Behavioral/Systems/Cognitive

Temporal Activity Patterns in Thermosensory Neurons of Freely Moving *Caenorhabditis elegans* Encode Spatial Thermal Gradients

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Our understanding of the operation of neurons and neuronal circuits has come primarily from probing their activity in dissected, anesthetized, or restrained animals. However, the behaviorally relevant operation of neurons and neuronal circuits occurs within intact animals as they freely perform behavioral tasks. The small size and transparency of the nematode *Caenorhabditis elegans* make it an ideal system for noninvasive, optical measurements of neuronal activity. Here, we use a high signal-to-noise version of cameleon, a fluorescent calcium-binding protein, to quantify the activity of the AFD thermosensory neuron of individual worms freely navigating spatial thermal gradients. We find that AFD activity is directly coupled to the worm's exploratory movements in spatial thermal gradients. We show that the worm is able, in principle, to evaluate and guide its own thermotactic behaviors with respect to ambient spatial thermal gradients by monitoring the activity of this single thermosensory neuron.

Key words: **behavior; *C. elegans*; fluorescence microscopy; imaging; sensory neurons; temperature**

Introduction

A foremost goal in neuroscience is to connect neuronal activity to the natural behavior of animals. The major difficulties include making noninvasive recordings of neuronal activity in intact animals as they perform behavioral tasks and stabilizing the effects of motion artifacts. These difficulties may begin to be resolved by studying the behavior of small, transparent animals, such as the nematode *Caenorhabditis elegans*, using genetically encoded optical probes to manipulate and monitor neuronal activity (Miesenbock and Kevrekidis, 2005; Miyawaki, 2005).

C. elegans crawls on surfaces, so its unrestrained navigational behaviors can be tracked by using an *x–y* translational stage. The fluorescence of an individual neuron that expresses a genetically encoded optical probe can thus be quantified, because the movements of the neuronal cell body inside the tracked worm remain mostly within the focal plane of the microscope. Slight out-of-plane movements of the neuron in freely moving worms may affect the quantification of fluorescence and neural activity, but this motion artifact can be reduced with dual-wavelength ratiometric recording of certain optical probes. In particular, yellow cameleon changes the degree of fluorescence resonance energy transfer (FRET) from cyan fluorescent protein (CFP) to yellow fluorescent protein (YFP) versions of *Aequorea* green fluorescent protein (GFP) on binding Ca^{2+} (Miyawaki et al., 1997). The ratio of YFP to CFP emission is a measure of Ca^{2+} concentration, and such a ratio is relatively unaffected by changes in focus and movement artifacts.

To understand how an animal's behavior is encoded in its nervous system, we must first understand how its sensory neurons encode the animal's environment. Here, we examine the case of thermosensory behavior of unrestrained *C. elegans* in spatial thermal gradients and the operation of the AFD neurons, so far the only neuron in *C. elegans* that has been identified with thermosensory function (Mori and Ohshima, 1995; Chung et al., 2006). *C. elegans* thermotactic behavior is remarkably sophisticated, using experience-dependent plasticity to produce distinct modes of behavior (Hedgecock and Russell, 1975). On spatial thermal gradients, *C. elegans* will track isotherms at temperatures near its previous cultivation temperature. When navigating at temperatures above their previous cultivation temperature, *C. elegans* will actively crawl down the thermal gradients in what is called cryophilic movement (Hedgecock and Russell, 1975; Ryu and Samuel, 2002).

Recently, it has been shown that the AFD neuron exhibits intracellular calcium dynamics in response to temperature changes only at temperatures near and above the previous cultivation temperature (Kimura et al., 2004; Clark et al., 2006). The operating range of the AFD neuron overlaps with both the range of the isothermal tracking behavior as well as the range of the cryophilic behavior. Here, we characterize AFD activity in freely moving worms actively exploring their temperature environment with their crawling movements. We quantify the AFD thermosensory response in unrestrained worms and show that

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specific patterns of AFD activity provide the worm with sufficient thermosensory information to perform its thermotactic behaviors.

Materials and Methods

Strains. The strain kdkEx1518 (H13p:YC2.12) was a gift from I. Mori (Nagoya University, Nagoya, Japan). PY1157 was a gift from P. Sengupta (Brandeis University, Waltham, MA). Yellow cameleon YC3.60 (Nagai et al., 2004) was expressed in AFD by cloning the YC3.60 open reading frame into the pPD49.26 vector to provide the *unc-54* 3′ untranslated region. Expression was driven by the *gcy-8* promoter cloned upstream of the open reading frame. All worms were cultivated on OP50 bacterial food at 20°C using standard techniques (Brenner, 1974).

Imaging the AFD thermosensory response of immobilized worms. Individual worms were glued to agar surfaces using cyanoacrylate glue (Abbott Laboratories, Abbott Park, IL). We subjected individual immobilized worms to feedback-controlled heating and cooling and monitored the dual-wavelength fluorescence emission from yellow cameleon specifically expressed in the AFD neurons, following the methods of Clark et al. (2006).

Imaging the AFD thermosensory response of semirestrained or unrestrained worms on a spatial thermal gradient. Worms were placed on a thin agar pad on a sapphire window, covered with a coverslip, and imaged while moving in a spatial thermal gradient, imposed by thermal contact between the sapphire window and copper plates under feedback thermoelectric control. The worm was illuminated with wide-field fluorescence excitation at 440 nm to excite the CFP fluorophore and imaged with a 20× Nikon (Tokyo, Japan) apochromatic objective (0.75 numerical aperture). Standard emission filters and dichroic mirrors (Chroma, Rockingham, VT) were used to split the image of the moving worms and simultaneously project the yellow and cyan emission images onto two halves of a CCD camera (Coolsnap; Photometrics, Tucson, AZ). Images were acquired at 5 Hz with 200 ms exposure.

Worms were either glued by their tails or allowed to freely navigate the spatial thermal gradients. For the case of semirestrained worms, the field of view of the 20× objective allowed us to visualize the full extent of motion of the worm's head. For unrestrained worms, we kept the worm's head within the field of view with a joystick-controlled motorized *x–y* stage and motorized focus. The custom-built *x–y* microscope stage could move as fast as 0.4 mm/s, driven by screw actuators under joystick control (Thorlabs, Newton, NJ). To account for a nonhorizontal plane, the focus was automatically adjusted based on the instantaneous *x–y* coordinates of the moving worm. The trajectory of each worm was determined after each experiment from the stage position and head position within the field of view. To determine crawling headings, the instantaneous measurements of the worm's head were smoothed with a one-pole, 0.1 Hz low-pass Butterworth filter.

Image analysis. Image analysis was performed with a custom-written code in Matlab (Mathworks, Natick, MA). The soma of the AFD neuron was sufficiently bright that it could be automatically identified in most frames. The dual-wavelength fluorescence emission was calculated from a region of interest of radius 28 μm around the soma. Within the region of interest, the total fluorescence intensity was calculated as the mean of the brightest 28 pixels ($\sim$25 μm^2) minus the 10th percentile of intensities within the region (local background subtraction). The ratiometric emission signal was calculated by dividing the total yellow fluorescence emission by the total cyan fluorescence emission in the region of interest of each video frame. The ratiometric inhomogeneity across the microscope field of view was estimated to be <5%, based on measurements using a uniformly fluorescent gel. We excluded from analysis measurements in which the AFD soma moved within 28 μm of the edges of the field of view. As the baseline ratiometric emission signal (R_0) for the data produced by each freely moving worm, we used the 25th percentile of the magnitude of all of its ratiometric emission signals.

Behavioral analysis. Individual worms were simultaneously tracked while moving on a 0.5°C/cm agar gradient under dark-field illumination. Worm tracks were reconstructed, and reorientation events were flagged as described by Clark et al. (2007). Reorientation probability was calculated as a function of worm heading with respect to the direction of the spatial thermal gradient. To exclude isothermal tracking behavior, worms cultivated at 25°C were analyzed between 15 and 20°C (to quantify atactic movements at temperatures below the previous cultivation temperature), and worms cultivated at 15°C were analyzed between 20 and 25°C (to quantify cryophilic movement at temperatures above the previous cultivation temperature).

Results

Yellow cameleon 3.60 provides strong signals of intracellular Ca^{2+} dynamics within the AFD thermosensory neuron

Intracellular Ca^{2+} dynamics within the AFD thermosensory neuron have been monitored using the fluorescent protein yellow cameleon (YC2.12) (Kimura et al., 2004; Biron et al., 2006; Clark et al., 2006). At temperatures near and above the previous cultivation temperature of *C. elegans*, temperature upsteps (downsteps) evoke an increase (decrease) in levels of intracellular Ca^{2+} within AFD. Thus, when the worm is subjected to a temperature waveform consisting of a sinusoidal oscillation added to a positive linear ramp, the levels of intracellular Ca^{2+} in the AFD thermosensory neuron become phase-locked to the sinusoidal component of the waveform at temperatures near and above the previous cultivation temperature. These Ca^{2+} dynamics may be inferred from changes in the ratiometric emission signal (i.e., the ratio of yellow to cyan fluorescence emission) from cameleon protein that is specifically expressed in the AFD neurons of transgenic animals. We define T^*_{AFD} as the lower bound of AFD activity. For worms cultivated at 20°C, T^*_{AFD} is $\sim$17°C (Clark et al., 2006).

Nagai et al. (2004) have developed an improved version of yellow cameleon, YC3.60, seeking to augment the Ca^{2+}-dependent change in the relative orientation and distance between its component CFP and YFP fluorophores. Their improvements conferred YC3.60 with sixfold increases in its ratiometric emission signal over conventional constructs like YC2.12. Thus, YC3.60 exhibits a greatly enhanced signal-to-noise ratio for detecting and quantifying intracellular Ca^{2+} dynamics. We compared the ratiometric emission signals obtained with YC2.12 and YC3.60 from the AFD neurons of worms subjected to temporal changes in temperature (Fig. 1). We confirmed that temperature waveforms evoke highly stereotyped patterns of intracellular Ca^{2+} dynamics, based on recordings of the ratiometric emission signal of YC2.12 and YC3.60. Both ratiometric emission signals become phase-locked to the sinusoidal component of a temperature waveform at temperatures above T^*_{AFD}. However, whereas YC2.12 exhibited typical maximum changes in the ratiometric emission signal of $\sim$30%, YC3.60 exhibited typical maximum changes of $\sim$200%. These data confirm the sixfold signal enhancement in measuring Ca^{2+} dynamics afforded by YC3.60 in the AFD neuron.

Imaging intracellular calcium dynamics in the AFD thermosensory neurons of semirestrained worms

Next, we sought to determine whether enhanced measurements with YC3.60 would allow us to quantify the intracellular Ca^{2+} dynamics in the AFD thermosensory neuron of a moving worm in a spatial thermal gradient. Thermosensation in the AFD neuron occurs at the ciliated sensory endings near the worm's nose (Satterlee et al., 2001; Chung et al., 2006; Clark et al., 2006). Thus, in principle, when the worm moves up or down a spatial thermal gradient or swings it nose during its undulating gait, the putative thermosensor at the worm's nose will encounter temporal changes in temperature.

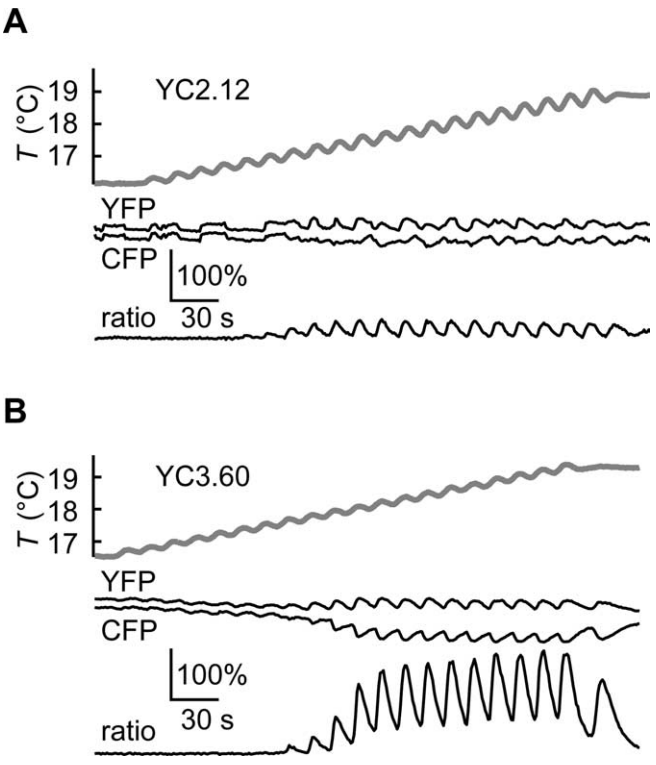


Figure 1. Temporal changes in temperature evoke ratiometric emission signals from yellow cameleon expressed in the AFD neuron of immobilized worms. ***A***, In an immobilized worm expressing YC2.12 in the AFD neuron, as the temperature increases above a threshold of ~17°C, the ratiometric emission signal from YC2.12 changes by ~30% in a representative experiment, indicating changes in the level of intracellular calcium in the AFD neuron. ***B***, The ratiometric emission signal from YC3.60 in the AFD neuron reports the same qualitative changes in the level of intracellular calcium, but with changes in the ratiometric emission signal of 200% in a representative experiment.

Any recording of neuronal activity in a moving animal will have a component that is functionally relevant as well as a component that is motion artifact. The goal in recording activity in moving animals is to ensure that the functionally relevant component can be discriminated from the motion artifact. We tethered individual worms by gluing their tails to flat agar surfaces and covered them with a glass coverslip, thus allowing free movement of the anterior portion of their body within the focal plane of a fluorescence microscope. A defined linear spatial thermal gradient was imposed on the agar surface in the perpendicular direction to the average orientation of the worm's body. Thus, with each body undulation, the thermosensor at the nose would encounter rhythmic changes in temperature, which in turn could be manipulated by changing the direction or steepness of the imposed spatial thermal gradient.

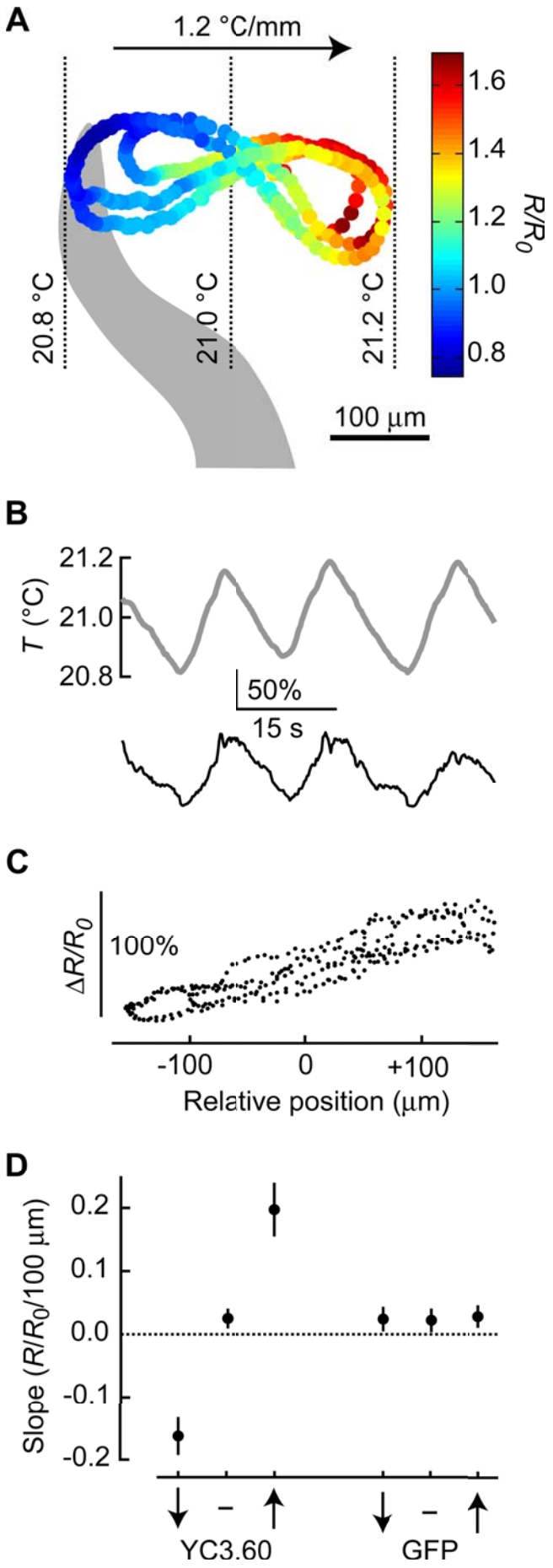
We quantified the ratiometric emission signals from the AFD neuronal cell bodies expressing YC3.60 in these semirestrained, tethered worms (Fig. 2*A*) (see also supplemental movie 1, available at www.jneurosci.org as supplemental material). We note that certain features of the experimental setup reduced the problem of motion artifacts. First, by placing the animal between a coverslip and the flat agar surface, the movements of the worm's body were always parallel to the focal plane of the microscope, thus keeping the neuronal cell body mostly within focus throughout each experiment. Second, dual-wavelength ratiometric imaging is relatively insensitive to out-of-plane movements of the neuronal cell body and smearing of the image because of movement during the exposure, both of which change intensity in both channels equally.

Moreover, our setup allowed us to perform straightforward experiments to confirm that the ratiometric emission signals that we observed were predominated by thermosensory function and not motion artifacts or artifacts of our experimental setup. First, the intracellular calcium dynamics that we observed using semirestrained worms in spatial thermal gradients were consistent with our expectations based on previous measurements using immobilized worms subjected to temporal thermal gradients. In immobilized worms, increases and decreases in temperature evoke increases and decreases in the level of AFD intracellular calcium concentration. Correspondingly, when semirestrained worms moved their nose toward higher and lower temperatures, they caused positive and negative changes in the ratiometric emission signal of YC3.60 in the AFD neuron (Fig. 2*B*,*C*). Because we could change the direction of the spatial thermal gradient within each experiment, we could verify that these ratiometric changes were not an artifact of the geometry of the semirestrained worms or the optics: (1) turning off the spatial thermal gradient nearly abolished position dependence in the ratiometric emission signal; and (2) reversing the direction of the gradient reversed this position dependence (Fig. 2*D*).

As an additional control experiment, we analyzed the ratiometric fluorescence emission of AFD neurons expressing GFP. The fluorescence emission of GFP overlaps with the two channels of YC3.60 fluorescence emission, but without dependence on the level of intracellular calcium. We verified that the ratiometric emission signals of AFD-expressing GFP in semirestrained worms exhibited no position dependence, with or without a spatial thermal gradient (Fig. 2*D*). Together, these results suggest that the ratiometric emission signal of YC3.60 is a reliable reporter of intracellular calcium dynamics and AFD thermosensory function in moving worms and that worm motion artifacts are minimal in our experimental setup.

Imaging intracellular calcium dynamics in the AFD thermosensory neurons of unrestrained worms

Next, we sought to quantify the thermosensory function of the AFD neuron in unrestrained worms navigating spatial thermal gradients. We used a motorized microscope stage to follow the movements of young adult *C. elegans* as they crawled on a square arena (18 × 18 mm) with an imposed linear spatial thermal gradient (0.3°C/mm). Thus, the worm's head was kept within the field of view under 20× magnification throughout the 2 min of each experiment, and the path of the moving worm was reconstructed after each experiment from the history of *x*–*y* displacements of the motorized stage and position of the soma within the field of view in each frame. Within each video frame, we quantified the ratiometric emission signal from the AFD neuron. Representative data of a worm navigating at temperatures near T^*_{AFD} are shown in Figure 3*A* (see also supplemental movie 2, available at www.jneurosci.org as supplemental material). Each data point is colored to represent an instantaneous measurement of ratiometric emission from the AFD neuronal cell body; the position of each data point is the instantaneous position of the unrestrained worm AFD soma on the spatial thermal gradient. In Figure 3*B*, we show the experimental data of Figure 3*A*, but plotted as the thermosensory input and the ratiometric emission signal over time. As with immobilized and semirestrained worms, intracellular calcium dynamics in the AFD neuron at $T > T^*_{AFD}$ are tied to temporal changes in temperature, such that increasing temperature tends to raise the level of intracellular calcium in AFD and



decreasing temperature tends to lower the level of intracellular calcium. Short-term adaptation in AFD neuronal activity may be deduced from relaxation in the ratiometric emission signals after the worm has been at a certain temperature for ~20 s (Fig. 3*A*,*B*).

Intracellular calcium dynamics in the AFD neuron are only evoked by temporal changes in temperature at temperatures near and above the worm's previous cultivation temperature. In particular, when *C. elegans* is grown at 20°C and subjected to temperature changes, the AFD neuron is only activated by temporal changes in temperature above ~17°C (Fig. 1) (Biron et al., 2006; Clark et al., 2006). We took data from worms grown at 20°C crawling at a variety of absolute temperatures (Fig. 3*C*). The ratiometric emission signals that we measure from unrestrained worms correspond closely with those measurements taken in semirestrained worms. Indeed, when these worms move toward increasing temperature at temperatures above ~17°C ($T > T^*_{AFD}$), the ratiometric emission signal from the AFD neuron tends to increase. At $T < T^*_{AFD}$, the ratiometric emission signal from the AFD neuron does not exhibit position dependence in the spatial thermal gradient.

Short-term adaptation and the broad operating range of the AFD thermosensory neurons

To quantify the operating range of the AFD thermosensory response, based on the experiments shown in Figure 3*C*, we measured the ratiometric emission signals for worms navigating spatial thermal gradients at different absolute temperatures. The lowest ratiometric emission signals did not vary with absolute temperature, showing that the signals generated by the cameleon indicator are not, for example, saturated at temperatures higher or lower than T^*_{AFD} (Fig. 4*A*). We found that the ratiometric emission signal from the AFD neuron is correlated with thermosensory input with a strong positive slope at $T > T^*_{AFD}$ (Fig. 4*B*). As an additional control, we measured the ratiometric emission signal from AFD neurons expressing GFP and confirmed that this ratiometric signal exhibited no position or temperature dependence during navigation on the spatial thermal gradients at any temperatures.

The response properties of the AFD neuron suggest that it exhibits high sensitivity over broad operating ranges. Using immobilized worms subjected to small temperature steps, we quantified the temporal dynamics of short-term adaptation in thermosensory signal transduction in the AFD neuron (Fig. 4*C*). After an upstep in temperature, the level of intracellular calcium in the AFD neuron rises, peaks within ~10 s, and returns to baseline within ~40 s. Next, we asked whether the same mathematical transformation, the mapping between a defined stimulus

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Figure 2. AFD neuronal activity in semirestrained worms moving in spatial thermal gradients. ***A***, The intracellular calcium dynamics of the AFD neuron were quantified in semirestrained worms. The worm tails were glued, allowing free undulating motion of the anterior part of the worm's body in imposed spatial thermal gradients. Data from a single experiment are shown, with color and spatial position indicating the time-varying ratiometric emission signal and position of the AFD soma, respectively, over several undulation cycles. To indicate scale, the gray silhouette shows the relative size and orientation of the anterior portion of the semirestrained worm at one time point during this experiment. ***B***, In the top and bottom panels, the data from ***A*** are plotted as temperature over time and ratiometric emission signal over time, respectively. ***C***, The ratiometric emission signal from ***A*** is plotted parametrically against head position. ***D***, The slope of the ratiometric emission signal with respect to position was calculated for worms either expressing YC3.60 or GFP in the AFD neurons, with spatial thermal gradients pointed in opposite directions or with no gradient at all. Error bars indicate 1 SEM. $n = 14–27$ worms were used for each measurement.

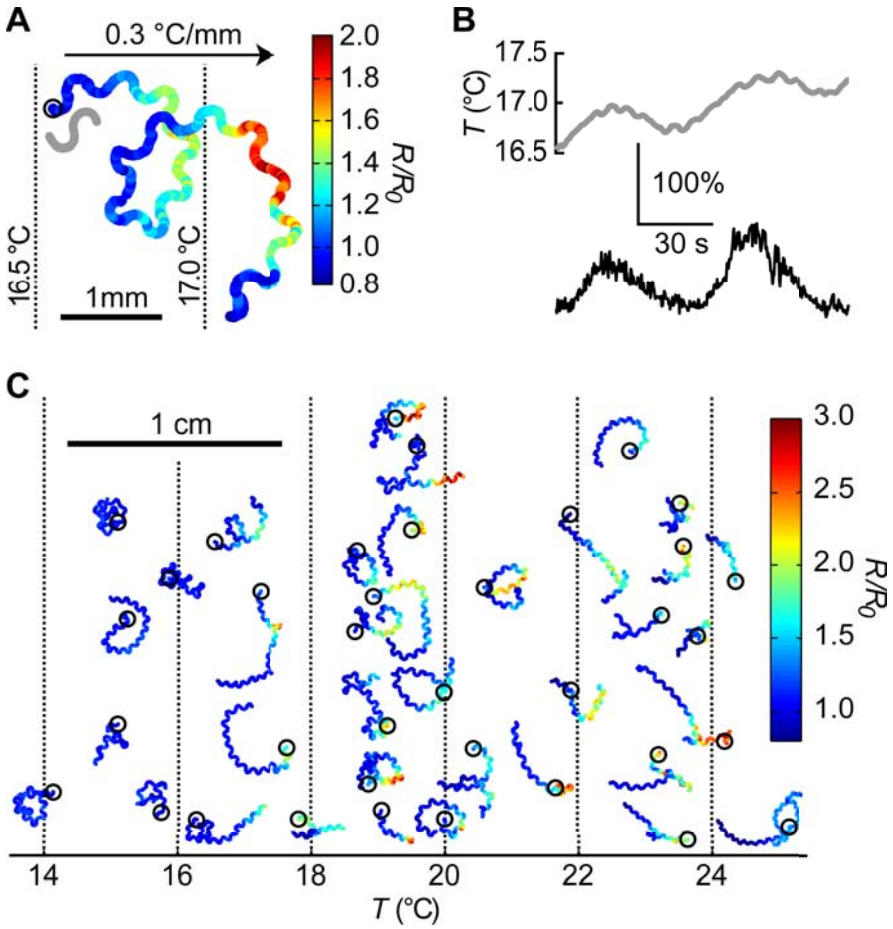

Figure 3. AFD neuronal activity in unrestrained worms navigating spatial thermal gradients. ***A***, The undulating path of an unrestrained worm crawling ~8 mm on a spatial temperature gradient in the vicinity of T^*_{AFD}. The color and position of each data point represent the ratiometric emission signal and position of the AFD thermosensory neuron of the moving worm, respectively. The black circle indicates initial position. To indicate scale, the gray silhouette shows the approximate relative size of the worm body. ***B***, The top and bottom panels show the time-varying temperature at the worm's head and the ratiometric emission signal from AFD for the data shown in ***A***. ***C***, A compilation of data showing the ratiometric emission signals for worms cultivated at 20°C navigating spatial thermal gradients at different absolute temperatures from ~15 to ~25°C. The ratiometric emission signal exhibits position dependence only at temperatures above ~17°C (i.e., $T > T^*_{AFD}$). A black circle indicates the initial position of each data trace.

waveform of thermosensory input and the response waveform of AFD intracellular calcium dynamics, occurs within the AFD neurons of freely moving worms. Using our full data set of the ratiometric emission signals of freely moving worms on spatial thermal gradients at $T > T^*_{AFD}$, we calculated the functional form of the least-squares-fit linear kernel that would best map the set of thermosensory inputs experienced by each worm to the ratiometric emission signals exhibited by the AFD neuron of each worm. Then, using the best-fit linear kernel, we estimated the AFD response to a temperature upstep. Our result established a close correspondence between the transfer function that we measured by subjecting immobilized worms to temperature upsteps and the transfer function that we estimated based on the AFD responses of freely moving worms on spatial thermal gradients. In both cases, an upstep in temperature evokes a transient increase in activity that gradually adapts to prestimulus levels on the timescale of ~40 s. The calculated kernel is smaller in amplitude than the measured one primarily because it averaged over all worms, including worms with small or no signals. The observed short-term adaptation renders the AFD neuron sensitive to the time derivative of thermosensory input and thus enables the AFD neuron to respond to small temperature changes over broad operating ranges.

The AFD neuron encodes the behaviorally relevant thermosensory information required for thermotactic behavior

The ratiometric emission signal of the AFD neuron can be viewed as a lower limit on the thermosensory information that the AFD neuron could actually encode and thus is a lower limit on the thermosensory information available to the worm to execute thermotactic navigation. We asked whether the AFD neuron encodes sufficient thermosensory information to allow the worm to produce thermotactic navigation. For this, we examined the ratiometric emission signals of individual worms crawling along different trajectories.

Thermotactic navigation in *C. elegans* has two distinct modes (Hedgecock and Russell, 1975; Ryu and Samuel, 2002). When worms navigate at temperatures near their previous cultivation temperature, they are able to crawl along isotherms in prolonged periods of forward movement (Ryu and Samuel, 2002; Luo et al., 2006). When worms navigate at temperatures above their previous cultivation temperature, they execute what is called cryophilic movement. Cryophilic movement is a biased random walk toward colder temperatures. Worm movement may be characterized as a sequence of runs that are interrupted by reorientation maneuvers. During cryophilic movement, the worm extends those runs that are directed toward colder temperatures and shortens those runs that are directed toward higher temperatures (Ryu and Samuel, 2002; Clark et al., 2007).

The worm requires a minimum amount of thermosensory information to implement either the strategy for isothermal tracking or the strategy for cryophilic movement. To track isotherms, the worm must be able to correct any deviation from isothermal alignment by appropriately adjusting the heading of its forward movement. One possibility is that the worm analyzes temporal variations in the activity of a thermoreceptor at the worm's nose. When the worm has perfect isothermal alignment, the activity of a thermoreceptor will be driven by rhythmic cycles of warming and cooling as the nose swings from side to side about the isothermal track. We recently showed that, in principle, the worm could use such a thermosensory signal to implement a specific isothermal tracking strategy, systematically altering its heading to offset any net warming or cooling within each undulation cycle (Luo et al., 2006). For this strategy to work, a thermoreceptor at the nose must be able to phase-lock to the thermosensory signal driven by the propulsive undulations of a worm crawling at normal speed.

Figure 5*A* shows ratiometric emission signals from YC3.60 expressed in the AFD neuron of a worm moving across a spatial thermal gradient. The ratiometric emission signal is phase-locked to the

temporal variations in thermosensory input driven by the worm's self-movement in the spatial thermal gradient. When the worm's head swings toward warmer temperatures, the AFD neuron exhibits an increase in intracellular calcium concentration. When the head swings toward colder temperatures, the AFD neuron exhibits a decrease in intracellular calcium concentration. Thus, in principle, the worm could use the AFD neuron to detect its own isothermal alignment as well as implement a sensorimotor algorithm for isothermal tracking.

For the worm to implement the strategy for cryophilic movement, the worm must first determine whether it is navigating at temperatures above its previous cultivation temperature and, if so, determine whether the temperature is rising or falling during each period of forward movement. In other words, the worm must compute the time derivative of its temperature measurements. The fundamental feature of the adaptive thermosensory response represented in Figure 4*C* is that the ratiometric emission signal is effectively the first derivative of the temperature input (Clark et al., 2006, 2007). For an unrestrained worm moving on a spatial thermal gradient, the time derivative of the thermosensory input is dictated by the worm's orientation with respect to the direction of the thermal gradient. Thus, the ratiometric emission signal should be directly correlated with the instantaneous heading of the unrestrained worm at higher temperatures and should be uncorrelated at lower temperatures. In Figure 5*B*, we graph the ratiometric emission signals from the AFD neurons as a function of the instantaneous headings of worms with respect to the gradient. Indeed, at $T < T^*_{AFD}$, the AFD neuron appears to be unstimulated. At $T > T^*_{AFD}$, the ratiometric emission signal tends to be low when the worm is moving toward colder temperatures and high when the worm is moving toward warmer temperatures, varying continuously with worm heading with respect to the gradient.

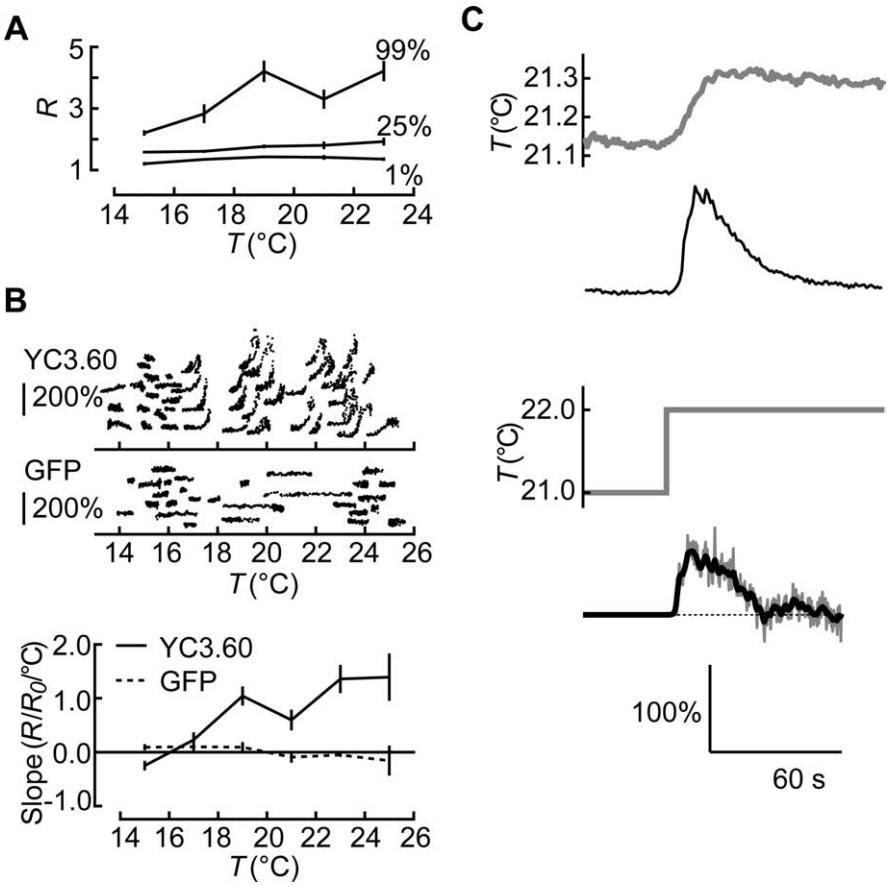

Figure 4. Operating range and short-term adaptation in the AFD thermosensory neuron. ***A***, The range of all ratiometric FRET measurements exhibited by worms freely navigating spatial thermal gradients at different absolute temperatures, showing the 1st, 25th, and 99th percentiles (mean ± SEM). The FRET measurements that correspond to the lowest intracellular calcium levels, the 1st and 25th percentiles, are invariant with absolute temperature ($p > 0.1$ using ANOVA). The 25th percentile exhibited by each worm was assigned as an arbitrary baseline for its ratiometric emission signals (R_0) (see Materials and Methods). ***B***, Top, Representative parametric plots of the ratiometric emission signal from the AFD neuron expressing YC3.60 and GFP against temperature for worms freely navigating spatial thermal gradients at different absolute temperatures. Bottom, The measurements of slope in the plot of ratiometric emission signal versus temperature of all data, calculated at different absolute temperatures (solid line, $n = 77$ worms expressing YC3.60; dotted line, $n = 31$ worms expressing GFP). ***C***, Using all worm tracks at $T > T^*_{AFD}$ ($n = 58$), we calculated the linear weighting of 75 s of thermosensory history that best fit the measured ratiometric emission signal. The top trace shows the ratiometric emission signal from an immobilized worm subjected to an actual step change in temperature. The bottom trace shows the predicted ratiometric emission signal to a temperature step, based on the best-fit calculation. The gray line shows the predicted response, and the black line shows the predicted response smoothed with a 2 s filter.

We also verified that worms are actually displaying cryophilic movement during the course of our physiological measurements by quantifying the statistics of the crawling trajectories (Fig. 3*C*). During cryophilic movement, the worm modulates its own reorientation probability depending on its heading with respect to ambient spatial thermal gradients, with low and high reorientation probabilities when the worm is oriented toward colder and warmer temperatures, respectively. Thus, worms exhibiting cryophilic movement should spend most of their time moving toward colder temperatures. For the experiments shown in Figure 3*C*, we found that worms spend 55% of their total time moving toward colder temperatures and 45% moving toward warmer temperatures (distinguishable from unbiased movement at $p < 0.05$).

Could the worm decode AFD activity to estimate its own heading in ambient spatial thermal gradients? The worm may be able to execute this biased random-walk strategy for cryophilic movement by linking reorientation probability to the level of AFD neuronal activity. Because the level of intracellular calcium dynamics in the AFD neuron represents a lower bound on the total information that the worm has about its thermosensory environment, we asked whether that information alone would be sufficient to create the biasing observed in worms performing cryophilic movement.

At $T < T^*_{AFD}$, the level of intracellular calcium in the AFD neuron does not vary with worm heading on spatial thermal gradients, and neither does the worm modulate its own reorientation probability as a function of heading during its random walk. However, at $T > T^*_{AFD}$, the level of intracellular calcium rises as the worm heading becomes oriented with the direction of the spatial thermal gradient, roughly in proportion to the change in the reorientation probability with worm heading (Fig. 5*C*). The computational problem that the worm is trying to solve is better conveyed by representing these experimental measurements as the probability that the worm has a particular heading

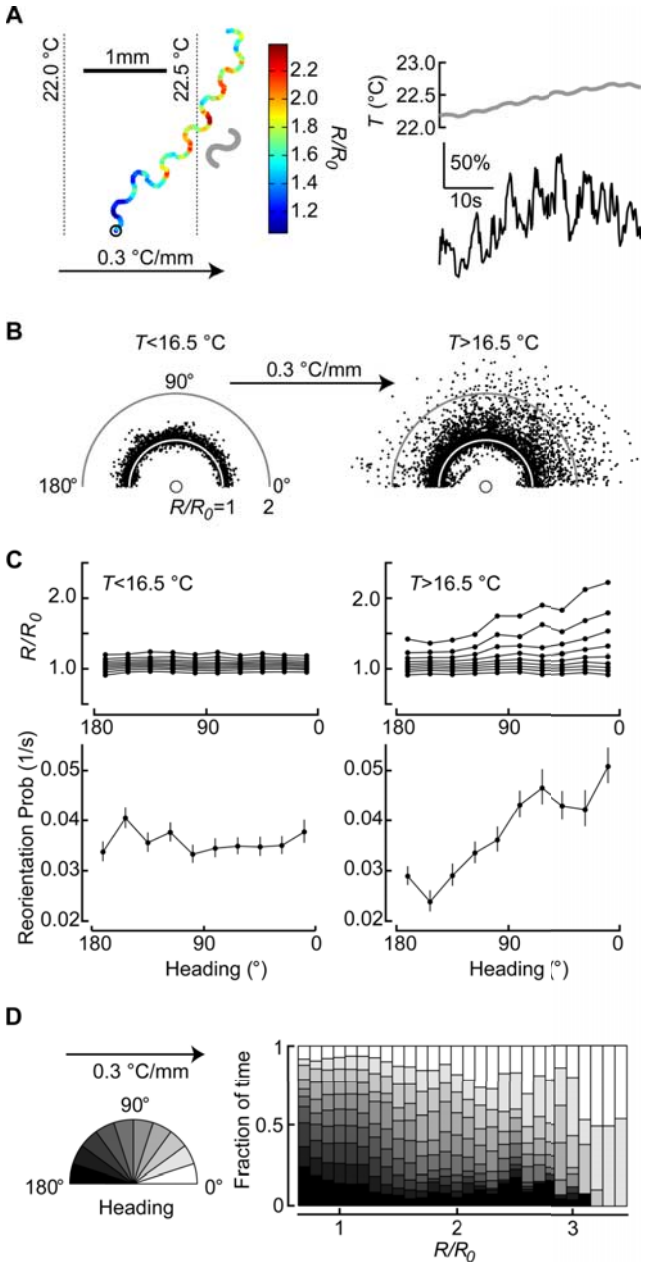

Figure 5. Representation of behaviorally relevant thermosensory information in the AFD neurons. ***A***, Approximately 40 s of data showing the ratiometric emission signal of a worm moving slantwise on a spatial thermal gradient, with intracellular calcium transients phase-locked to rhythm of the undulatory gait. Left, The color and position of each data point represent the ratiometric emission signal and position of the AFD thermosensory neuron of the moving worm. Right, The temperature at the worm's head and the ratiometric emission signal are plotted over time. ***B***, Ratiometric emission signals are plotted as a function of instantaneous heading with respect to the direction of the spatial thermal gradient for all worms cultivated at 20°C navigating at $T > T^*_{AFD}$ ($n = 58$; right) and at $T < T^*_{AFD}$ ($n = 19$; left). The distance from the center of each scatter plot indicates the magnitude of the measured ratiometric emission signal; the angular position represents instantaneous worm heading with respect to the direction of the spatial thermal gradient. ***C***, The distribution of measured ratiometric emission signals for each heading angle is shown by plotting every 10th percentile of measured values, from the 10th to 90th percentiles, both above and below T^*_{AFD}. The bottom panel shows behavioral measurements indicating the worm's reorientation probability as a function of worm heading on a spatial thermal gradient, both at temperatures above and below the previous cultivation temperature [what we call the thermotactic set point or T_S (Biron et al., 2006)]. Error bars indicate 1 SEM. ***D***, Using all measurements of worms navigating temperatures above T^*_{AFD}, we plot the relative likelihood that the worm has a particular heading conditioned on the measurement of a specific value of the ratiometric emission signal. Headings are indicated by gray scale (see legend to the left).

conditioned on a specific value of AFD neuronal activity (Fig. 5*D*). Thus, when the level of intracellular Ca^{2+} in AFD is high, the worm is more likely to be moving directly up the gradient; when the level of intracellular Ca^{2+} in AFD is low, the worm is equally likely to be heading in any direction. Together, these results show that the worm could use the activity of the AFD neuron to estimate its own orientation with respect to ambient spatial thermal gradients, modulate its reorientation probability accordingly, and execute the biased random-walk strategy for cryophilic movement.

Discussion

A current focus of technological development in neuroscience aims to quantify the activity of individual neurons in intact neural circuits within animals as they freely perform behavioral tasks, because this may be the only case in which the functionally relevant operation of a neuron may be definitively observed. Toward this end, major strides in instrumentation have been made in vertebrate neurophysiology, with miniature head-mounted microscopes that can be surgically inserted into an animal's brain, allowing stable imaging of neurons and neural activity in awake, freely moving animals (Helmchen et al., 2001); new techniques for dynamically stabilizing the position of recording electrodes that can be chronically implanted within the brain (Fee, 2000); and miniaturized fiber optic microscopy that allows the visualization of neuronal activity in deep brain areas (Jung et al., 2004).

Another route to completely noninvasive imaging of neuronal function is to apply genetically encoded optical probes of neural activity to transparent animals such as *C. elegans* or larval zebrafish. One reason for imaging neuronal activity in semirestrained or unrestrained animals is to understand how neuronal activity gives rise to behavioral decision making and observable motor output. Pioneering optical imaging experiments have been performed in restrained larval zebrafish, analyzing the activity of specific motor neurons and interneurons that mediate escape behavior (Higashijima et al., 2003), and in semirestrained *C. elegans*, correlating the activity of the ASH sensory neuron, which mediates avoidance behavior to Cu^{2+}, to reversals (Faumont and Lockery, 2006). However, it is equally important to understand how an animal's movement modifies, defines, and even optimizes its own sensory experience as it freely navigates its environment. To analyze this phenomenon, what may be called active perception, we must be able to image the neuronal activity of unrestrained animals exploring their different environments.

Sophisticated examples of active perception have been studied in the whisking behavior of rodents, which scan objects by actively sweeping their sensory vibrissa across surfaces (Mehta et al., 2007), and of bats, which use their sonar-related vocalizations during flight to locate targets in complex environments (Moss et al., 2006). However, a rudimentary form of active perception may be found in the nematode *C. elegans* as it navigates chemical and thermal gradients. The worm appears to use only the time-varying signals of chemosensory and thermosensory neurons that are located in the worm's head. Thus, to detect the direction of ambient chemical and thermal gradients, the worm must couple the temporal patterns in the activity of its sensory neurons to the worm's own movements, either its overall heading or the side-to-side movements of the tip of the worm's nose driven by its undulatory gait.

Thermosensory behavior in *C. elegans* comprises two navigational modes (Hedgecock and Russell, 1975; Ryu and Samuel, 2002). When worms navigate at temperatures above their previous cultivation temperature, worms execute a biased random walk depending on thermosensory perception of warming or cooling in what is called cryophilic behavior. This biased

random-walk strategy requires that the worm be able to compute the first derivative of a thermosensory input during periods of forward movement and modulate reorientation probability accordingly (Clark et al., 2007). When worms navigate at temperatures near their previous cultivation temperature, worms crawl along isotherms in strikingly persistent periods of forward movement. The strategy for isothermal tracking requires the worm to sense and actively maintain isothermal alignment. When the worm is perfectly aligned along an isotherm in a spatial thermal gradient, temporal variations in a thermosensory input at the worm's nose should be locked to the sinusoidal undulating rhythm of the worm's locomotory gait. If the worm veers from the isotherm, toward either the warm or cool side of a spatial thermal gradient, then, in principle, the worm could correct its alignment by curving its movement to offset the resultant warming or cooling (Luo et al., 2006).

We have shown, using unrestrained worms, that the AFD thermosensory neuron encodes the minimal information that the worm requires to execute either the cryophilic behavior or the isothermal tracking behavior. First, worms restrict the temperature range of each behavior based on absolute temperature. Worms only track isotherms at temperatures near their previous cultivation temperature and exhibit cryophilic movement at temperatures above their previous cultivation temperature. Here, we showed for unrestrained worms, as we previously showed for immobilized worms, that the AFD neuron exhibits an operating range spanning the temperature range of isothermal tracking and of cryophilic movement. For worms cultivated at 20°C, the AFD neuron is only sensitive to temperature changes above ~17°C ($T > T^*_{AFD}$). Thus, the worm may regulate the temperature range of its active thermotactic behaviors by regulating the operating range of the AFD thermosensory response.

When the worm crawls on a spatial thermal gradient, short-term adaptation in the AFD thermosensory response allows the worm to respond to the time derivative of the thermosensory input and thus to detect warming or cooling during periods of forward movement. We have shown that the information encoded in the AFD calcium signal of a moving worm provides enough information for the worm to detect whether it is heading up or down a gradient, such that it could bias its reorientation rate accordingly. When AFD is active, small temporal increases and decreases in temperature evoke short-term increases and decreases in the level of intracellular calcium in the AFD neuron. We have shown that these changes in activity can occur on a timescale comparable to the timescale of worm undulatory motion. Thus, the AFD neuron is able to phase-lock to the oscillating component of the thermosensory input of a worm moving isothermally in a spatial thermal gradient.

One outstanding question is why the operating range of the AFD neuron appears to span both the operating range of isothermal tracking behavior and of cryophilic behavior. One possibility is that AFD activity is different in the ranges of isothermal tracking and cryophilic behavior in a way that cannot be distinguished by imaging intracellular calcium dynamics. Another possibility is that the worm uses additional information from another, as yet unidentified, thermosensory neuron to distinguish the temperature ranges for isothermal tracking and cryophilic behavior. Even so, the worm relies on its shallow 302-neuron nervous system to encode all aspects of its behavior. Our observations suggest that the sophistication of navigational behavior in unrestrained *C. elegans* may be attributed to the sophisticated properties of individual neurons at its sensory periphery.

The next steps in understanding the neural circuit for thermotaxis in *C. elegans* will be to demonstrate how the thermosensory signal that is processed by the AFD neuron is systematically transformed into motor output through the hierarchy of interneurons, command motor neurons, and motor neurons that comprise the *C. elegans* nervous system. Systematic laser ablation analysis and genetic analysis should produce a more extensive and satisfactory map of the neural circuit for thermotaxis in *C. elegans* (Mori and Ohshima, 1995; Chung et al., 2006). Quantifying the activity of neurons downstream of the AFD neuron in freely behaving worms should allow us to follow the neural transformations that lead to thermotactic behavioral decision making and motor output.

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