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The *C. elegans* Thermosensory Neuron AFD Responds to Warming

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Summary

The mechanism of temperature sensation is far less understood than the sensory response to other environmental stimuli such as light, odor, and taste. Thermotaxis behavior in *C. elegans* requires the ability to discriminate temperature differences as small as ~0.05°C and to memorize the previously cultivated temperature [1, 2]. The AFD neuron is the only major thermosensory neuron required for the thermotaxis behavior [3]. Genetic analyses have revealed several signal transduction molecules that are required for the sensation and/or memory of temperature information in the AFD neuron [4–7], but its physiological properties, such as its ability to sense absolute temperature or temperature change, have been unclear. We show here that the AFD neuron responds to warming. Calcium concentration in the cell body of AFD neuron is increased transiently in response to warming, but not to absolute temperature or to cooling. The transient response requires the activity of the TAX-4 cGMP-gated cation channel, which plays an essential role in the function of the AFD neuron [5]. Interestingly, the AFD neuron further responds to step-like warming above a threshold that is set by temperature memory. We suggest that *C. elegans* provides an ideal model to genetically and physiologically reveal the molecular mechanism for sensation and memory of temperature information.

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Results and Discussion

The AFD neuron of *Caenorhabditis elegans* is one of the best-studied thermosensory neurons in terms of its behavioral and molecular characteristics [1], but the understanding of its physiological properties has been limited by difficulty in performing electrophysiological analysis on individual neurons in the animals. In the present study, we monitored the responsiveness of the AFD neuron in live animals using cameleon, a genetically encodable calcium sensor [8]. Cameleon is a chimeric protein containing CFP and YFP derivatives and a calcium binding domain. The binding of calcium to the calcium binding domain increases the fluorescence resonance energy transfer (FRET) between CFP and YFP, such that changes in calcium concentration can be monitored by changes in the fluorescence ratio between CFP and YFP. Cameleon and other genetically encodable calcium sensors have been successfully used to monitor changes in intracellular calcium concentration ($[Ca^{2+}]_i$) in muscles and mechanosensory neurons of *C. elegans* [9–12].

As shown in Figures 1 and 2A, warming from 15°C to 25°C caused a transient increase in the fluorescence ratio in cameleon YC2.12 [13] in the cell body of the AFD neuron. The maximum ramp rate of the warming was 1.5°C/s, and the transient increase in fluorescence ratios appeared to begin when the temperatures reached ~20°C (see below). In conjunction with the ratio increase, there was a reciprocal change in the fluorescence intensities of YFP and CFP (Figure 1B), indicating that the increase is due to a change in FRET and consistent with a change in calcium concentration. The transient increase was detected 16 times in 20 trials (Figure 2A and Table 1), and its value is similar to that observed in mechanosensory neurons in the animals stimulated by gentle touch [10]. In contrast, YC4.12, which has a lower affinity for calcium [8], gave a smaller transient increase than YC2.12 (Figure 2A and Table 1). This result also indicates that the transient increase is due to calcium changes in the AFD neuron and not to other physiological changes in the cameleon protein itself or in the intracellular environment.

In the *tax-4(p678)* null mutant, the transient increase in YC2.12 fluorescence was observed far less frequently and had a smaller magnitude (Figure 2A and Table 1) compared to wild-type animals. The TAX-4 cGMP-gated channel is essential for the temperature-sensing function and some specific gene expression within the AFD neuron in adult animals [5, 7]. cGMP- and cAMP-gated channels play a central role in visual and olfactory signal sensation in the sensory neurons of vertebrates and invertebrates [14]. Therefore, these results suggest that a major part, if not all, of the transient $[Ca^{2+}]_i$ increase in the AFD neuron depends on the temperature-sensing molecular pathway including TAX-4.

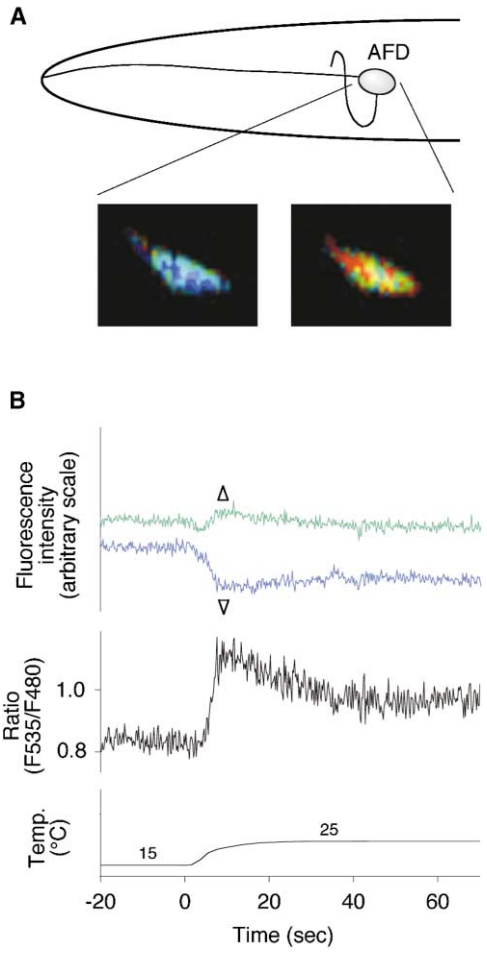

Figure 1. Imaging Response in the AFD Neuron

(A) The AFD neuron in *C. elegans*. A schematic diagram of the AFD neuron (top) and corresponding pseudocolor images depicting the fluorescence ratio before (left) and during (right) the transient response are shown. In these images, blue represents a lower and red represents a higher fluorescence ratio.

(B) An example of the fluorescence ratio change in the AFD neuron. Intensities of YFP (F535, green), CFP (F480, blue), the ratio (F535/F480, black), and temperature (thin black line at the bottom) are shown. Open arrowheads indicate the reciprocal change in fluorescence. The imaging started 60 s prior to the beginning of the temperature change (t = 0).

Baseline levels of the ratio before and after warming were similar between wild-type and *tax-4(p678)* backgrounds, suggesting that the baseline levels do not reflect the TAX-4-dependent responses associated with warming (Figure 2A and Table 1).

When temperature was decreased from 25°C to 15°C for a similar period of time as in the warming experiment, little transient change in the ratio was observed (Figure 2B and Table 1). Cooling in a step-like manner (see below) did not cause transient change in the ratio, either (data not shown). Two possibilities may explain this difference between warming and cooling. First, the AFD neuron may not respond to cooling. A second possibility is that the AFD neuron does respond to cooling, but cooling does not cause a detectable change in calcium levels (for discussion, see below).

Considering the possibility that the AFD neuron may only respond to artificially rapid warming (such as shown in Figures 1B and 2A), we also examined AFD response during a slower warming period. When temperature was raised about four times more slowly than in the previous experiments (maximum ramp rate at 0.35°C/s), a transient increase was still observed (Figure 2C). The transient response began at 37.3 ± 7.0 s (mean ± SD; n = 8) after the start of the warming period, which corresponds to a temperature of 20.6°C ± 0.6°C. Ramp rate during this period was about 0.08°C/s. During *C. elegans* movement, one "bending" of the animal (one unit of their forward movement in which they flip their head from one side to the other) takes almost 1 s, and its width is ~0.1 mm in length. In the typical thermotaxis assay in 9 cm plate, spatial gradient of temperature is roughly estimated as 0.02°C/0.1 mm (=25°C–17°C/4 cm). Therefore, the animals may recognize temperature differences between both sides of their body at a rate of ~0.02°C/s, which is a similar order to the ramp rate in this experiment. This result suggests that the transient $[Ca^{2+}]_i$ changes observed in these experiments are not artifactual and reflect responses of the AFD neuron in wild-type animals during thermotaxis. However, it is still ten times larger than the animal's sensitivity threshold to temperature change (0.008°C/s) [15].

In order to determine whether the AFD neuron responds to warming per se or to absolute temperature around 20°C, we next raised temperature in a step-like manner. When temperature was raised from 15°C to 25°C by 2°C increments, the AFD neuron of wild-type animals cultivated at 20°C responded to every warming above 19°C (Figure 3 and Table 2). This result indicates that the AFD neuron responds to warming above a threshold temperature but not to absolute temperature. Only one response was observed in the monotonous warming (Figures 1B, 2A, and 2C), while multiple responses were observed in the step-like warming. It also suggests that the AFD neuron responds to warming when the ramp rate is larger than a certain value.

Interestingly, the threshold temperature is modulated by memory. When animals had been cultivated overnight at 15°C or 25°C prior to imaging, the warming response of the AFD neuron exhibited a lower or higher threshold temperature than the ones cultivated at 20°C, respectively (Figure 3 and Table 2). This result indicates, for the first time to our knowledge, that the temperature memory is stored within a thermosensory neuron as a threshold temperature to thermal stimuli. Although the modulation of threshold occurs within the AFD thermosensory neuron, it is likely that it may require the activity of other neurons such as downstream neurons of the thermotaxis circuit [1].

In summary, we demonstrate here that the AFD thermosensory neuron responds to warming with high sensitivity. This result is consistent with the fact that the AFD neuron is essential for the thermotaxis behavior, which allows animals to discriminate small temperature differences during isothermal tracking [1, 2]. A previous study showed that synaptic release from the AFD neuron was low when ambient temperature was equal to cultivated temperature and high when they were different [16]. We do not consider that our and their results are conflicting

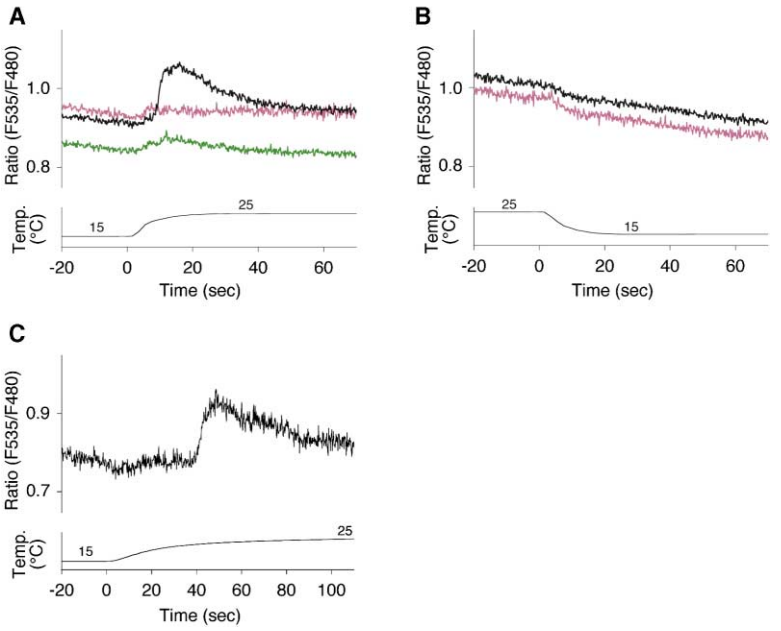

Figure 2. The AFD Neuron Responds to Warming, but Not to Cooling

(A) AFD response during warming. The average fluorescence ratio of YC2.12 or YC4.12 in wild-type animals (black line, n = 20; green line, n = 17, respectively) as well as YC2.12 in *tax-4(p678)* mutants (red line, n = 17) is shown. Note that the baseline level of YC2.12 in wild-type animals is similar to that of YC2.12 in the *tax-4(p678)* mutants. However, YC4.12 in wild-type animals has a lower baseline level, probably due to its lower affinity for calcium.

(B) AFD response during cooling. The average fluorescence ratio of YC2.12 in wild-type animals (black line, n = 16) or in *tax-4(p678)* mutants (red line, n = 10) is shown.

(C) An example of the AFD response observed during slow warming. YC2.12 in wild-type animals exhibited a transient increase in the ratio when temperature was around 20°C.

because synaptic release activity and $[Ca^{2+}]_i$ change in the cell body could be different and because temporal resolution of their experiment (more than 1 min) is lower than that of our experiments (0.2 s). Thus, it is likely that the responses of the previous study reflect basal activities of the AFD neuron dependent on ambient temperature although they are related to temperature memory. In contrast, we monitored acute response of the AFD neuron to thermal stimuli, which appears closely related to the ability of the animals to track isotherms as well as to memorize temperature.

The AFD neuron responds to warming but not to cooling, at least in our experimental conditions (Figure 2). A recent behavioral study suggests that the animals respond to higher temperature but not to lower temperature than cultivated temperature during or within several minutes after temporal temperature change [15], while another study suggests that the animals respond to up and down temporal thermal gradients in different ways [17]. Analyses using thermotaxis assay upon spacial thermal gradients suggest that the animals do move toward cultivation temperature by migrating up as well as down the thermal gradients [2, 3, 5, 6]. Hence, it is essential to clarify the biological link between our results presented here and the different conclusions derived from these behavioral studies. Although the AFD thermosensory neuron is responsible for the major part of the thermotaxis behavior, laser ablation and genetic studies suggest that another unidentified thermosensory neuron "X" also plays a certain role in cryophilic drive during the thermotaxis behavior [1]. The fact that the AFD neuron only responds to warming suggests that X may respond to cooling.

Our data suggest that the properties of the AFD neuron are analogous, at least in part, to the properties of the warm fibers in the mammalian peripheral nervous system, which sense innoxious warm temperatures on the skin [18, 19]. The mammalian warm fibers show a transient active response to rapid temperature increases but do not respond to mechanical stimulus. This is in contrast to the peripheral nociceptive neurons in mammals, which are capable of responding to both noxious heat and mechanical stimuli [19, 20]. Similar to the warm fiber, the AFD neuron is not responsible for the sensation of noxious heat or mechanical stimuli of *C. elegans* [21, 22].

What kind of molecules are involved in the sensation of warming? Although members of the mammalian TRP ion channel family are sensitive to various temperature ranges in vitro [23, 24], their physiological role in sensa-

Table 1. Averages of the Fluorescence Ratio Change during Monotonous Temperature Changes

Temperature	YC in Strain	Baseline at 1st Temp.	Baseline at 2nd Temp.	Transient Change (%)
15°C to 25°C	YC2.12 in wild type (n = 20)	0.91 ± 0.02	0.95 ± 0.02	13.0 ± 1.0 (n = 16)
	YC4.12 in wild type (n = 17)	0.85 ± 0.02*	0.84 ± 0.02**	6.4 ± 0.9** (n = 10)
	YC2.12 in *tax-4(p678)* (n = 17)	0.93 ± 0.02	0.94 ± 0.03	3.3 ± 0.6** (n = 3)
25°C to 15°C	YC2.12 in wild type (n = 16)	1.01 ± 0.02	0.93 ± 0.02	n.d.
	YC2.12 in *tax-4(p678)* (n = 10)	0.97 ± 0.02	0.89 ± 0.03	n.d.

Values are mean ± SE. Single asterisk indicates $p < 0.05$, and double asterisks indicate $p < 0.005$ compared to the corresponding values of YC2.12 in wild-type. The values of transient change (%) were calculated comparing to the baseline at second temperature. Parentheses under "transient change (%)" represents numbers of the experiments where the transient change was detected. n.d., not detected.

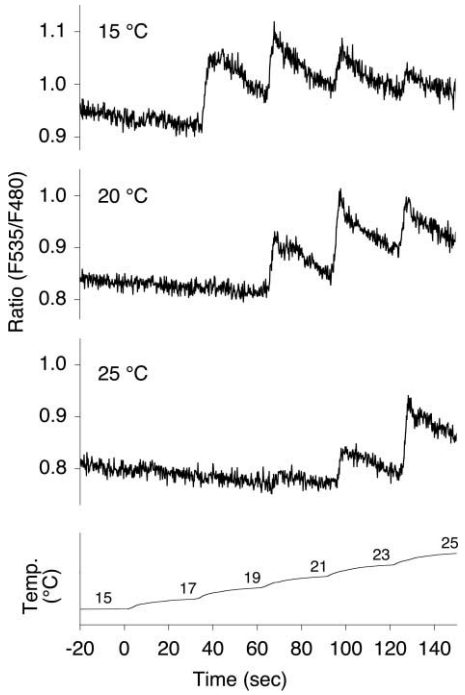

Figure 3. The AFD Neuron Responds to Step-like Warming above a Threshold Temperature

Wild-type animals expressing YC2.12 in the AFD neuron were grown at 20°C until the day before the imaging. The animals were then cultivated at 15°C (top), 20°C (middle), or 25°C (bottom) overnight. The average ratio change during step-like warming is shown in each panel (n = 5). All the animals cultivated at 15°C or 20°C responded to each warming step above 17°C or 19°C, respectively. When the animals were cultivated at 25°C, four out of five animals responded above 21°C and one above 19°C (Table 2). This variety of threshold temperatures may reflect the fact that the animals migrate toward a preferred temperature less accurately than they track isotherms at preferred temperature [2].

tion of temperature is not understood. Interestingly, any of the four TRPV channel family members found in *C. elegans* are not expressed in the AFD neuron, and at least two of them are not activated by heat in vitro [25]. Although genetic analyses in *C. elegans* have revealed that signal transduction molecules, such as cGMP-gated cation channels TAX-2/TAX-4, calcineurin ortholog TAX-6, and calcium/calmodulin-dependent protein kinase CMK-1, function in the AFD neuron [4–7], the way that temperature signal is transduced is still unclear. Further genetic and physiological studies of *C. elegans* may reveal the evolutionarily conserved, yet novel, mechanism of the signaling pathway for the sensory response to warming, including temperature receptors.

Experimental Procedures

Molecular Biology

Details of the plasmid construction are available upon request. YC4.12 was generated from YC2.12 using site-directed mutagenesis to create an E31Q substitution in the calcium binding domain [8]. Cameleon was expressed specifically in AFD neuron using an AFD-specific promoter from K01H12.3, which includes the first 2 amino acid residues and upstream 1612 sequences (according to WormBase freeze WS120; http://ws120.wormbase.org/). The promoter and YC2.12 or YC4.12 sequences were ligated into the pPD49.26 vector containing *unc-54* 3′ UTR as a general 3′ UTR sequence (a gift from A. Fire) to create AFDp::YC plasmids.

Strain Maintenance and Germline Transformation

The standard procedure for culturing and handling wild-type N2 and the mutant animals was described by Brenner [26]. Germline transformation was performed by coinjecting the AFDp::YC plasmid (10–25 ng/μl), the injection marker *ges-1::NLS GFP* (50 ng/μl, [6]), and pBluescript vector as carrier (to a total DNA concentration of 100 ng/μl) into the gonad of wild-type or *tax-4(p678)* animals [27]. Transgenic animals were identified by intestinal GFP fluorescence as a result of the expression marker. Two or three independent transgenic lines were established and used for the imaging.

Sample Preparation and Ratio Imaging

Several transgenic adult animals expressing YC were fixed onto a 2% agar pad (0.5 mm thick) on a large cover glass (24 × 60 mm, 0.12–0.17 mm thickness, Matsunami, Japan) using a small amount of the cyanoacrylate glue, Aron Alpha A (Sankyo, Japan). Gluing the tip of the animal's nose was avoided because it abolishes transient calcium changes. The fixed animals were immersed in M9 buffer, covered by a smaller cover glass (24 × 24 mm, 0.12–0.17 mm thickness, Matsunami, Japan), and subjected to imaging. General room temperature was kept at ~25°C, and the sample preparation was completed within 30 min. For temperature regulation, a Peltier-based thermocontroler ThermoPlate MATS-555 (Tokai Hit, Japan) was placed on the stage of an upright microscope E600 with air 40× objective (Nikon). The sample was then placed on the ThermoPlate, kept at the first temperature of the imaging (e.g., at 15°C for the warming experiments) for 10 min, and then exposed to excitation light using a bandpass filter (435/10), a ND32 filter, and a dichroic mirror (455). The fluorescence was introduced into W-View optics system (Hamamatsu, Japan) containing two dichroic mirrors (510) and bandpass filters (480/30 and 535/25). F480 and F535 images split by W-View were simultaneously captured by a CCD camera HiSCA (Hamamatsu, Japan). Images were taken at 203 ms exposure time for 3–3.5 min with 4 × 4 binning. One AFD neuron was subjected to the imaging only once, and just one of a pair of AFD neurons (left or right) were used from one animal. Temperature change caused a slight change in the volume of the Peltier unit, which led to a change in the focal plane. However, this slight change in focal plane could be re-adjusted by hand and did not cause a problem in the ratio imaging. The temperature in the graph was calculated as the average of two surface temperatures: that of the bottom large cover glass and that of the top small cover glass above the worms and agar pad. The temperature was measured with thermistor temperature sensor D617 with temperature probe BYZ-53 (Technol Seven, Japan). Each temperature was monitored every 2 s, and the differences of the two were about 0.5°C (fast warming) or 1°C (step-like warming) at maximum. Time lags due to the time constant of the temperature probe (2.5 s) were adjusted in the graph.

Table 2. Averages of the Transient Ratio Increase (%) during Step-like Warming

Cultivation Temperature	15°C to 17°C	17°C to 19°C	19°C to 21°C	21°C to 23°C	23°C to 25°C
15°C	n.d.	7.5 ± 1.8	9.5 ± 2.2	7.9 ± 1.7	4.6 ± 1.5
20°C	n.d.	n.d.	7.6 ± 0.9	10.2 ± 1.0	10.4 ± 1.5
25°C	n.d.	n.d.	7.8*	6.9 ± 1.2	9.5 ± 1.0

Values are mean ± SE. All the numbers are the averages of five trials except for data indicated with an asterisk, where the transient ratio increase was detected only once out of five trials. n.d., not detected.

As control experiments for the ratio imaging, YC2.12 or YC3.12 were expressed in pharyngeal muscles of wild-type animals using the *myo-2* promoter of pPD30.69 (a gift from A. Fire). The change in the fluorescence ratio within the terminal bulb was monitored during pharyngeal pumping, which was induced by serotonin (1 mg/ml). The magnitude of the ratio change from YC2.12 and 3.12 were 5%–6% and 3%–4%, respectively, which is comparable to previously reported data [9].

Data Analysis

For each imaging, fluorescence intensities of F535 and F480 from the AFD cell body and its corresponding background were measured using AquaCosmos (Hamamatsu). To determine the maximum ratio of imaging during monotonous temperature change, the average ratio from 11 successive frames was calculated, and the approximate baseline value of second temperature was subtracted as background (Table 1). For the baseline comparison, the baseline values were determined as the average ratio from 51 successive frames (center $\pm$ 25 frames) 6 s before (first temperature) or 50 s after (second temperature) the beginning of the temperature change (Table 1). For the step-like warming experiment, the maximum ratio in each warming was compared to the ratio just before the next warming as a baseline value (Table 2). The Mann-Whitney U test was used to calculate all the significance values.

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