Whole-Body Imaging of Neural and Muscle Activity during Behavior in *Hydra vulgaris*: Effect of Osmolarity on Contraction Bursts

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Visual Abstract

Robust effect of osmolarity on *Hydra* activity

- **Low Osmolarity**
  - Locomotion
  - CB Neuronal Activity

- **High Osmolarity**
  - Locomotion Contraction
  - Ectoderm Muscle
  - CB Neuronal Activity
Significance Statement

We imaged whole-body muscle and neuronal activity in Hydra in response to different physiological and environmental conditions. Osmolarity bidirectionally altered Hydra contractile behavior in a reflexive fashion. These changes were accompanied by specific changes in the activity of one neuronal circuit and one set of muscles. By providing neurobiological mechanisms for a reflex in a cnidarian, this work is a step toward comprehensive deciphering of the mechanisms of animal behavior by measuring the activity of all neurons and muscle cells.

The neural code relates the activity of the nervous system to the activity of the muscles to the generation of behavior. To decipher it, it would be ideal to comprehensively measure the activity of the entire nervous system and musculature in a behaving animal. As a step in this direction, we used the cnidarian Hydra vulgaris to explore how physiological and environmental conditions alter simple contractile behavior and its accompanying neural and muscle activity. We used whole-body calcium imaging of neurons and muscle cells and studied the effect of temperature, media osmolarity, nutritional state, and body size on contractile behavior. In mounted Hydra preparations, changes in temperature, nutrition state, or body size did not have a major effect on neural or muscle activity, or on contractile behavior. But changes in media osmolarity systematically altered contractile behavior and foot detachments, increasing their frequency in hypo-osmolar media solutions and decreasing it in hyperosmolar media. Similar effects were seen in ectodermal, but not in endodermal muscle. Osmolarity also bidirectionally changed the activity of contraction burst (CB) neurons, but did not affect the network of rhythmic potential (RP) neurons in the ectoderm. These findings show osmolarity-dependent changes in the activity of CB neurons and ectodermal muscle, consistent with the hypothesis that CB neurons respond to media hypo-osmolarity, activating ectodermal muscle to generate CBs. This dedicated reflex could serve as an excretory system to prevent osmotic injury. This work demonstrates the feasibility of studying an entire neuronal and muscle activity in a behaving animal.

Introduction

Calcium imaging of neuronal circuits (Yuste and Katz, 1991) has enabled recent investigations of the circuit basis of animal behavior in a number of transparent organisms such as Caenorhabditis elegans, Drosophila larvae, and zebrafish embryos (Nagel et al., 2005; Liewald et al., 2008; Honjo et al., 2012; Cong et al., 2017; Kim et al., 2017). While these studies have focused on particular parts of the nervous system, to systematically understand the neural code, i.e., the relation between the activity of a nervous system and behavior, it would be ideal to measure the activity of the entire nervous system and the entire muscular tissue during the entire behavioral repertoire of an animal. This is now possible with the transparent fresh-water cnidarian Hydra vulgaris, using transgenic strains that express calcium indicators in every neuron (Dupre and Yuste, 2017) and every muscle cell of the body (Szymanski and Yuste, 2019), and applying machine learning to systematically analyze its behavior (Han et al., 2018). Hydra has a simple body consisting of ectoderm and endoderm myoepithelial cells. Muscular processes, myonemes, run longitudinally in the ectoderm and radially in the endoderm. Thus, each myoepithelial layer can have distinct functions in different behaviors, but can also become coactive during sustained contractions (Szymanski and Yuste, 2019).

Hydra has one of the simplest nervous system in evolution, with several hundreds to a few thousand neurons, depending on the size of the animal (Hadzi, 1909; Parker, 1919; Westfall et al., 1991). The simplicity of Hydra’s system gives hope that systematic measurements of the neural and muscular activity of behaving Hydra could be used to decipher the mechanisms of behavior. Hydra neurons are believed to be multifunctional. A sensory neuron with sensory cilia also synapses with epithelial cells as a motor neuron (Westfall, 1973). These neurons are organized in two independent nerve nets, in the ectoderm and endoderm (Dupre and Yuste, 2017). Hydra’s nerve nets are distributed throughout the body of the animal, without any cephalization (Epp and Tardent, 1978). Several independent neuronal circuits, interspersed within the nerve nets, are active synchronously in an oscillating manner. The
main ones named contraction burst (CB) and rhythmic potential (RP1) circuits, involve independent groups of ectoderm neurons, whereas a third circuit, the RP2 circuit, involves endodermal cells (Dupre and Yuste, 2017). These three circuits are associated with three different motor behaviors: CBs (CB circuit), elongation (RP1), and egestion (RP2; Dupre and Yuste, 2017).

Hydra is a fresh-water animal living in ponds, lakes and streams. Because of this, Hydra experiences fluctuations in temperature and osmolarity as well as the amount of food available, which determines its body size. Previous research has described Hydra responses to changes in environmental and physiological conditions. Those include decreases in contractions with increased osmolarity (Benos and Prusch, 1973) and after feeding (Grosvenor et al., 1996; Rushforth and Hofman, 1972) and necrosis after acute increases in temperature (Bosch et al., 1988). These past studies show that external modification of Hydra behavior is possible.

Motivated by this work, we explored systematically how different environmental conditions affect Hydra behavior, focusing on body contractions. Do so do so, we performed measurements of Hydra behavior under standard conditions in mounted and freely behaving animals and used calcium imaging to measure how neurons and muscular cells respond to physiological and environmental conditions important for their survival. Experimental conditions included high or low osmolarity (control, 50 mM sucrose or diH2O), temperature (23°C or 30°C), food (zero, one, and four shrimp per day for a week), and body size (mature vs newly released buds). In each of these conditions, we measured the number of contractions and foot detachments in behavior assays, the ectodermal and endodermal muscle activity, and the activity of the CB and RP1 neuronal circuits.

We expected to see major changes in behavior, neuronal, and muscle activity, as the chosen conditions are essential to Hydra survival. But surprisingly, in mounted preparations, we only found robust effects due to osmolarity. Increased osmolarity decreased contractions frequency, consistent with Benos and Prusch (1973), decreased foot detachments and also decreased the activity of CB neurons and ectodermal muscle cells, whereas decreased osmolarity had opposite effects, as a reflex. Our results indicate that Hydra’s CB circuit senses osmolarity to control ectodermal muscle and generate contraction behaviors, revealing a specific neuro-muscular reflex that probably evolved for osmoprotection.

Materials and Methods

Materials
Sucrose and sea salt were purchased from Sigma. Brine shrimp, Artemia nauplii, were obtained from Brine Shrimp Direct. We used transgenic Hydra expressing GCaMP6s in neurons (Dupre and Yuste, 2017) or in ectoderm/endoderm muscle cells (Szymanski and Yuste, 2019).

Hydra culture

Hydra were maintained in media composed of 1.3 mM CaCl2, 0.02 mM MgCl2, 0.03 mM KNO3, 0.5 mM NaHCO3, and 0.08 mM MgSO4 in an 18°C incubator. Hydra were fed brine shrimp three times a week and were starved for 2 d before an experiment.

Environmental or physiological conditions

The following conditions were used. (1) Food: Hydra were fed zero, one, or four shrimp every day for a week. Hydra were starved for 1 d before an experiment. (2) Size: Hydra with large (~1 cm) or small (~0.3 mm) sizes, chosen after bud separation, were fed once. (3) Temperature: room (23°C) or high temperature (30°C). (4) Osmolarity: Hydra were imaged in media with low osmolarity (diH2O, 0 mOsm/l), control medium (control, Hydra media, 5 mOsm/l, fresh water is usually between 2 and 8 mOsm/l), or high (50 mM sucrose, 50 mOsm/l) osmolarity.

Calcium imaging

Wide-field calcium imaging of Hydra was conducted at 2 Hz using a fluorescence dissecting microscope (Leica M165) equipped with a long-pass GFP filter set (Leica filter set ET GFP M205FA/M165FC), 1.63× Plan Apo objective, and a sCMOS camera (Hamamatsu ORCA-Flash 4.0). A mercury arc lamp was used to illuminate the sample. Hydra were mounted between coverslips with 100- to 200-μm spacers, depending on animal thickness. All imaging was conducted at a room temperature ~23°C unless indicated.

Behavior analysis

The number of contractions and foot detachments were manually scored from calcium imaging movies (mounted Hydra between coverslips) or movies of freely moving Hydra in glass-bottom dishes (MatTek). Five animals were placed per well (depth is 700–750 μm) for 1-h recordings.

Analysis of neural and muscular activity

Values for whole-body fluorescent intensity in each frame over time were obtained with ImageJ and used to detect CB and RP1 pulses using a semi-automated program in MATLAB. Whole-body muscle activity was analyzed in the same manner.

Analysis of body column width

Hydra were imaged at 0.5 Hz using a dissection microscope (Leica M165), 1.63× Plan Apo objective, and sCMOS camera (Hamamatsu ORCA-Flash 4.0). Hydra were mounted between coverslips with around 200-μm spacer in control media or in high-osmolarity solution (50 mM sucrose). To measure width, the body column of Hydra was fitted into ellipse using a program written by MATLAB. The lowest values from each cycle were used to calculate average width at the end of the elongation.

Statistical methods

Data are shown as average ± SEM in figures and in the text. Two-tailed unpaired Student’s t test or one-way
Table 1: Statistical tests and results

| Figure | Description | Methods | 95% CI of difference | Significant | p value |
|--------|-------------|---------|----------------------|-------------|---------|
| 1B     | Food: 0 vs 1 | 1       | -2.355 to 6.718      | No          | 0.4707  |
|        | Food: 0 vs 4 | 1       | -3.537 to 5.357      | No          | 0.8506  |
|        | Food: 1 vs 4 | 1       | -5.718 to 3.355      | No          | 0.7981  |
|        | Osmo: Ctr vs low | 1 | -6.364 to 4.864 | No | 0.9432 |
|        | Osmo: Ctr vs high | 1 | 0.2450 to 10.25 | Yes | 0.038  |
|        | Osmo: Low vs high | 1 | 0.3148 to 11.69 | Yes | 0.0367 |
|        | Size: Ctr vs small | 2 | -9.991 to -2.937 | No | 0.0008 |
|        | Temp: Ctr vs high | 2 | -0.5233 to 6.023 | No | 0.958  |
| 1C     | Food: 0 vs 1 | 1 | -2.198 to 1.335 | No | 0.8207 |
|        | Food: 0 vs 4 | 1 | -1.448 to 2.085 | No | 0.898  |
|        | Food: 1 vs 4 | 1 | -0.9775 to 2.478 | No | 0.5411 |
|        | Osmo: Ctr vs low | 1 | -2.688 to 0.3822 | No | 0.1728 |
|        | Osmo: Ctr vs high | 1 | 1.034 to 3.682 | Yes | 0.0003 |
|        | Osmo: Low vs high | 1 | 1.958 to 5.064 | Yes | <0.0001 |
|        | Size: Ctr vs small | 2 | 0.08979 to 2.894 | Yes | 0.0378 |
|        | Temp: Ctr vs high | 2 | -0.9724 to 1.722 | No | 0.5716 |
| 1E     | Food: 0 vs 1 | 1 | -1.740 to 2.407 | No | 0.9195 |
|        | Food: 0 vs 4 | 1 | 0.3931 to 4.540 | Yes | 0.0164 |
|        | Food: 1 vs 4 | 1 | 0.05976 to 4.207 | Yes | 0.0426 |
|        | Osmo: Ctr vs low | 1 | 0.7542 to 6.579 | No | 0.01 |
|        | Osmo: Ctr vs high | 1 | -0.2806 to 4.642 | No | 0.0925 |
|        | Osmo: Low vs high | 1 | -3.947 to 0.9758 | Yes | 0.3223 |
|        | Size: Ctr vs small | 2 | 4.300 to 21.17 | No | 0.0059 |
|        | Temp: Ctr vs high | 2 | -6.122 to -2.412 | Yes | <0.0001 |
| 1F     | Food: 0 vs 1 | 1 | -1.026 to 0.09217 | No | 0.1178 |
|        | Food: 0 vs 4 | 1 | -1.426 to -0.3078 | Yes | 0.0014 |
|        | Food: 1 vs 4 | 1 | -0.9588 to 0.1588 | No | 0.2029 |
|        | Osmo: Ctr vs low | 1 | 1.610 to 3.724 | Yes | <0.0001 |
|        | Osmo: Ctr vs high | 1 | 0.6877 to 2.474 | Yes | 0.0002 |
|        | Osmo: Low vs high | 1 | -1.979 to -0.1925 | Yes | 0.0134 |
|        | Size: Ctr vs small | 2 | -0.1413 to 0.9413 | No | 0.1413 |
|        | Temp: Ctr vs high | 2 | -2.683 to -0.9838 | Yes | <0.0001 |
| 2C     | Food: 0 vs 1 | 1 | -176.2 to 327.3 | No | 0.8033 |
|        | Food: 0 vs 4 | 1 | -257.1 to 281.1 | No | 0.9991 |
|        | Food: 1 vs 4 | 1 | -315.3 to 188.2 | No | 0.8705 |
|        | Osmo: Ctr vs low | 1 | -147.0 to 148.8 | No | 0.9998 |
|        | Osmo: Ctr vs high | 1 | 12.02 to 307.8 | Yes | 0.0356 |
|        | Osmo: Low vs high | 1 | -6.375 to 324.4 | No | 0.0588 |
|        | Size: Ctr vs small | 2 | -138.6 to 167.4 | No | 0.8303 |
|        | Temp: Ctr vs high | 2 | -132.3 to 152.1 | No | 0.8738 |
| 2D     | Food: 0 vs 1 | 1 | -318.4 to 280.4 | No | 0.981 |
|        | Food: 0 vs 4 | 1 | -473.7 to 125.1 | No | 0.2655 |
|        | Food: 1 vs 4 | 1 | -475.4 to 164.8 | No | 0.378 |
|        | Osmo: Ctr vs low | 1 | -174.6 to 237.8 | No | 0.9107 |
|        | Osmo: Ctr vs high | 1 | -106.8 to 282.0 | No | 0.4681 |
|        | Osmo: Low vs high | 1 | -150.2 to 262.2 | No | 0.7494 |
|        | Size: Ctr vs small | 2 | 2.523 to 332.7 | Yes | 0.0473 |
|        | Temp: Ctr vs high | 2 | -20.35 to 199.1 | No | 0.0955 |
| 2E     | Food: 0 vs 1 | 1 | -13.68 to 17.47 | No | 0.939 |
|        | Food: 0 vs 4 | 1 | -18.72 to 15.75 | No | 0.948 |
|        | Food: 1 vs 4 | 1 | -20.83 to 13.08 | No | 0.8034 |
|        | Osmo: Ctr vs low | 1 | -19.22 to 0.7527 | No | 0.0686 |
|        | Osmo: Ctr vs high | 1 | -6.431 to 13.54 | No | 0.5872 |
|        | Osmo: Low vs high | 1 | 1.625 to 23.96 | Yes | 0.0273 |
|        | Size: Ctr vs small | 2 | -7.207 to 13.24 | No | 0.5081 |
|        | Temp: Ctr vs high | 2 | -4.729 to 13.86 | No | 0.2836 |

(Continued)
| Figure | Description | Methods | 95% CI of difference | Significant | p value |
|--------|-------------|---------|----------------------|-------------|---------|
| 2F     | Food: 0 vs 1 | 1       | -19.97 to 13.83      | No          | 0.9455  |
| 2F     | Food: 0 vs 4 | 1       | -30.51 to 3.289      | No          | 0.1296  |
| 2F     | Food: 1 vs 4 | 1       | -28.61 to 7.526      | No          | 0.3429  |
| 2F     | Osmo: Ctr vs low | 1 | -18.81 to 7.069 | No | 0.4634 |
| 2F     | Osmo: Ctr vs high | 1 | -7.909 to 16.49 | No | 0.6216 |
| 2F     | Osmo: Low vs high | 1 | -2.777 to 23.11 | No | 0.1307 |
| 2F     | Size: Ctr vs small | 2 | 2.745 to 22.78 | Yes | 0.0188 |
| 2F     | Temp: Ctr vs high | 2 | 0.5891 to 18.07 | Yes | 0.0396 |
| 2G     | Food: 0 vs 1 | 1       | -1.613 to 1.854      | No          | 0.9773  |
| 2G     | Food: 0 vs 4 | 1       | -0.8072 to 2.899     | No          | 0.2839  |
| 2G     | Food: 1 vs 4 | 1       | -0.8077 to 2.659     | No          | 0.3176  |
| 2G     | Osmo: Ctr vs low | 1 | 1.379 to 4.514 | Yes | 0.0017 |
| 2G     | Osmo: Ctr vs high | 1 | 0.5862 to 3.721 | Yes | 0.0108 |
| 2G     | Osmo: Low vs high | 1 | -0.9592 to 2.545 | No | 0.4373 |
| 2G     | Size: Ctr vs small | 2 | -7.207 to 13.24 | No | 0.5081 |
| 2G     | Temp: Ctr vs high | 2 | -4.729 to 13.86 | Yes | 0.2936 |
| 2H     | Food: 0 vs 1 | 1       | -2.115 to 3.010      | No          | 0.8669  |
| 2H     | Food: 0 vs 4 | 1       | -2.517 to 2.607      | No          | 0.9985  |
| 2H     | Food: 1 vs 4 | 1       | -3.142 to 2.336      | No          | 0.9032  |
| 2H     | Osmo: Ctr vs low | 1 | -0.4909 to 5.189 | No | 0.1103 |
| 2H     | Osmo: Ctr vs high | 1 | -2.518 to 2.609 | No | 0.9988 |
| 2H     | Osmo: Low vs high | 1 | -5.037 to 0.4289 | No | 0.1028 |
| 2H     | Size: Ctr vs small | 2 | -2.405 to 1.561 | No | 0.6416 |
| 2H     | Temp: Ctr vs high | 2 | -2.067 to 1.073 | No | 0.4785 |
| 3C     | Food: 0 vs 1 | 1       | -168.3 to 323.3      | No          | 0.6736  |
| 3C     | Food: 0 vs 4 | 1       | -276.1 to 190.3      | No          | 0.8709  |
| 3C     | Food: 1 vs 4 | 1       | -353.6 to 112.8      | No          | 0.3699  |
| 3C     | Osmo: Ctr vs low | 1 | -448.7 to -9.334 | Yes | 0.0406 |
| 3C     | Osmo: Ctr vs high | 1 | 5.853 to 341.4 | Yes | 0.0422 |
| 3C     | Osmo: Low vs high | 1 | 192.3 to 612.9 | Yes | 0.0005 |
| 3C     | Size: Ctr vs small | 2 | -96.12 to 287.7 | No | 0.288 |
| 3C     | Temp: Ctr vs high | 2 | -173.1 to 196.4 | No | 0.8855 |
| 3D     | Food: 0 vs 1 | 1       | -880.9 to 180.7      | No          | 0.2575  |
| 3D     | Food: 0 vs 4 | 1       | -594.9 to 429.8      | No          | 0.9638  |
| 3D     | Food: 1 vs 4 | 1       | -198.1 to 743.2      | No          | 0.3624  |
| 3D     | Osmo: Ctr vs low | 1 | -398.8 to 148.8 | No | 0.4752 |
| 3D     | Osmo: Ctr vs high | 1 | -285.7 to 221.3 | No | 0.9411 |
| 3D     | Osmo: Low vs high | 1 | -160.7 to 346.3 | No | 0.6139 |
| 3D     | Size: Ctr vs small | 2 | -189.6 to 358.8 | No | 0.497 |
| 3D     | Temp: Ctr vs high | 2 | -430.9 to 270.0 | No | 0.5946 |
| 3E     | Food: 0 vs 1 | 1       | -20.65 to 14.51      | No          | 0.8669  |
| 3E     | Food: 0 vs 4 | 1       | -31.19 to 3.966      | No          | 0.1246  |
| 3E     | Food: 1 vs 4 | 1       | -29.33 to 8.249      | No          | 0.2875  |
| 3E     | Osmo: Ctr vs low | 1 | -18.11 to 16.66 | No | 0.9932 |
| 3E     | Osmo: Ctr vs high | 1 | -9.921 to 18.47 | No | 0.7082 |
| 3E     | Osmo: Low vs high | 1 | -12.39 to 22.38 | No | 0.7294 |
| 3E     | Size: Ctr vs small | 2 | -4.836 to 10.90 | No | 0.406 |
| 3E     | Temp: Ctr vs high | 2 | -4.654 to 17.22 | No | 0.2095 |
| 3F     | Food: 0 vs 1 | 1       | -878.5 to 168.3      | No          | 0.1957  |
| 3F     | Food: 0 vs 4 | 1       | -583.0 to 417.9      | No          | 0.8911  |
| 3F     | Food: 1 vs 4 | 1       | -187.2 to 732.3      | No          | 0.2734  |
| 3F     | Osmo: Ctr vs low | 1 | -23.83 to 22.78 | No | 0.998 |
| 3F     | Osmo: Ctr vs high | 1 | -25.81 to 12.84 | No | 0.5477 |
| 3F     | Osmo: Low vs high | 1 | -28.53 to 16.61 | No | 0.7608 |
| 3F     | Size: Ctr vs small | 2 | -6.952 to 12.37 | No | 0.536 |
| 3F     | Temp: Ctr vs high | 2 | -4.654 to 17.22 | No | 0.2095 |

(Continued)
Figs. 2

the code was used to analyze neural and muscular activity in osmolarity (Shimizu and Fujisawa, 2003), and excreting excess water from the body (Macklin et al., 1973). Another common behavior of Hydra is locomotion, i.e., translocation of the foot from one place to another. This is initiated by “foot detachment,” where the basal disk detaches from a substrate’s surface (Rodrigues et al., 2016).

We first tested how these two simple behaviors of Hydra were affected by various physiological and environmental conditions. Conditions included amount of food, osmolarity or temperature of media, and the size of an animal. For the amount of food, Hydra was starved for 1 d before an experiment. For each condition, the frequency and duration of contractions and foot detachments were measured. In mounted preparations, where specimens are place in a microscope chamber with a spacer, osmolarity or body size robustly changed the frequency of contractions (Fig. 1A–C; see Materials and Methods). High-osmolarity media significantly decreased the frequency of contractions compared with control (Fig. 1B, \( p = 0.0380 \)) or low-osmolarity conditions (Fig. 1B, \( p = 0.0367 \)). Similarly, high-osmolarity media significantly decreased the number of foot detachments compared with control (Fig. 1C, \( p = 0.0003 \)) or low-osmolarity conditions (Fig. 1C, \( p < 0.0001 \)). Also, smaller size Hydra had more contractions (Fig. 1B, \( p = 0.0008 \)) but fewer foot detachments (Fig. 1C, \( p = 0.0378 \)).

As mounting restricts Hydra behavior, because of compression of body between glass coverslips, we also imaged freely moving Hydra under widefield illumination in the same conditions (Movie 1). Consistent with results in mounted preparations (Fig. 1B,C), in free moving animals, high osmolarity also decreased the number of contractions compared with low osmolarity (Fig. 1E, \( p = 0.0100 \)) and the number of foot detachments, compared with control (Fig. 1F, \( p = 0.0134 \)) or low-osmolarity conditions (Fig. 1F, \( p < 0.0001 \)). But, unlike mounted preparations, well-fed (four shrimp per day) Hydra did not show any difference in behavior, comparing with control conditions. (Fig. 1B, \( p = 0.8506 \) for contractions; Fig. 1C, \( p = 0.8980 \) for detchaments). Also, in well-fed freely moving Hydra, the number of contractions decreased (Fig. 1E, \( p = 0.0164 \)), while the number of foot detachments increased (Fig. 1F, \( p < 0.0001 \)) in freely moving animals. Overall, osmolarity was the only parameter that robustly changed behavior in both freely moving and mounted specimens. As motor behaviors must be generated as a result of contractile force derived from muscle, we next assessed how these changes in behaviors are accounted for the activity of muscle cells. For these experiments, we used exclusively mounted preparation, as it is yet not feasible to image and reconstruct the activity of neurons and muscle cells in freely moving animals.

Table 1: Continued

| Figure | Description | Methods | 95% CI of difference | Significant | \( p \) value |
|-------|-------------|---------|----------------------|-------------|--------------|
| 3G    | Food: 0 vs 1 | 1       | \(-0.6621 to 3.852\) | No          | 0.1787       |
|       | Food: 0 vs 4 | 1       | \(-1.813 to 2.470\)  | No          | 0.908        |
|       | Food: 1 vs 4 | 1       | \(-3.408 to 0.8747\) | No          | 0.2816       |
|       | Osmo: Ctr vs low | 1 | \(-6.867 to –1.087\) | Yes         | 0.0066       |
|       | Osmo: Ctr vs high | 1 | \(-0.5830 to 4.411\) | No          | 0.1499       |
|       | Osmo: Low vs high | 1 | 3.165 to 8.437 | Yes | <0.0001 |
|       | Size: Ctr vs small | 2 | \(-1.356 to 3.915\) | No          | 0.3006       |
|       | Temp: Ctr vs high | 2 | \(-0.8085 to 4.736\) | No          | 0.1378       |
| 3H    | Food: 0 vs 1 | 1       | \(-9.974 to 2.307\)  | No          | 0.2484       |
|       | Food: 0 vs 4 | 1       | \(-6.661 to 4.990\)  | No          | 0.919        |
|       | Food: 1 vs 4 | 1       | \(-2.828 to 8.823\)  | No          | 0.3722       |
|       | Osmo: Ctr vs low | 1 | \(-389.9 to 139.9\) | No          | 0.4301       |
|       | Osmo: Ctr vs high | 1 | \(-382.1 to 229.7\) | No          | 0.7785       |
|       | Osmo: Low vs high | 1 | \(-257.1 to 354.7\) | No          | 0.901        |
|       | Size: Ctr vs small | 2 | \(-3.063 to 4.773\) | No          | 0.6283       |
|       | Temp: Ctr vs high | 2 | \(-6.933 to 3.432\) | No          | 0.4402       |
| 4D    | High vs Ctr | 2       | 5.575 to 43.27       | Yes         | 0.0193       |

Method 1 indicates ordinary one-way ANOVA, Tukey’s multiple comparison test, and method 2 indicates unpaired t test. The four conditions used were food (Food), osmolarity (Osmo), size (Size), and temperature (Temp). Control medium (ctr).

ANOVA with Tukey’s multiple comparison test were conducted in GraphPad Prism software (Table 1).

Code accessibility

All code is available as Extended Data 1. The MATLAB code was used to analyze neural and muscular activity in Figs. 2–4.

Results

**Hydra’s contractile behavior affected by media osmolarity**

*Hydra* has a small repertoire of highly stereotypical behaviors (Han et al., 2018). One of the most noticeable ones is spontaneous periodic contractions, known as “contraction bursts” (Wagner, 1905; Reis and Pierro, 1955; Passano and Mccullough, 1964). Possible roles of contractions by *Hydra* include foraging, protection by retraction (Miglietta et al., 2000; Swain et al., 2015), food digestion (Shimizu and Fujisawa, 2003), and excreting excess water from the body (Macklin et al., 1973). Another common behavior of *Hydra* is locomotion, i.e., translocation of the foot from one place to another. This is initiated by “foot detachment,” where the basal disk detaches from a substrate’s surface (Rodrigues et al., 2016).

We first tested how these two simple behaviors of *Hydra* were affected by various physiological and environmental conditions. Conditions included amount of food, osmolarity or temperature of media, and the size of an animal. For the amount of food, *Hydra* was starved for 1 d before an experiment. For each condition, the frequency and duration of contractions and foot detachments were measured. In mounted preparations, where specimens are place in a microscope chamber with a spacer, osmolarity or body size robustly changed the frequency of contractions (Fig. 1A–C; see Materials and Methods). High-osmolarity media significantly decreased the frequency of contractions compared with control (Fig. 1B, \( p = 0.0380 \)) or low-osmolarity conditions (Fig. 1B, \( p = 0.0367 \)). Similarly, high-osmolarity media significantly decreased the number of foot detachments compared with control (Fig. 1C, \( p = 0.0003 \)) or low-osmolarity conditions (Fig. 1C, \( p < 0.0001 \)). Also, smaller size *Hydra* had more contractions (Fig. 1B, \( p = 0.0008 \)) but fewer foot detachments (Fig. 1C, \( p = 0.0378 \)).

As mounting restricts *Hydra* behavior, because of compression of body between glass coverslips, we also imaged freely moving *Hydra* under widefield illumination in the same conditions (Movie 1). Consistent with results in mounted preparations (Fig. 1B,C), in free moving animals, high osmolarity also decreased the number of contractions compared with low osmolarity (Fig. 1E, \( p = 0.0100 \)) and the number of foot detachments, compared with control (Fig. 1F, \( p = 0.0134 \)) or low-osmolarity conditions (Fig. 1F, \( p < 0.0001 \)). But, unlike mounted preparations, well-fed (four shrimp per day) *Hydra* did not show any difference in behavior, comparing with control conditions. (Fig. 1B, \( p = 0.8506 \) for contractions; Fig. 1C, \( p = 0.8980 \) for detchaments). Also, in well-fed freely moving *Hydra*, the number of contractions decreased (Fig. 1E, \( p = 0.0164 \)), while the number of foot detachments increased (Fig. 1F, \( p < 0.0001 \)) in freely moving animals. Overall, osmolarity was the only parameter that robustly changed behavior in both freely moving and mounted specimens. As motor behaviors must be generated as a result of contractile force derived from muscle, we next assessed how these changes in behaviors are accounted for the activity of muscle cells. For these experiments, we used exclusively mounted preparation, as it is yet not feasible to image and reconstruct the activity of neurons and muscle cells in freely moving animals.

**Bidirectional effects of osmolarity on ectodermal muscle activity**

*Hydra*’s body is composed of two layers of cells: ectodermal and endodermal epitheliomuscular tissues. Both
epithelia are separated by an extracellular matrix called mesoglea. Inside these epithelial layers, there is a gastrovascular cavity that functions as a both gut and vasculature and carries nutrients to the entire body (Shimizu and Fujisawa, 2003). Both ectoderm and endoderm epithelio-muscular tissues generate action potentials (Dupre and Yuste, 2017; Szymanski and Yuste, 2019), which likely propagate through gap junctions (Westfall et al., 1980). These muscle cells contract in a calcium-dependent manner through myonemes, intracellular muscle processes that run longitudinally along the ectoderm and radially in

Figure 1. Effect of experimental conditions on contractions and locomotion behavior. Data from mounted preparations in A-C and from 1-h freely moving Hydra in D-F. A, Upper images, Changes in body length during longitudinal contractions. Lower images, Foot detachment. Scale bar, 500 µm. Number of contractions (B) and foot detachments (C) were counted. D, Upper images depict changes in body length during longitudinal contractions. Lower images depict foot detachment followed by locomotion. Scale bar, 1 mm. Number of contractions (E) and foot detachment/locomotion (F) were counted. The four conditions used were food (Food), osmolarity (Osmo), size (Size), and temperature (Temp). Control medium (ctr). Error bars shown as mean ± SEM, with symbol marks denoting data points from individual Hydra (N = 9–16 for B, C; N = 15–30 for E, F). Tukey’s multiple comparisons tests were performed following one-way ANOVA for osmolarity experiment, and Student’s t test was performed for others: ns, p ≥ 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

Movie 1. Freely moving Hydra in control media. Animals were allowed to move freely in a Petri dish. Video was taken at 2 Hz and speed up 40-fold. Scale bar, 1 mm. [View online] Movie 2. Ectoderm muscle activity in control media. The animal was allowed to move between coverslips in mounted configuration. Video was taken at 2 Hz and speed up 20-fold. Scale bar, 500 µm. [View online]
the endoderm (Otto, 1977). Thus, Hydra generates motor behavior such as contractions and elongations by coordinating the activity of these two layers of muscle (Szymanski and Yuste, 2019). However, how their activity is affected by physiological and environmental conditions has not been characterized. To test the effect of environmental manipulations on muscle activity, we used transgenic Hydra that express genetically-encoded calcium indicator GCaMP6s in every ectoderm or endoderm muscle cell (Szymanski and Yuste, 2019). With these transgenic animals, 2-h-long calcium imaging sessions were conducted (Movie 2) to explore how each physiological or environmental condition changes muscle activity (Fig. 2A).

Widespread activation of the entire body musculature was observed when Hydra contracted, as described previously (Szymanski and Yuste, 2019), with transient calcium increases that synchronously occurred in the entire muscle tissue. These activations usually appeared as a burst during each contraction event, faithfully reflecting behavioral CBs (Passano and McCullough, 1963, 1964). To analyze the spatiotemporal dynamics of these muscle pulses and bursts, we used a computer program to semi-automatically detect events from whole-body fluorescence intensity measurements (Fig. 2B). In agreement with behavioral data (Fig. 1), in ectoderm muscle tissue, high osmolarity decreased the number of pulses (Fig. 2C), burst duration (Fig. 2E), and frequency (Fig. 2G, \( p = 0.0017 \)), as compared with low osmolarity. In contrast, we detected no change in endoderm muscle activity in response to osmolarity changes, previously (Szymanski and Yuste, 2019).
although increases in endodermal muscle activity were observed during contractions, and changes of that baseline rate was also observed in smaller Hydra, or with increased temperature (Fig. 2D,F,H).

We concluded that osmolarity altered ectodermal muscle activity in the same way as it changed contractile behavior but did not affect endodermal muscle. This is consistent with the hypothesis that ectodermal muscle generates CBs in the animal, responding to medium osmolarity. To search for the origin of their response, we then examined the neural activity, presumable controlling of this muscle activation.

**Bidirectional effect of osmolarity on CB neuronal circuit activity**

Hydra’s nerve nets lie at the base of both ectodermal and endodermal epithelial layers (Sarras et al., 1991) and are divided functionally into non overlapping circuits (Dupre and Yuste, 2017). Two of such circuits are the CB and RP1 networks (Dupre and Yuste, 2017). These circuits activate in synchronous and oscillatory manner during Hydra’s spontaneous contraction (CB) or during elongation (RP1; Passano and McCullough, 1963; Rushforth and Burke, 1971; Dupre and Yuste, 2017).

However, while these circuits likely have a combination of sensory and motor neurons, the exact role of these cells is still unclear. Similar to bilaterian species, the cnidarian Hydra has neuromuscular junctions (Chapman et al., 2010), and there is evidence suggesting direct interaction of muscle cells and neurons. First, gap junctions are found between muscle cells and neurons (Westfall et al., 1980). Second, Hydra contractions are greatly reduced after chemically eliminating neurons (Campbell et al., 1976), suggesting that muscle activity in Hydra are initiated and coordinated by neurons. We therefore set out to study neural activity in Hydra to account for the observed changes in the muscle activity and behavior under different conditions.

Similarly to muscle imaging experiments (Fig. 2), 2-h calcium imaging sessions were conducted in mounted preparations using Hydra expressing GCaMP6s in the entire nerve net (Movie 3; Fig. 3A; Dupre and Yuste, 2017). Then, the spatiotemporal dynamics of the CB and RP1 pulses for the entire neuronal populations were semi-automatically extracted using a computer program from whole-body fluorescence measurements (Fig. 3B), and events frequencies were calculated. Results showed that low osmolarity increased the number of neuronal CB pulses compared with control, while high osmolarity decreased them (p = 0.0422) compared with control or low osmolarity (p = 0.0005; Fig. 3C), with no significant change in neuronal CB burst duration (Fig. 3E). In addition, high osmolarity decreased CB pulse frequency, compared with low osmolarity (p < 0.0001), while low osmolarity increased CB pulse frequency compared with controls (p = 0.0066; Fig. 3G). Other experimental conditions (food, temperature, and body size) did not significantly alter the activity of CB neurons. These results indicate that CB neuronal activity is inversely proportional to osmolarity: lower osmolarity increases neuronal CB activity while higher osmolarity decreases it.

In contrast to these results in CB neurons, none of the condition altered the activity of RP1 neurons, thought to be responsible for body elongation (Fig. 3D,F,H; Dupre and Yuste, 2017). These results suggest that the activity of RP1 neurons are not affected by the environmental conditions tested. Overall, osmolarity consistently altered contractions, ectoderm muscle activity, and CB neuronal activity, with hypo-osmolarity leading to increases and hyperosmolarity to decreases in all these three physiological outputs. These results suggest that the neuronal CB circuit is the origin on the osmolarity response and the generation of CB muscle activity and CB contractions.

**Discussion**

In this study, we examined the effect of internal and external experimental factors on the contractile behavior and activity of muscle and neural tissue of H. vulgaris. We established imaging and analysis methods to measure the activity of all neuron and muscle cells during behavior in mounted preparations, under different physiological and environmental conditions. Among the conditions tested (amount of food, osmolarity or temperature of media, and size of animal), osmolarity consistently affected three functional readouts, in both free behaving and mounted preparations: contractile behavior, ectoderm muscle activity, and neuronal activity of the CB circuit. For foot detachments, ectoderm muscle CB duration and neuronal CB frequency, these effects were bidirectional, inversely related to osmolarity. Thus, Hydra appears to respond to osmolarity by specifically changing its neural and muscular activity, which presumably then changes behavior.

In both mounted and freely moving preparations, the number of contractions of Hydra in high osmolarity significantly decreased compared with low osmolarity (Fig. 1B,E), consistent with previous behavioral findings (Benos and Pruscha, 1973). Changes of Hydra behavior with osmolarity are thought to be triggered by increased water accumulation in Hydra’s gastrovascular cavity, causing Hydra to swell. As Hydra cells are highly permeable to...
water (Lilly, 1955), water could follow the concentration gradient between media (~5 mOsm/l) and Hydra tissue (~120 mOsm/l), accumulating in the gastrovascular cavity (~60 mOsm/l), which serves as an excretory pathway in these basal metazoans that lack excretory systems (Benos and Prusch, 1972). Furthermore, previous reports have suggested that the speed of water accumulation in Hydra tissues depends on osmolarity (Kücken et al., 2008; Soriano et al., 2009). Using regenerating hollow spheres of Hydra tissues, made of two epithelial layers as in intact Hydra, the speed of sphere swelling because of water accumulation decreased linearly with increasing osmolarity (Kücken et al., 2008; Soriano et al., 2009). Our results are in excellent agreement with this previous work, demonstrating concomitant changes in the ectodermal muscle and CB neuronal circuits, thus providing a neurobiological pathway that mediates this osmolarity reflex. By contracting its body, Hydra would be “wringing” itself periodically, eliminating excess water from its cells.

What are the mechanisms by which Hydra alters the contractions with osmolarity? One possibility is a mechanosensory system that could sense tissue pressure. Mechansensory responses in Hydra have been characterized in cnidocytes (Kass-Simon and Scappaticci, 2002), which use neurons to regulate their activation. Hydra is expected to express a set of potential osmoregulatory genes and mechanosensory receptor genes such as TRP channels, integrin (Pedersen et al., 2011; Siebert et al., 2019), and it will be interesting to examine the functions of these proteins in regulating neuronal and muscular activity during behavior.

We propose the following model (Fig. 4A): Hydra undergoes a spontaneous cycle of elongation and contraction. In low osmolarity, this cycle speeds up because of...
increases in water accumulation and activation of mechanosensory receptors in the tissue. In contrast, in high osmolarity, this cycle slows down because of decrease in water accumulation and lesser activation of mechanosensory receptors. As a first test of this model, we found that high-osmolarity solution (50 mM sucrose) significantly shortens the width of the body column, as if water accumulation was indeed reduced (Fig. 4B-D). According to our results, body contractions would be generated by ectodermal muscles, themselves under the control of CB neurons. But while responses were indeed altered in an osmolarity-dependent manner in both CB neurons and ectoderm muscle tissue, our data also showed no change in endoderm muscle activity with osmolarity. CB neurons localize within the ectoderm layer, so their activity and those of ectoderm muscle are mutually consistent (Figs. 2, 3). Thus, CB neurons could be the motor neurons that forms synapse onto ectodermal muscle cells and activate them. On the other hand, endoderm muscle appears not to contact CB neurons or ectoderm muscle (Rushforth and Burke, 1971; Dupre and Yuste, 2017), behaving as a separate system, somehow unaffected by changes in osmolarity. Future experiments could examine ectoderm and endoderm muscle activity together, with simultaneous calcium imaging of both tissues with two different color indicators. Also, simultaneous imaging of neurons and muscle cells using transgenic Hydra that expresses different color calcium sensors in both sets of cells could explore the relationship between CB neurons and ectoderm muscle. Furthermore, future analysis based on the activity of individual neurons, which still requires the development of robust tracking software, could reveal additional neuronal mechanisms of how osmolarity altered various behavior at single-neuron resolution.

We also found conditions that changed contractions in free behavior without altering neuronal or muscle activity in mounted preparations. Although they were not the direct object of our study, as they did not occur in conditions where we could perform calcium imaging of the neuronal and muscle cells, it is still interesting to comment on them. For instance, during free behavior, high temperature (30°C) increased the number of contractions and

Figure 4. Proposed model and effect of osmolarity on body width. A, Schematic model depicting how Hydra changes body width depending on osmolarity. Light-blue arrows indicate the direction and speed of water accumulation, which swells Hydra’s body and activate mechanosensory system and contractions. B, Representative images showing width of Hydra’s body column at the end of elongation cycle, under control media (blue, above) or high-osmolarity solution (red, below). C, Representative traces showing changes in width over time under control media (blue) or high-osmolarity solution (red). D, Width of body column in control media (blue, 70.962 ± 6.560) or high-osmolarity solution (red, 46.540 ± 4.036). Line depicts the same animal in each condition. Error bars are shown as the mean ± SEM, with symbol marks denoting data points from individual Hydra (N=4). Student’s t test was performed: *p < 0.05.
foot detachments (Fig. 1E,F). Above 25°C, Hydra activates heat shock protein pathways leading to apoptosis; 30°C is eventually lethal to Hydra (Bosch et al., 1988), so increased locomotion could reflect an escape behavior, likely absent in mounted preparations. We also found that well-fed freely behaving animal (four shrimp per day) had fewer contractions overall but increased locomotion, as measured by foot detachments (Fig. 1E). It is not clear what could be the physiological function of these behaviors and why these conditions did not alter the activity of neurons or muscles in mounted preparations. The activity of CB neurons and contractions is inhibited during Hydra’s feeding behavior, while the activities of CB neurons and contractions increased right after the feeding behavior (Grosvenor et al., 1996). In the current study, rather than measuring at the immediate effect by feeding, we tallied changes in behavior of Hydra that had been fed various amount of food constantly for a week, and the experiments were conducted after starving for 1 d. Therefore, our conditions were not exactly comparable to those of Grosvenor et al. (1996), and measurements revealed Hydra did not alter muscle or neuronal activity depending on their energy state. Finally, it also remains possible that the differences between free-behaving and mounted animals could be that mechanical restrictions of Hydra may have disrupted physiological responses of neurons and muscles to heat and food. This effect should be reexamined by imaging neurons and muscle activity of freely moving Hydra, perhaps with wide-field 3D high-speed scanning systems (Cong et al., 2017; Kim et al., 2017).

In summary, using Hydra, we measured and analyzed the activity of the entire neuronal and muscle tissue in an animal during behavior. We find that osmorality controls the activity of a selective group of neurons and muscle cells, without affecting others, leading to changes in contractile behavior. This approach, measuring the entire neuronal and muscle activity during a simple behavior in an accessible preparation, could be used systematically in Hydra and other animals to understand how neuronal and muscle function generates behavior.

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