We computed the coefficients of determination for each motor component in real time with jaw width and then again after normalization by duration of the bite in which it occurred. A plot of these coefficients of determination against one another (Fig. 4E3) shows that correlations are improved or are identical for 70% of the motor components after normalization. Furthermore, those motor components that fall below the line are still very close to the line, so even when there is a reduction in correlation it is quite small.

These results suggest that it is possible to reduce the impact of variability from animal to animal and the effect of variability from response to response by normalizing motor components to the duration of the motor program in which they occur. It also suggests that normalization will not reduce, and may even enhance, the ability to relate motor components to behavioral efficacy. Indeed, these results suggest that if normalization is not done, variations in the duration of responses alone among different animals may overwhelm any other variations in the motor patterns and obscure their relationship to behavioral efficacy.

**Animals Have Distinct “Motor Program Styles” After Normalization**

After normalization by behavior duration, do interanimal differences remain in motor components? We examined motor components in biting and swallowing responses normalized by behavior duration to address this question. The normalized duration of the firing of the B4/B5 neurons on BN3 is shown for two bites and two swallows in two different animals in Fig. 5. Although these responses are now on a common timescale (% of behavior duration), the percentage that B4/B5 is active in animal 5 is clearly longer than in animal 1 in both biting (Fig. 5A1) and swallowing (Fig. 5A2). Raster plots and probability densities for the two animals (Fig. 5, B1 and B2; arrows indicate the location of the data shown in Fig. 5, A1 and A2) show that animals 1 and 5 clearly differ from one another in bites and swallows; furthermore, differences in normalized B4/B5 percentage are seen among all seven animals [Fig. 5, B1 and B2; gray lines show the probability density functions for all the other animals; average AUC for all pairwise comparisons among the 7 animals in biting is 0.78 (\( P < 0.05 \)) and in swallowing is 0.83 (\( P < 0.05 \)).

Since B4/B5 duration is only one of many components of the motor pattern, we determined how many motor components discriminate on average among all seven animals in biting or in swallowing. We determined statistical significance of AUCs using approximate randomization tests (see MATERIALS AND METHODS). We found that 64 of 65 motor components had mean AUC values that were significant in biting (Fig. 5C1; arrow indicates location of B4/B5 mean AUC for biting) and 59 of 65 motor components had mean AUC values that were significant in swallowing (Fig. 5C2; arrow indicates location of B4/B5 mean AUC for swallowing).

Thus, even after normalization, many motor components can distinguish animals as individuals. These results suggest that an important source of variability is an animal’s “motor pro-
gram style,” i.e., the way in which it deploys components of its motor patterns, which can be used to discriminate an individual animal from other animals, even when the durations of the motor components have been normalized by behavior duration. This variability from animal to animal is found in both biting and swallowing behavior.

What Variations in the Motor Program Affect Motor Behavior?

We have shown that variability can be due to variations from response to response as well as variability in individual animals and that it is possible to reduce some variability by normalizing motor components by behavior duration. We have also shown that, even after normalization, variations in normalized motor program components can still distinguish animals as individuals in either biting or swallowing behaviors. Can components of the motor program discriminate between biting and swallowing behavior?

To address this question, it is important to understand the critical biomechanical features of the different behaviors. Our laboratory has done extensive studies on the biomechanics and neural control of biting and swallowing through recordings in intact, behaving animals while taking video of behavior (Morton and Chiel 1993a), in vivo MRI measurements of muscle...
movements (Neustadter et al. 2002a, 2007), computer simulation of the kinematics (Neustadter et al. 2002b, 2007) and kinetics (Sutton et al. 2004) of the behaviors, characterization of motor patterns for variants of swallowing (Ye et al. 2006a), and studies of biting and swallowing in a reduced, semi-intact preparation (McManus et al. 2012). Thus it is possible to define biomechanical features that are likely to discriminate between the two behaviors.

In Aplysia, biting is defined as an attempt to grasp food that does not succeed whereas swallowing is defined as ingestion of food that has been successfully grasped (Kupfermann 1974). The same muscular structures are deployed differently to achieve these two different tasks. For biting the most critical motor component is strong protraction of the grasper to grasp food. In contrast, for swallowing the most critical motor component is strong retraction of the closed grasper to pull food into the buccal cavity (Evans and Cropper 1998; Neustadter et al. 2007).

The difference in the critical motor components leads to differences in deployment of the buccal mass musculature (Fig. 6; this schematic is based on in vivo MRI measurements; see Neustadter et al. 2007). In biting, the I2 muscle (protractor muscle) is activated to initiate the protraction movement of the grasper (Fig. 6, top, 1st to 2nd panel; Hurwitz et al. 1996). During and shortly after the peak of protraction, the grasper closes in an attempt to grasp food (Fig. 6, top, 3rd panel; Neustadter et al. 2007). Closure of the grasper is due to activation of the B8a/b motor neurons, which appear as the largest units on the RN (Morton and Chiel 1993b).

As the I2 muscle is activated it shortens, and thus it loses mechanical advantage and its force is reduced because of its percentage of the swallowing behavior (Fig. 1, top trace), and that I2 activity would occupy a larger percentage of the biting behavior, since the protraction phase is crucial for biting (Fig. 1A, top trace), and that 2) the burst of large motor neuronal activity (due to activation of the B8a/b motor neurons that induce grasper closure; Morton and Chiel 1993a, 1993b) in RN would overlap the I2 activity, so that the grasper can close to aid the I2 in protraction and to grasp food (Fig. 1A, top and 2nd traces). Since retractions are weak in bites unless food has been grasped (Fig. 6, top, 4th and 5th panels; contrast Fig. 6, bottom, 4th and 5th panels, which show the much stronger retraction of swallowing), biting magnitude is not significantly affected by the duration of grasper closure after peak protraction (i.e., the duration of RN activity after peak protraction) or the duration of activation of the muscles that aid retraction, the I1/I3 muscle, whose motor neurons project via BN2 (Lu et al. 2013).

In swallowing, the grasper is protracted open to grasp more food but cannot be protracted too strongly or it may push food out of the buccal cavity (Fig. 6, bottom, 1st through 3rd panels). In contrast, it is critical for swallowing that the grasper be strongly closed and retracted to pull food into the buccal cavity (Fig. 6, bottom, 4th and 5th panels). In turn, this implies that I2 activation (protraction) may occur for a shorter percentage of the swallowing behavior (Fig. 1B, top trace), 2) RN activation (grasper closure) may only slightly overlap the end of I2 activation to start closure prior to the onset of retraction (if the grasper closes too early, it will push food out of the buccal cavity; Fig. 1B, top 2 traces), and 3) the duration of RN activation (grasper closure) and BN2 activation (retrac-

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Fig. 6. Biomechanical constraints distinguish biting from swallowing. Cross sections of the feeding apparatus based on in vivo MRI studies of intact, freely behaving animals (Neustadter et al. 2002a, 2002b, 2007). Top: bites, which are an attempt to grasp food, have a stronger protraction phase than swallows to improve the chances of contacting seaweed, and the grasper closes just before the peak of protraction (dashed box indicates peak of protraction). The grasper must close if food is to be grasped and pulled into the buccal cavity; however, if a bite does not succeed in grasping food, the grasper retracts more weakly than in swallowing. Bottom: swallows occur once an animal has succeeded in grasping food and is attempting to pull it into the buccal cavity. The initial position of the grasper in swallowing is more retracted than in biting. The grasper protracts open so that it can be in a position to pull food in, but the peak protraction of a swallow is weaker than that of a bite to reduce the likelihood that the grasper will push food out of the buccal cavity. The grasper closes at or shortly after peak protraction; if closing occurs early in protraction, food will be pushed out of the buccal cavity. Since the retraction phase of swallowing is the power phase, during which the animal exerts force to pull seaweed into the buccal cavity, the peak retraction phase of swallowing is stronger than in biting (dashed box indicates peak of retraction).
nation) will be longer than in biting to ensure a strong retraction (Fig. 1B, 2nd and 3rd traces).

Examining a bite and a swallow from a single animal normalized by behavior duration, we observe all of these features (Fig. 7A): 1) The I2 protraction is a larger percentage of behavior duration in biting than in swallowing; 2) the RN duration (duration of grasper closure) is a larger percentage of behavior duration in swallowing than in biting; 3) the overlap of the RN and I2 activity (enhancing protraction, attempting to grasp food) as a percentage of behavior duration is larger in biting than in swallowing (Fig. 7A).

Each of these components of the motor program can be used to discriminate bites from swallows across all animals (Fig. 7B; vertical lines beneath each x-axis show the actual values of the 3 motor program components in each bite or swallow, and the corresponding probability density function is plotted above the axis; arrows indicate the specific values for the bite and swallow shown schematically in Fig. 7A). The AUCs for each of these motor components are significant at the $P = 0.05$ level (the critical value, based on approximate randomization tests, is 0.598). Finally, if one plots each of the measures in a three-dimensional plot (Fig. 7C), it is clear that many bites and many swallows occupy distinct regions in the three-dimensional space defined by these three motor components.

These results demonstrate that biomechanically relevant features of the two motor programs can effectively discriminate bites from swallows. It is clear that many bites and many swallows occupy distinct locations in the space defined by their motor components, but it is also clear that there is some overlap. This is not surprising, because we have previously shown that swallows may have variants with different strengths of protraction, making them more similar to bites (Ye et al., 2006a).

**Are Motor Components That Discriminate Bites from Swallows Associated with Behavioral Efficacy?**

How well do the normalized motor components that discriminate bites from swallows correlate with the actual effectiveness of either biting or swallowing behavior? Above (Fig. 4E3) we compared the correlations of all normalized and non-normalized biting motor components with a measure of biting efficacy, protraction strength, as measured by jaw width. Now that we have identified motor program components that effectively discriminate biting from swallowing, we can examine how they correlate with biting or swallowing effectiveness.

For biting, we again use jaw width as a measure of the strength of protraction. For swallowing, effectiveness is the total amount of seaweed that is drawn into the buccal cavity. In turn, this is the inward movement of the seaweed during retraction minus the outward movement during the immediately preceding protraction. Thus minimizing outward and maximizing inward movement generates a larger net inward movement. We therefore examine the relationship of the three motor components that we have shown discriminate
barring from swallowing—I2 duration, RN duration, and I2/RN overlap—with the outward or the inward movement of seaweed.

Plots of the motor components versus jaw width (Fig. 8, top) show that I2 activation (i.e., activation of the protractor muscle) as a percentage of a biting duration is correlated with jaw width (Fig. 8, top left). The overlap of RN activation with I2 activation as a percentage of biting duration (onset of grasper closure prior to end of protraction) is also correlated with jaw width (Fig. 8, top right). In contrast, normalized RN percentage does not correlate with jaw width (Fig. 8, top center). These results are consistent with the biomechanical analysis presented above (Fig. 6).

Plots of the motor components versus outward seaweed movement (Fig. 8, middle) show that I2 activation as a percentage of swallowing duration is correlated with outward seaweed movement (Fig. 8, middle left), as is I2/RN overlap (Fig. 8, middle right). Thus the larger the percentage of the behavior occupied by these motor components, the less effective a swallow is likely to be, again consistent with the biomechanical analysis (Fig. 6). Indeed, we found that there was a significant negative correlation with normalized I2 duration and net seaweed movement, suggesting that the negative effects of strong protractions in swallowing are overpowering any subsequent inward movement ($R^2 = 0.12, P < 0.0031$). The other motor component (normalized RN duration) is not significantly correlated with outward seaweed movement (Fig. 8, middle center).

In contrast, plots of the motor components versus inward seaweed movement (Fig. 8, bottom), show that RN activation (grasper closure) as a percentage of a swallowing behavior is correlated with inward seaweed movement (Fig. 8, bottom center), and neither of the other motor components is correlated with inward seaweed movement, again consistent with the biomechanical analysis (Fig. 6).

These data suggest that biomechanically relevant motor components that discriminate biting from swallowing are correlated with behavioral efficacy. Interestingly, which discriminating measures are correlated with behavioral efficacy may change from biting to swallowing. Furthermore, some correlated motor components can reduce the efficacy of the behavior rather than increasing it. Indeed, in swallowing the motor...

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**Fig. 8.** Correlations between behavioral output and motor measures that discriminate bites from swallows are behavior specific. Top: jaw width, a behavioral measure of protraction that distinguishes large from small bites, is significantly correlated with both normalized I2 % (left; $n = 49$ bites from all 7 animals, $R^2 = 0.47, P < 0.001$) and normalized I2/RN overlap % (right; $n = 49$ bites from all 7 animals, $R^2 = 0.22, P < 0.001$). Biting jaw width was not correlated with normalized RN % (center; $R^2 = 0.00, P = 0.99$). Middle and bottom: swallows had the opposite correlations. Total inward seaweed movement (bottom) correlated with RN duration (bottom center; $n = 53$ swallows from all 7 animals, $R^2 = 0.02, P < 0.001$) but not I2 or I2/RN overlap (bottom left and right; $R^2 = 0.02, P = 0.22$ and $R^2 = 0.00, P = 0.98$, respectively). Outward movement of seaweed (which should be minimized for effective swallowing) was correlated with the normalized I2 % (middle left; $n = 53$ swallows from all 7 animals, $R^2 = 0.33, P < 0.001$) and the I2/RN normalized overlap % (middle right; $n = 53$ swallows from all 7 animals, $R^2 = 0.21, P < 0.001$) but not with normalized RN % (middle center; $R^2 = 0.04, P = 0.11$).
components that reduce efficacy are exactly those that increase efficacy in biting (I2 duration and I2/RN overlap). Finally, the data suggest that there may be important nonlinearities in the relationships between motor components and motor efficacy. For example, note that the minimum normalized I2 percentage starts at \( \sim 30\% \) of the behavior duration for biting (Fig. 8, top left) and starts at \( \sim 15\% \) of the behavior duration for swallowing (Fig. 8, middle left). Because of the properties of the I2 muscle, if it is activated for too short a time the grasper will not protract through the jaws (Yu et al. 1999), and so it will not be possible to grasp food at all. These nonlinearities may limit the ability to establish strong linear correlations between motor components and behavioral efficacy.

**What Is the Relationship Between Motor Components That Discriminate Behaviors and Those That Discriminate Individual Animals?**

The results from the previous analysis suggest that if we examine the same normalized motor component in a behavior in which it is biomechanically relevant, we will see values that are more similar across animals. In contrast, when the same motor component is not essential to the behavior, it may vary more from animal to animal. We can test this hypothesis by determining how well a motor component discriminates one animal from another in all bites, or in all swallows. We first do this for one motor measure in this section and then for all of the motor measures in the next section.

Since the biomechanical analysis (Fig. 6) and the efficacy data (Fig. 8) suggest that RN duration plays a very significant role in inward seaweed movement during swallowing but no significant role in biting, we examined the normalized RN duration in different animals during swallowing and biting (Fig. 9). The normalized duration of RN activity was very different during bites in two animals (Fig. 9A1; animal 1 and animal 2) but was much more similar during swallowing (Fig. 9B). We examined all bites and all swallows for all seven animals (Fig. 9B1 and B2). The raster plots at the bottom of Fig. 9B1 show the normalized RN durations for animal 1 and animal 2 (arrows indicate the specific data shown in Fig. 9A1), and the plots above the raster plots show the probability density functions (gray lines show all the other animals). The distributions are significantly different from one another. In contrast, the distributions of the normalized durations of RN activity in swallowing completely overlap (Fig. 9B2), and the animals cannot be distinguished from one another statistically with this motor component. Thus when the motor component becomes important for the behavior, it varies much less across animals.

**Are Discriminators of Behavior Better Correlated with Behavioral Efficacy Than Discriminators of Individual Animals?**

We have examined a few motor components that discriminate individual animals or discriminate between biting and swallowing, and these have led to the hypothesis that discriminators of behavior are more likely to be correlated with behavioral efficacy, whereas discriminators of individual animals are not. Is this hypothesis true in general?

To examine this question for all motor components, rather than for the few described above, we did the following: First, we determined the ability of each motor component to discriminate bites versus swallows (i.e., calculated an AUC value for that motor component as a discriminator of bites vs. swallows). Second, we computed a coefficient of determination (\( R^2 \) value) for behavioral efficacy. For biting, this was jaw width; for swallowing, this was net seaweed ingested. Third, we chose the higher of the two coefficients of determination since, as shown in Fig. 8, a motor component might be correlated with one but not both behaviors. Our hypothesis is that a motor measure that is a good discriminator between biting and swallowing should be correlated with some measure of motor efficacy. However, a particular motor measure may be correlated with biting, whereas another motor measure may be correlated with swallowing. There is no reason to assume, a priori, that a discriminator must be correlated with both behaviors. Thus we chose the higher of the two coefficients of determination for either biting or swallowing. Finally, we plotted the discrimination value for behavior (AUC value) against the correlation with behavioral efficacy (coefficient of determination value) for each motor component.

The results support the hypothesis that better discriminators of bites versus swallows are associated with behavioral efficacy (Fig. 10A). It is clear that effective discriminators (higher AUC values) are, in general, also associated with higher correlations with behavioral efficacy. The specific examples examined above (Figs. 7, 8, and 9), i.e., I2 duration, RN duration, and I2/RN overlap, are represented by the black, white, and gray boxes, respectively (Fig. 10A). Overall, the correlation between AUC values that discriminate bites from swallows and coefficients of determination for behavioral efficacy is significant.

To test the other half of the hypothesis, i.e., that motor components that are good discriminators of individual animals will not, in general, be associated with behavioral efficacy, we did the following: First, we determined the ability of each motor component to discriminate among all the individual animals (i.e., calculated a mean AUC value for that motor component as a discriminator of animals). We did this separately for biting and for swallowing since the features that discriminate animals in biting are not necessarily the same as those that discriminate animals in swallowing. Second, for each motor component, we computed a coefficient of determination for behavioral efficacy. For biting the measure was jaw width, and for swallowing the measure was net inward seaweed movement. Finally, we plotted the discrimination value for individual animals (AUC value) against the measure of behavioral efficacy (coefficient of determination value) for either biting or swallowing for each motor component.

The hypothesis states that there should be no relationship between the ability to discriminate individual animals and behavioral efficacy, but we in fact observed a stronger result (Fig. 10, B1 and B2): Motor components that had higher measures of individuality were associated with lower correlations in behavioral efficacy.

These results support the hypothesis that, in general, if a motor component is a good discriminator of bites versus swallows, it is also likely to be highly correlated with behavioral efficacy. They also support the hypothesis that, in general, if a motor component is effective at discriminating one animal from another, it also likely to be associated with a low correlation with behavioral efficacy. Finally, although many of the motor components are consistent with these general observa-
tions, there are clearly many exceptions (i.e., the correlations are far from perfect). This is not surprising, since the coefficient of determination relating the motor component to behavioral efficacy may not capture the complex relationship between some motor components and behavioral output.

Separating Behavioral and Individual Variability

Can the variability in motor components due to behavioral differences be separated from the variability in the same motor components due to individual differences? To do this, we can examine two individual animals and two motor components that are positively correlated with one behavior and not with the other behavior. In particular, we chose to look at I2 duration, which is positively correlated with biting efficacy and positively correlated with outward seaweed movement, and RN duration, which is positively correlated with swallowing efficacy but not correlated with biting efficacy (Fig. 8).

Figure 11 shows the distributions of these motor components when they are plotted against each other for two animals, both in biting and in swallowing (animal 2 and animal 3). While only two of the seven animals are shown for clarity, the comparisons made here were similar for all seven animals. During biting behaviors, the normalized I2 durations are very similar in the two animals; in contrast, during swallowing behaviors, the values of normalized I2 duration are both shorter in the two animals than what was observed in biting. This can be seen in Fig. 11 in the alignment of the two animals’ bite distributions and their separation from the two animals’ swallow distributions along the x-axis.

Focusing on the RN durations reveals different results. During swallowing, the values of the normalized RN duration...
are very similar in the two animals; in contrast, during biting behaviors, the animals have distinctly different normalized RN durations. This can be seen in Fig. 11 in the alignment of the two animals’ swallow distributions and the separation of the bite distributions along the y-axis.

These results strongly suggest that once appropriate subcomponents are identified, it is possible to distinguish variations in motor patterns that are critical for expression of motor behavior from those that vary as a function of an animal’s unique “motor program style.” Moreover, the data suggest that many of the features of the motor program that vary from animal to animal are those that are not critical for expression of a particular motor behavior, and thus may change depending on the specific motor behavior that the animal is doing at a particular time.

**DISCUSSION**

To our knowledge, this is the first study of the variability of a significant fraction of individual, identified motor neurons mediating different behaviors monitored during normal behavior in fully intact animals. Furthermore, this may also be the first study to demonstrate that individual variability in motor neurons is regulated by the specific needs of different behaviors. The rationale for not beginning with a semi-intact preparation, which would permit multiple intracellular recordings, is that previous studies have demonstrated that the levels of variability in reduced preparations may differ from those observed in vivo. As one of many examples, work by Selverston and colleagues demonstrated that, in isolation, the stomatogastric nervous system generated a highly regular, rhythmic pattern (Miller and Selverston 1985). In contrast, when extracellular recordings were made from key nerves in vivo the patterns were highly irregular (Heinzel 1988). By using the variability observed in intact animals as a gold standard, it will now become possible to do subsequent analyses of the detailed cellular, synaptic, molecular, and genetic sources of variability reviewed above.

To briefly summarize the results, normalizing by behavior duration helps to distinguish variability due to individual response durations from other motor component variations (Fig. 4). Normalized motor program components discriminate individual animals (Fig. 5) and discriminate biting from swallowing (Fig. 7). Motor components that discriminate behaviors more effectively are, in general, more likely to be associated with higher behavioral efficacy (Fig. 8, Fig. 10), whereas motor components that best discriminate individuals are, in general, associated with lower behavioral efficacy (Fig. 10). Sources of variability can be related to differences among individual animals or differences among the biomechanical requirements of different behaviors (Fig. 11). The framework that we present in this paper for the analysis of variability may be of considerable value for analyzing variability, and its relationship to behavior, in a wide variety of other preparations.

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**Fig. 10.** Behavior discrimination strength is positively correlated with behavioral output, whereas individual discrimination strength is negatively correlated with behavioral output. 
**A:** the ability of each of the 65 motor program features to discriminate between biting and swallowing (AUC) is positively correlated with that motor feature’s best ability to predict one of the motor behavior measurements ($R^2$)—for jaw width (protraction amplitude) or inward or outward movement of seaweed. The 3 behavior discriminators from Figs. 7 and 8 are highlighted (black square, I2 duration; white square, RN duration; gray square, I2/RN overlap). There is a significant correlation between how well a motor measure discriminates bites from swallows with the coefficient of determination of that same motor measure with a behavioral outcome (no. of motor components = 65, $R^2$ = 0.5, $P < 0.001$). 
**B:** for the same set of motor measures, individual discrimination AUCs for bites (R1) and swallows (R2) have a negative relationship with behavioral outcomes (jaw width $R^2$ values and the net inward seaweed movement $R^2$), respectively. For the biting behavioral outcome the overall $R^2$ is 0.14 ($P < 0.02$), and for the swallowing behavioral outcome the overall $R^2$ is 0.10 ($P < 0.01$). Although the trend is the same for both biting and swallowing, where each motor measure falls is behavior specific. For example, note that RN duration (white square) switches from a high individuality AUC and low behavioral correlation in biting to a low individuality AUC and a stronger behavioral correlation in swallowing (consistent with the data shown in Fig. 9).
Variability, Individuality, and Effective Behavior

Variability in Animals and Responses and Its Reduction by Normalization

Duration of behavior varies in individual Aplysia (Fig. 4A). Other taxa show individual variations in feeding rate and duration. In Drosophila melanogaster, fast and slow larval feeding rates are heritable, and slower-feeding larvae become adults with greater longevity than faster-feeding larvae (Foley and Luckinbill 2001). More generally, variation in behavior duration can distinguish individuals. For example, great tits (Parus major) vary in how quickly they explore their environment. These differences are correlated with other behavioral traits (e.g., aggressiveness), are heritable, and have differential effects on fitness depending on environmental factors (Dingemanse and Réale 2005).

Response duration can change within individual animals (Fig. 4D1). Successive biting responses in Aplysia are shorter and stronger, in part because of serotonin released from the MCC (Kupfermann 1979). Neurmodulation has been shown to increase contraction strength and relaxation rates of muscles in Aplysia (Cropper et al. 2004; Hurwitz et al. 2000; Weiss et al. 1992). Neurmodulation is also important for vertebrate motor control. In vertebrate smooth muscle, neurmodulators act directly on the muscle (Burnstock 1985). In skeletal muscle, persistent inward currents in motor neuronal dendrites are modulated by serotonin and norepinephrine (Heckman et al. 2008) and, given appropriate excitatory and inhibitory synaptic input, regulate motor output (Powers et al. 2012).

Since the first bite in a sequence tends to have a longer real-time I2 duration (Fig. 4D2) but also has a behavioral efficacy comparable to shorter real-time duration bites occurring later in the sequence (Fig. 4E1), neurmodulation must increase the intensity of muscle responses to motor neuronal output to compensate for a decrease in the overall behavioral duration. Normalized I2 percentage is a better predictor of protraction amplitude (Fig. 4, E2 vs. E1), suggesting that percentage of I2 activity is regulated more tightly than real-time duration. More intense activation of the B31/B32 interneurons mediating the protraction phase of biting (Hurwitz et al. 1996) could generate a shorter protraction-phase burst, and more intense synaptic output from protraction-phase interneurons could in turn induce a faster retraction phase.

Although most motor pattern components become better predictors of behavioral output after normalization, not all do (Fig. 4E3). If a motor component is relatively fixed in its real-time duration, then normalization may increase variability, reducing behavioral output correlation. The differential effects of normalization have been seen in vertebrate systems, such as cat walking (Herzog et al. 1994).

Finally, our results suggest that animals may have their own “motor program styles,” i.e., they may show significant individual variations in outputs of the motor system that are both consistent within a given animal and differ from one individual to another (Fig. 5). Variations in “motor program styles” have been observed in a wide variety of animals (e.g., leech; Calabrese et al. 2011) and in humans. For example, a recent study in humans showed that regulation of individual temporal variability in motor output could enhance learning speed (Wu et al. 2014).

Variability, Biomechanics, and Behavior

Variability in behavior has been noted and studied for many years in human beings. When Bernstein defined some of the key problems for motor systems, he pointed out that behavior was generally topologically rather than metrically defined, using as an example a request for an individual to draw 10 five-pointed stars in succession: “I doubt if it is at all possible to make a metrically perfect copy of a similar object without the help of a compass and a ruler, that is, the human motor system cannot attain any high degree of metric proficiency, but it can be said that our motor system is very sensitive to topological distinctions...” (Bernstein 1967, p. 45). A recent book has characterized the many aspects of variability seen both in normal movements (e.g., postural sway and responses to support perturbations) and in trained athletes. Analyzing the movements and strategies of individuals has revealed that there are multiple ways in which a problem can be solved. The best solutions need to take into account the unique properties of the individual’s anatomy as well as the changing motor task, and the broad framework of dynamical systems theory may be helpful for better understanding these issues (Davids et al. 2006).
Biomechanics constrains the behavioral relevance of motor neuronal outputs but may not require precise timing or sequencing (Fig. 6). Indeed, in *Aplysia* feeding, combinations of key motor outputs vary in both biting and swallowing (Fig. 7C). Furthermore, although there are significant correlations between motor components and behavioral efficacy, the coefficients of determination account for only 20–47% of the variance (Fig. 8). What could account for this variability? Understanding how “motor commands” generate behavior requires an understanding of variability within the nervous system, within the neuromuscular transform, and within the geometry of the body and the implications of a multifunctional system for overall behavioral fitness.

Variability in motor neuronal outputs may reflect variations in the nervous system. In studying the motor control of leech heartbeat, Calabrese and colleagues showed that high variability in the synaptic inputs to heart motor neurons led to the conclusion that “individual animals appear to arrive at individual solutions to produce functional motor outflow from the heartbeat CPG” (Calabrese et al. 2011).

Do variations in motor neuronal output always generate changes in motor output? An endoscopic study of the key feeding structures in crab by Heinzel and colleagues demonstrated that changes in motor neuronal activity caused specific, and sometimes relatively nonintuitive, changes in movement (Heinzel et al. 1993). In contrast, however, many parameter sets can generate similar neuronal outputs (Prinz et al. 2004), and such variations provide multiple “solutions” to the problem of remaining robust to environmental perturbations such as temperature variation (Caplan et al. 2014). Furthermore, neurons can regulate their intrinsic conductances so that their patterns of electrical activity remain similar even if their synaptic inputs change (Davis 2006). Finally, Nargeot and colleagues (Nargeot and Simmers 2012) have shown that, with reinforcement, biting and the output of the feeding pattern generator in *Aplysia* can become highly regular. Thus the neural network controlling behavior may only be one of several sources of variability.

Motor neuronal output is expressed through effects on muscle, and this can introduce another source of variation. The “neuromuscular transform” (Brezina et al. 2000) describes the nonlinear transformations that occur between motor neurons and muscles. There is no simple, linear relationship between motor neuronal activity and muscle force (Hooper and Weaver 2000). Moreover, fitting data to models requires unique parameters for each individual (Blümel et al. 2012). During biting, the I2 muscle does not activate immediately even if stimulated at high frequency (Hurwitz et al. 1996; Yu et al. 1999). If I2 is activated for too short a time, protraction will not occur; if it is activated for too long, protraction will saturate and retraction cannot occur (note the absence of data points below 10% and above 75% of the behavior duration for all I2 graphs in Fig. 8).

Once muscles have generated force, the actual behavior that they generate depends on the geometry of the body, which introduces yet another source of variation. Combined deployment of DOF within the complex geometry of the body may lead to equivalent motor outputs. Natural selection acts on overall behavior, so equivalent DOF may not be tightly regulated, a concept known as the uncontrolled manifold (Scholz and Schöner 1999). Consequently, any single DOF deployed as part of a combination may be less correlated with motor output than if it were deployed alone. For example, there are trade-offs on how soon the grasper should close to enhance protraction [too early, and the closed grasper cannot grasp food; too late, and it provides no assistance to I2 (Novakovic et al. 2006)]. Many combinations may be essentially equivalent; thus I2/RN overlap may not correlate strongly with behavioral efficacy.

Correlations between motor components and behavior might improve if more features of the *Aplysia* motor system were included in the analysis. In particular, it might be useful to include the activity of the motor neurons for the intrinsic muscles of the odontophore such as B15/B16, the motor neurons for the accessory radular closer (ARC) muscle [Cohen et al. 1978; the ARC muscle is also known as I5 (Howells 1942)], or B48, the motor neuron for the I7–I10 muscles, which contribute to opening of the radula (Evans et al. 1996). Although we were unable to distinguish these motor neurons on BN3, in future studies it might be possible to combine the suspended buccal mass preparation (McManus et al. 2012) with voltage-sensitive dyes (Hill et al. 2010; Morton et al. 1991) to obtain a more complete picture of the full activity of all the motor neurons during feeding. Including features that did not always occur in every motor program, but were more likely to occur during swallowing than during biting, might also improve discrimination, such as activity in the B38 motor neurons, which are more active in swallowing than in biting (McManus et al. 2014), and the activity of the B3 motor neurons, which are more active in swallowing than in biting (Lu et al. 2013). Another good discriminator of biting and swallowing was the amount of overlap of RN activity as B6/B9 were activated (i.e., the conjunction of closure and retraction during swallowing). Our results also identified poor discriminators: B43 activity had similar durations in biting and swallowing (Lu et al. 2013), and B4/B5 activity could not effectively discriminate biting from swallowing. Despite these limitations, the results reported in this study are likely to remain valid.

Neuronal and biomechanical variations have important implications for motor control. The nonlinearities of motor control systems create “good enough” and “don’t care” regions in the solution space. The range of “good enough” solutions may be larger and more accessible than a single global optimum and equally effective for survival or reproduction (Horn et al. 2004; Loeb 2012). As long as motor patterns are within the “good enough” region, there may not be strong selection for a particular motor pattern. Furthermore, some variations in the activity of a motor neuron may have no behavioral effect (a “don’t care” region; Beer et al. 1999), for example, when its target muscle is slack or exerting maximal force. Thus defining biomechanical context is crucial for determining whether variability in the neural motor program reduces behavioral effectiveness or directly affects behavior.

Finally, evolution may not select for a particular behavioral trajectory but rather for the ability to flexibly switch among a sequence of behaviors to achieve a goal. For example, *Aplysia* rapidly switch from biting (if they have failed to grasp food) to swallowing (once food is in the buccal cavity), but during swallowing they may switch to reposition movement to reposi-

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swallowing in Aplysia form a continuum: Weak bites overlap strong swallows and cannot be discriminated from them (Fig. 7C). The “common ground” for both biting and swallowing is closure of the radula during the retraction phase [unlike rejection, in which the radula closes during the protraction phase (Morton and Chiel 1993a)]. Since biting is an attempt to grasp food, the animal must be able to rapidly switch from biting to swallowing once it does grasp food. Similar flexibility is seen in multifunctional vertebrate systems, such as the turtle scratch system (Berkowitz 2010; Stein 2005).

Controlling and Exploiting Neuronal and Biomechanical Variability

If a particular motor component does not play a significant behavioral role, it can contribute to an individual “motor program style” (Fig. 5, Fig. 9, A1 and B1). When the same component does play a role in behavior, it may no longer vary among individuals (Fig. 9, A2 and B2). More generally, motor components that are most effective at discriminating different motor behaviors (Fig. 7) are generally correlated with behavioral efficacy (Fig. 10A), whereas motor components that are most effective at discriminating different individuals are generally not correlated with behavioral efficacy (Fig. 10, B1 and B2). What mechanisms could induce these relationships?

Sensory feedback may “sculpt” neuronal activity for different behavioral contexts. Thus before food is grasped the activation of RN during retraction may vary (Fig. 9, A1 and B1), but once food is grasped variations in RN duration may be reduced by sensory feedback (Fig. 9, A2 and B2), since grasper closure is critical for swallowing (Fig. 6). Proprioception has been shown to play a role in altering RN activity as the animal switches from biting to swallowing (Evans and Cropper 1998). Modeling work suggests that sensory feedback may play critical roles in lengthening components of behavior (e.g., the retraction phase, mediated in part by grasper closure, i.e., RN activity) due to the dynamics of Aplysia’s feeding pattern generator (Shaw et al. 2014). Furthermore, sensory input may move the system into and through the correct “solution space” for swallowing behavior. As constraints are added to a task, there may be a greater convergence on similar solutions, as was seen in a finger pressing task in humans (Zhang et al. 2008).

Our results provide guidance for exploring the neural mechanisms by which sensory feedback shapes motor neuronal activity. First, by using a reduced preparation in which all but proprioceptive input from the feeding apparatus has been removed (McManus et al. 2012), or by using a preparation in which the entire musculature has been disconnected (the isolated buccal ganglion), it may be possible to characterize the effects of removing much or all of the sensory input on motor neuronal variability. Second, because many of the key sensory neurons that provide chemoafferent or mechanoafferent inputs to the feeding apparatus have been characterized (Evans et al. 2003; Evans and Cropper 1998; Rosen et al. 2000a, 2000b), working out the cellular mechanism by which sensory feedback can shape motor neuronal activity can focus on both identified sensory and motor neurons. Finally, the studies by Nargeot and his colleagues have elegantly demonstrated that it is possible to work out, at the level of individual identified neurons, the mechanisms by which variability can be reduced in the feeding neural circuitry (Sieling et al. 2014), suggesting that similar studies can be done to understand how variability is regulated by sensory feedback. Studies by Benjamin and his colleagues have demonstrated the power of invertebrate systems for exploring the neural mechanisms of behavioral hierarchy (Pirger et al. 2014), and the extent to which central pattern generators are hierarchical or distributed systems (Benjamin 2012).

By compensating for some forms of variability (e.g., by normalization), and by then examining other components of variability, variations that are essential for behavior can be clarified and distinguished (Fig. 11). The methodology described in this report is likely to be of general use for the study of motor systems, muscle synergies, the role of sensory feedback, how humans may exploit variability to improve in motor tasks, and behavioral discrimination (e.g., Kim and Shadlen 1999; Tresch and Jarc 2009; Wu et al. 2014).

REFERENCES

AUTHOR CONTRIBUTIONS

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AUTHOR CONTRIBUTIONS

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