Motor control by precisely timed spike patterns

Kyle H. Srivastava, Georgia Institute of Technology
Caroline M. Holmes, Emory University
Michiel Vellema, University of Southern Denmark
Andrea R. Pack, Emory University
Coen P.H. Elemans, University of Southern Denmark
Ilya Nemenman, Emory University
Samuel Sober, Emory University

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A fundamental problem in neuroscience is understanding how sequences of action potentials ("spikes") encode information about sensory signals and motor outputs. Although traditional theories assume that this information is conveyed by the total number of spikes fired within a specified time interval (spike rate), recent studies have shown that additional information is carried by the millisecond-scale timing patterns of action potentials (spike timing). However, it is unknown whether or how subtle differences in spike timing drive differences in perception or behavior, leaving it unclear whether the information in spike timing actually plays a role in brain function. By examining the activity of individual motor units (the muscle fibers innervated by a single motor neuron) and manipulating patterns of activation of these neurons, we provide both correlative and causal evidence that the nervous system uses millisecond-scale variations in the timing of spikes within multispike patterns to control a vertebrate behavior—namely, respiration in the Bengalese finch, a songbird. These findings suggest that a fundamental assumption of current theories of motor coding requires revision.

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Biomedical Engineering Doctoral Program, Georgia Institute of Technology and Emory University, Atlanta, GA 30322; Department of Physics, Emory University, Atlanta, GA 30322; Department of Biology, Emory University, Atlanta, GA 30322; Department of Biology, University of Southern Denmark, DK-5230 Odense, Denmark; and Neuroscience Doctoral Program, Emory University, Atlanta, GA 30322

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The brain uses sequences of spikes to encode sensory and motor signals. In principle, neurons can encode this information via their firing rates, the precise timing of their spikes, or both (1, 2). Although many studies have shown that spike timing contains information beyond that in the rate in sensory codes (3–5), these studies could not verify whether precise timing affects perception or behavior. In motor systems, rate coding approaches dominate (6, 7), but we recently showed that precise spike timing in motor cortex can predict upcoming behavior better than spike rates (8), showing that spike timing carries information in motor as well as sensory cortex. However, as in sensory systems, it remains unknown whether spike timing in motor systems actually controls variations in behavior (9, 10). Resolving this question, therefore, requires examining the code used by the neurons that innervate the muscles, because discovering an apparent spike timing code in any brain area upstream of motor neurons is subject to the same ambiguity about whether spike timing patterns actually affect behavior.

A spike timing-based theory of motor production predicts that millisecond-scale fluctuations in spike timing, holding other spike train features constant, will causally influence behavior. We tested this prediction by analyzing the activity of single motor units (that is, the muscle fibers innervated by a single motor neuron), focusing largely on the minimal patterns that have variable spike timing but fixed firing rate, burst onset, and burst duration: sequences of three spikes (“triplets”), where the third spike is a fixed latency after the first, but the timing of the middle spike varies. We examined timing codes in songbirds by focusing on respiration, which offers two key advantages. First, breathing is a relatively slow behavior (cycles last ~400–1,000 ms), so the existence of timing codes is not a priori necessary; however, the precise control of breathing during singing (11, 12) suggests that timing may play a role. Second, we developed an electrode system which allowed us to collect spiking data over >50,000 breaths, yielding the large dataset sizes necessary to decipher the neural code.

We recorded electromyographic (EMG) signals from the expiratory muscle group (EXP) (13) using a flexible microelectrode array (Fig. L1) to isolate spikes from single motor units. Precise timing codes might be implemented by individual spikes (1, 14, 15) or timing of spikes within a multispike pattern. Thus, we first verified whether single motor unit spike trains contain multispike features at high temporal resolution. Analysis of interspike intervals (ISIs) revealed that spiking was more regular than expected from a Poisson process (SI Appendix). Although not sufficient to establish the existence of a spike timing code, such regularity is crucial if the brain were to use spike timing patterns to control behavior, and hence output spikes in a controlled fashion. By showing that ISIs are more regular than expected in a Poisson process (in which the ISIs are independent), we establish the possibility of a timing code, and therefore the necessity of performing the more detailed analyses described below.

Next, we quantified the timescale on which the nervous system controls spikes within triplets by measuring the mutual information between consecutive ISIs in anesthetized birds. The nonzero value of information (Fig. 1C, red circle) suggests that consecutive ISIs (and hence, spike triplets) are controlled in the neural code. To understand the characteristic timescale of this control, we jittered the timing of each ISI by a Gaussian random number with SD $\sigma$, and again estimated the consecutive ISIs mutual information. We found that the information only approaches its unjittered values for $\sigma \sim 1$ ms (Fig. 1C, blue), showing that spike trains have millisecond-scale features. Similar findings were obtained in awake birds (SI Appendix, Fig. S1).

We then asked whether these millisecond-scale features predict behavior by simultaneously recording single motor units and air...
pressure within the respiratory system. Because respiration is controlled by ensembles of motor units, we did not expect a single motor neuron to drive the breathing cycle and instead expected it to only affect fluctuations around the mean. We therefore subtracted the mean respiratory pressure waveform from the recorded pressure (Fig. 1A) and investigated the relationship between such pressure residuals and the preceding spike train (Fig. 2A) using an estimator of mutual information (16) that we developed specifically for this purpose. This method separates the total mutual information between spikes and pressure residuals into contributions from spike count and spike timing:

\[ I(\text{spikes, pressure}) = I(\text{spike count, pressure}) + I(\text{spike timing, pressure}). \]

Seven of eight birds tested (all but EMG3) had statistically significant information in spike timing, which was of the same order of magnitude as the information in spike rate (Fig. 2A). We quantified discriminability of the PTAs using the \( d' \) statistic (17) and found \( d' = 0.108 \pm 0.011 \) (SD) \( 17 \) ms after triplet onset (Fig. 2C). The same effect is present across all six birds (Fig. 2D). Notably, although \( d' \) traces are similar across animals, the PTAs themselves are not (SI Appendix, Fig. S2). Therefore, although the discriminability of different patterns is consistent (Fig. 2D), the encoding of pressure differs across individuals. Wavelet-based functional ANOVA (wfANOVA) (18) (SI Appendix) revealed a consistent significant effect between the PTAs across birds after accounting for intersubject variability (SI Appendix, Fig. S3 and Table S1). Therefore, millisecond-scale changes in timing of a single spike in a multispike pattern at a fixed firing rate predict significant changes in air sac pressure. This result agrees with our previous findings that cortical neurons upstream of vocal and respiratory muscles also use spike timing to encode behavior (8).

Although the above results show that precise spike timing predicts behavioral variations, they cannot reveal whether timing affects muscle output. To test this hypothesis, we extracted muscle fiber bundles from EXP and measured force production (e.g., \( N = 23,991 \) and \( 11,558 \) for the two patterns in bird EMG1, or \( 11 \) and \( 5\% \) of all spike triplets of \( \leq 20\text{-ms duration}, \) respectively).

We found that PTAs after the \('10–10\) and \('12–8\) spike triplets were significantly different (Fig. 2B). We quantified discriminability of the PTAs using the \( d' \) statistic (17) and found \( d' = 0.018 \pm 0.011 \) (SD) \( 17 \) ms after triplet onset (Fig. 2C). The same effect is present across all six birds (Fig. 2D). Notably, although \( d' \) traces are similar across animals, the PTAs themselves are not (SI Appendix, Fig. S2). Therefore, although the discriminability of different patterns is consistent (Fig. 2D), the encoding of pressure differs across individuals. Wavelet-based functional ANOVA (wfANOVA) (18) (SI Appendix) revealed a consistent significant effect between the PTAs across birds after accounting for intersubject variability (SI Appendix, Fig. S3 and Table S1). Therefore, millisecond-scale changes in timing of a single spike in a multispike pattern at a fixed firing rate predict significant changes in air sac pressure. This result agrees with our previous findings that cortical neurons upstream of vocal and respiratory muscles also use spike timing to encode behavior (8).

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in vitro while stimulating using three-pulse patterns with 10–10- and 12–8-ms interpulse intervals (IPIs). Changing the timing of the middle pulse by 2 ms significantly altered force output (Fig. 3B); wFANOVA identified significant differences in the force evoked by these patterns (SI Appendix, Fig. S4 and Table S1). Furthermore, d’ values evoked by 10–10 and 12–8 triplet stimulations are several SDs from zero (Fig. 3C). The same effect holds for other pairs of similar triplets, such as 2–18- and 4–16-ms IPIs (SI Appendix, Fig. S5 A and B). Therefore, our in vitro experiments establish that the small, precisely regulated differences in motor neuron spike patterns observed in vivo cause muscles to produce different forces.

We next explored whether different spike patterns not only correlate with behavior and drive distinct force production but also cause distinct behaviors in vivo. We recorded air sac pressure while simultaneously applying temporally patterned electrical stimulation to EXP, again using 10–10- and 12–8-ms stimulation triplets (Fig. 4A). Moving the middle pulse from 10 to 12 ms after the first evoked distinct pressure waveforms (Fig. 4B) consistently across all six birds tested (Fig. 4C). wFANOVA identified significant differences between the effects of these triplets (SI Appendix, Fig. S6A and Table S1). Finally, we comprehensively investigated the effects of moving the middle pulse from 2 to 18 ms after the first pulse (in steps of 2 ms), which resulted in significant differences in the mean pressure (Fig. 4D) (P < 0.001 for all 36 pairs of these stimulation patterns). These experiments thus show a causal link between millisecond-scale timing of muscle activation and the ensuing behavior.

Overall, we have shown that respiratory motor unit activity is controlled on millisecond timescales, that precise timing of spikes in multispike patterns is correlated with behavior (air sac pressure), and that muscle force output and the behavior itself are causally affected by spike timing (all on similar temporal scales) (Figs. 2D, 3C, and 4C). These findings provide crucial evidence that precise spike timing codes casually modulate vertebrate behavior. Additionally, they shift the focus from coding by individual spikes (1, 14, 19) to coding by multispike patterns and from using spike timing to represent time during a behavioral sequence (20, 21) to coding its

Fig. 3. Millisecond-scale stimulation patterns affect force production. (A) In vitro muscle fiber preparation (Materials and Methods). (B) Force output (full width of the line indicates mean ± SEM; normalized to peak force during tetanic contraction) differs significantly between 10–10- and 12–8-ms IPI stimulation triplets (wFANOVA) (SI Appendix, Fig. S4A and Table S1). (C) Discriminability (d’; full width of the line indicates mean ± SD; bootstrapped) of force profiles in five birds (birds IV1–IV5) after stimulation with the same stimuli as in B.

Fig. 4. Millisecond-scale differences in stimulation patterns modulate air pressure in vivo. (A) Three-pulse stimulation was delivered during respiration. (B) The 10–10- and 12–8-ms triplets (as in Figs. 2 and 3) caused significant differences in air sac pressure residuals (mean ± SEM) (SI Appendix, Fig. S4A and Table S1). (C) Discriminability (d’; mean ± SD; bootstrapped) of pressure residuals after 10–10- and 12–8-ms triplets across birds pSTIM1-pSTIM6. (D) Responses to all stimulation patterns tested (mean ± SEM). In B–D, time is relative to the onset of the stimulation pattern. Note that the reddish brown trace is the response to only one stimulus pulse and that the gray trace is the response to two pulses separated by 20 ms.
structural features. Put another way, although it is clear that earlier activation of neurons would lead to earlier activation of muscles, this relationship only accounts for encoding when a behavior happens (10, 22). Here, we show that changing the timing of a single spike within a burst by ~1 ms can also affect what the animal will do, not just when it will do it. Furthermore, we showed that the effect of moving a single spike is stable across animals (Fig. 2). We believe that this precise spike timing code reflects and exploits muscle nonlinearities: spikes less than ~20 ms apart generate force supralinearly (SI Appendix, Fig. S12), with stronger nonlinearities for shorter ISIs. Thus, changing the first ISI from 12 to 10 ms significantly alters the effect of the spike pattern on air pressure (Fig. 2B). Such nonlinearities in force production as a function of spike timing have been observed in a number of systems (23). To quantify these nonlinearities, we started by examining the nonlinearity of our single unit spike timing codes in the motor systems of other animals. Importantly, our findings show that the nervous system uses millisecond-timescale changes in spike timing to control behavior by exploiting these muscle nonlinearities, even though the muscles develop force on a significantly longer timescale (tens of milliseconds as shown in Fig. 3B).

The surprising power of spike timing to predict behavior might reflect synchrony between motor units in the respiratory muscles (26), so that timing variations in one motor unit co-occur with timing variations in others. Resolving this question requires examining temporal population codes in motor systems, a subject not yet explored. Furthermore, respiration is driven by a brainstem central pattern generator but modified by descending inputs from the forebrain (12, 27). It remains unknown which of these is the source of timing precision/variability. Because respiration is critical to vocalization in songbirds, it will be of special interest to record respiratory timing patterns during singing and determine how the temporal code used by neurons in upstream area RA (the robust nucleus of the arcopallium, which sends inputs to respiratory networks) is transduced to the motor periphery (8). Furthermore, our findings suggest that anisotropy might contribute to differences in functional roles among spike trains in the respiratory network. Zhu and colleagues (13) found that we are recording motor units from the most superficial muscle: m. obliquus externus abdominis. Because all three muscles have similar functional roles involving contraction during respiration (28), recording a motor unit from any of these muscles would not affect our interpretation.

Materials and Methods

Surgical Procedure. We used EMG and electrical stimulation to determine the importance of motor timing in the EXP for avian respiration. All procedures were approved by the Emory University Institutional Animal Care and Use Committee. Before surgery, adult male Bengalese finches (~90-d old) were anesthetized using 40 mg/kg ketamine and 3 mg/kg midazolam injected (i.m. Proper levels of anesthesia were maintained using 0–3% (vol/vol) isoflurane in oxygen gas.

Subjects. Our studies used a total of 24 adult (~90-d old) Bengalese finches. Eight birds (which we refer to as birds EMG1–EMG8) underwent EMG recordings of single motor units, in which one single unit was isolated in each animal (that is, birds EMG1–EMG8 each contributed a single unit to the study, and data from all eight are used in the analyses in Fig. 1C). Concurrent with EMG recordings, the pressure within the air sac was continuously monitored (see below for detailed descriptions of procedures). Of these eight birds, six (EMG1–EMG6) yielded sufficient neural and pressure data, such that we were able to compute pressure waveforms conditional on the occurrence of a particular spike pattern (these subjects are the birds shown in Fig. 2A and D). Furthermore, we recorded EMG spike trains (but not air pressure, because we were unable to find a pressure meter that was both sensitive enough to detect small respiratory pressure changes and lightweight enough for an awake bird to carry) from four awake birds, which we refer to as birds EMG9–EMG12, and they provided the data shown in SI Appendix, Fig. S1.

Additionally, muscle fiber bundles (one bundle per bird) were extracted from each of five birds for in vitro testing of force production. We refer to these birds (data are shown in Fig. 3) as birds I1–I5. We also examined the effect of electrically stimulating the expiratory muscles in vivo in six anesthetized birds, which we refer to as birds pressure stimulation 1 (pSTIM1) to pSTIM6. Results from these subjects are shown in Fig. 4. Lastly, four birds (C1–C4) were used to examine the effect of curare on both EMG activity and the efficacy of muscle stimulation. The results are shown in SI Appendix, Fig. S7.

EMG Recordings. To optimize our ability to isolate individual motor units, we developed microscale, flexible, high-density electrode arrays that sit on the surface of individual muscles to record EMG signals (Fig. 1). The gold electrodes were fabricated on 20-μm-thick photo-definable polyimide with a range of contact sizes and spacing (Premtice). The electrode exposures ranged from 25 to 300 μm in diameter and were separated by as little as 25 μm. Several alignments of electrodes (16 per array) were fabricated, including a 4 × 4 grid and 4 tetrodes. To record from the EXP, an incision was made dorsal to the leg attachment and rostral to the pubic bone. After spreading fascia on the muscle group, an electrode array was placed on its surface. The other end of the array connects to a custom-designed Omnetics adapter to interface with a digital amplifier (RHD2132; Intan Technologies). The Intan evaluation board delivered the EMG signals to the computer at 30 kHz.

With these arrays, we were able to acquire high-quality EMG recordings from 16 locations simultaneously during quiet respiration in eight male Bengalese finches. The increased number of channels allows the experimenter to decide which channels should be subtracted from each other to create bipolar signals. Because of the high specificity and impedance of individual fibers, we were able to extract single fibers even in those cases (Materials and Methods, Data Analysis and Fig. 1). Because we can record 16 unipolar signals in a very small area, we have increased the probability of recording a single motor unit while allowing us to test how different i.m. segments are differentially recruited. Although EXP is made up of three sheet-like overlapping muscles (musculus [m.] obliquus externus abdominis, m. obliquus internus, and m. transversus abdominis), we presume that we are recording motor units from the most superficial muscle: m. obliquus externus abdominis. However, because all three muscles have similar functional roles involving contraction during respiration (28), recording a motor unit from any of these muscles would not affect our interpretation.

Pressure Recording. Thoracic air sac pressure was monitored using a Silastic Tube (Dow Corning) inserted in the same manner as previous studies (11, 29, 30). In vivo pressure recordings were obtained with a custom-made system (Fig. 2C). (B) Simultaneous EMG (black) and pressure recordings (gray) from the same subject as in the in vivo experiments. Fiber bundles were then isolated from the surface of m. obliquus externus abdominis (the most superficial muscle in the EXP group). The fiber bundles were then mounted in a test chamber while continuously being flushed with oxygenated Ringers solution at 39 °C. One end of the muscle was fixed to a servomotor (although it was not used) using silk suture, whereas the other end was mounted on a force transducer (Model 400A; Aurora Scientific). The muscle fibers were then stimulated through the solution using parallel platinum electrodes (Model 701C; Aurora Scientific). For each muscle preparation, both stimulation current and preparation length were optimized for maximum force generation. A single 300-μs pulse was used for stimulus optimization followed by a 200-Hz, 100-ms tetanic stimulation for length optimization. To test the importance of motor timing, we stimulated the muscle with three 300-μs pulses at optimal current, with the first and third pulses separated by 20 ms and the middle pulse being placed 2, 4, 10, or 12 ms after the first pulse. Additional trials were conducted with only a single pulse as a control. These five stimulation patterns were repeated in random order with 60 s between each trial and after five such iterations, followed by a 200-Hz, 100-ms tetanic stimulation. After this procedure, we measured a total of 25 iterations, taking ~135 min. To account for muscle fibers dying over the course of the experiment, force measurements were normalized to the maximum tetanic force at 200 Hz and linearly interpolated for each stimulus. Force transducer and stimulation signals were digitized at 20 kHz with an NI DAQ Board (PCI-MIO-16E4; National Instruments).
In Vivo Muscle Stimulation. Stimulation of EXP was performed in six male Bengalese finches using two fine-wire electrodes made of insulated multi-strand copper wire (50-μm diameter, Phoenix Wire Inc.). Pressure residuals were used to trigger stimulation with custom-written LabVIEW code when the pressure crossed a user-defined threshold. Generally, the stimulation pulse train was targeted for 100 ms after the pressure crossed zero. In birds in which the initial upswing of the respiratory cycle was slower than normal, stimulation was delayed up to 50 ms longer to prevent stimulation from occurring during the upswing itself. We wanted to avoid stimulating during that interval because it was more difficult to extract the pressure effects of stimulation. Stimulation between 100 and 150 ms after the zero crossing also mirrored the timing of spikes found in EMG recordings. The LabVIEW code then sent a stimulation pattern to an external stimulator (Model 2100; A-M Systems), which was connected to the fine-wire electrodes in EXP.

To test the importance of motor timing on behavior, three stimulation pulses (biphasic, 250-μs pulse duration, and 250-μA current) were delivered, with the first and third separated by 20 ms and the middle pulse placed in 2-ms increments across the duration (nine different patterns) in addition to a single pulse and no pulse control stimuli. All 11 patterns were randomly interleaved during the experiment. Pressure and trigger times were recorded at 32 kHz using the LabVIEW code.

The selection of an appropriate current was important for interpreting the results of these experiments. To properly compare them with EMG recordings, it is important to use a current that activates the axons of motor neurons but does not activate muscle fibers directly. One previous study that stimulated songbird muscles used currents as great as 2 mA (31), whereas a more recent one posited that currents below 500 μA were likely activating nerve fibers (32). We therefore selected a current of 250 μA for EXP stimulation for the figures in the text to ensure robust effects on air sac pressure.

To test that we were only stimulating the axons of motor neurons, we applied curare which locally blocks the neuromuscular junction, to EXP and compared both EMG and stimulation effects with those when saline was used to paralyze EXP would endanger the wellbeing of the animal. However, confirming that the initial upswing of the respiratory cycle was slower than normal, we were able to compare the same patterns for a single-millisecond shift in a single spike (SI Appendix, Fig. S8), and significant differences were seen in some birds.

To isolate the effects of EXP stimulation on air sac pressure, the mean pressure waveform of 20 previous unstimulated (catch) respiratory periods was similarly subtracted from the stimulated pressure waveform. A trailing window was used to eliminate the possibility of future effects of stimulation affecting our mean subtraction. For this pressure residual calculation, waveform forms were aligned to the phase of the respiratory pattern at which the stimulation, instead of the spike pattern, occurred. All catch-subtracted pressure waveforms were averaged within a given stimulation pattern, with the SE calculated at each time point. To compare responses to different stimuli, d’ and its estimated error were calculated as above.

In vitro force measurements were compared following different stimulation patterns using the same d’ analysis as both for the recording and the in vitro stimulation analyses above. Because of difficulty obtaining Bengalese finches in Denmark (where our in vitro studies took place), we used two male and three female wild birds for analysis. Stimulation waveforms were only being conducted on male Bengalese finches, no qualitative differences were observed between sexes aside from normal intersubject variability (SI Appendix, Fig. S9). Because the sample size was small for each sex, we could not perform a statistical comparison between the two groups.

Mutual Information: Consecutive ISIs. To estimate the scale of temporal structures in the neural code, we evaluate the mutual information between subsequent ISIs that are ≤ 30 ms-long (and hence fall into the same breathing cycle) and also between these ISIs corrupted by a Gaussian noise with various SDs (Fig. 1C). Mutual information between two continuous variables x and y is defined as

\[
m(x, y) = \int dx dy P(x, y) \log \frac{P(x, y)}{P(x)P(y)}
\]

and a sum replaces the integral for discrete variables. Mutual information is a measure of statistical dependency that does not assume normality of the underlying distribution in contrast to the more familiar correlation coefficient, and it measures all statistical dependences between the two variables, such that it is zero if and only if the two variables are completely statistically independent. Because ISIs are non-Gaussian distributed, using mutual information is more appropriate than simpler dependency measures. Mutual information is measured in bits. Measurement of x provides one bit of information about y (and vice versa) if the measurement of x allows us to answer one binary (yes/no) question about y.

Estimation of mutual information from empirical data is a complex problem (16, 35). To solve this problem for mutual information between two real-valued consecutive ISIs, we use the k-nearest neighbors estimator (36). The method detects structures in the underlying probability distribution by estimating distances to the k-nearest neighbors of each data point. By varying k, one explores structures in the underlying data on different scales. We choose which k to use by calculating the mutual information for varying amounts of data (using different size subsets of the full data) and detecting the (absence of the) sample size-dependent bias (5, 37, 38). The joint distribution of consecutive ISIs is smooth, and hence a broad range of k near k = 10 produces unbiased information estimates. To identify possible sample size-dependent biases and calculate the error bars, we divided the dataset of N samples into nonoverlapping subsamples of size N/m, with the inverse data fraction m = 2, …, 10. We calculated mutual information in each subsample and then evaluated the SD of the estimates for a given m, where 10 independent partitions were done for each m (SI Appendix, Fig. S10A). We then fitted these empirical variances to the usual 1/(sample size) law by performing a linear regression log s²(m) = A + log m. We then estimated the variance for the full dataset by setting m = 1 (SI Appendix, Fig. S10B). The same analysis was performed for both the original dataset and the jittered datasets to the extent that the error bars for different sample sizes (different values of m) agree with each other within the error bars, the estimate of the mutual information likely does not have a sample size-dependent bias (SI Appendix, Fig. S10A).
Mutual Information: Spikes and Pressure. We calculated the mutual information between 20-ms-long spike trains and 100-ms-long pressure residuals (Fig. 2B). These time scales were chosen because spikes have to be closer to each other than about 20 ms to cause supralinear effects in muscle activation (SI Appendix, Fig. S12) and because effects of spikes on pressure appear to last less than 100 ms (Fig. 2C). We focused on spike trains that began at the phase $\varphi \approx 0.8\pi$ in the breathing cycle (Materials and Methods, Data Analysis), because the most spikes occurs near that phase (nearby choices reveal similar results). For the pressure patterns, we started 10 ms after 0.8$\pi$ phase point, because it takes about 10 ms to affect behavior (Fig. 4D). Importantly, although the starting point of patterns/pressures was chosen based on the phase, the time within the patterns and the pressure traces were not rescaled. The autocorrelation time of pressure residuals is about 10 ms for all EMG birds, and therefore, we chose to describe the 100-ms pressure residuals using $p = 11$ real-valued data points spread 10 ms apart (SI Appendix, Fig. S11A).

With this choice, we decided to calculate the mutual information between an 11D pressure vector ($\mathbf{y}$ in Eq. 1) and the spiking vector ($\mathbf{x}$ in Eq. 1). For this calculation, we modified the $k$-nearest neighbor mutual information estimator (36) in the following way. We rewrote the mutual information between the spikes and the pressure as

$$ I(x,y) = I(n,y) + \sum_{n} P(n) I(x,y|n), $$

where $n$ is the number of spikes in the 20-ms spike train. The first term on the right-hand side of Eq. 2 is because the information between the firing rate alone and the pressure, and the second is the information between the timing alone and the pressure. This partitioning allowed us to automatically estimate the relative contribution of each of these terms.

We estimated each of the information quantities $I(n,y)$ and $I(x,y|n)$ using the $k$-nearest neighbors estimator [recall that $I(x,y)=0$, where $x$ is an $n$-dimensional vector of spike timings for a fixed spike count $n$ (we did not discretize time for this analysis)]. Because mutual information is reparameterization-invariant, we rescaled the number of spikes to have zero mean and unit variance, and then additionally reparameterized the spike times and the pressure values to have normal marginal distributions, so that the $i$ th value of the variable in a set of $N$ samples was mapped into the value that corresponds to the cumulative distribution of a unit variance normal being equal to $(i−1)/N$. Having unit variances ensured that every variable contributes similarly to determining nearest neighbors of data points. Furthermore, making variables to have exactly normal marginal distributions decreased the influence of outliers (we have verified that this reparameterization is a negligible effect data with no outliers).

As in Materials and Methods, Mutual Information: Consecutive ISIs, we found the value of $k$ that produced no sample size-dependent drift in the estimate of $I(x,y)$; for EMG1, this value was $k = 3$ (SI Appendix, Fig. S11 A and B). We similarly estimated error bars by subampling the data, estimating the variance of each subsample, and extrapolating to the full dataset size. For some birds, no value of $k$ produced estimates with zero sample size-dependent bias within error bars. In these cases, we chose the $k$ that resulted in the smallest sample size-dependent drift. In all such cases (bird identification EMG3–EMG6) (included by empty boxes in Fig. 2A), the drift was upward as the sample size increased, indicating that the value of the mutual information calculated at the full sample was an underestimate. Underestimating the mutual information makes it harder to show that spike timing contains information about the behavior, and yet, seven of eight birds in Fig. 2A showed statistically significant information in spike timing.

We additionally verified that $p = 11$ points for characterization of the pressure is the right choice by estimating $I(n,y)$ at different values of $p$. As $p$ increases from one, more features in the pressure get sampled, uncovering more information. Information plateaus near $p \sim 10$, indicating that all relevant features in the data have been recovered, and in some cases, it finally leaves the plateau at larger $p$ because of undersampling, indicating that such a reparameterization should be discarded.

Finally, we note that in $I(n,y)$, $n$ is a discrete variable, making the use of the $k$-nearest neighbors estimator problematic. We address the issue by injecting each discrete datum with small Gaussian random noise. All data shown used the noise with $SD = 10^{-4}$, but other values in the range $10^{-6} \ldots 10^{-2}$ were tried with no discernible differences.

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