

**Analysis on regulation of neural activity and behavior
by glutamate neurotransmission
in the thermotaxis neural circuit of *C. elegans***

(線虫 *C. elegans* の温度走性を司る神経回路におけるグルタミン酸を
介した神経伝達による神経活性と行動の制御機構の解析)

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Summary

Elucidation of the mechanism by which massive information is processed in complex neural circuits of the brain is a fundamental problem in neuroscience. Here, we show that glutamate signals from two distinct sensory neurons bidirectionally affect the same postsynaptic interneuron, thus producing the opposite behaviors of the nematode *Caenorhabditis elegans*. EAT-4/VGLUT dependent glutamate signals from AFD thermosensory neuron inhibit the postsynaptic AIY interneurons through activation of GLC-3/GluCl inhibitory glutamate receptor and behaviorally drive migration toward colder temperature. By contrast, EAT-4 dependent glutamate signals from AWC thermosensory neurons stimulate the AIY neurons to induce migration toward warmer temperature. Alteration of the strength of glutamate signals from AFD and AWC leads to significant changes of AIY activity, resulting in drastic modulation of behavioral outputs. We thus provide an important insight on information processing, where two glutamate transmissions encoding opposite information flows regulate neural activities to produce a large spectra of behavioral outputs.

Introduction

Animals can sense a vast numbers of environmental stimuli and give rise to approximate behavioral outputs through processing immense neural information in complex neural circuits of the brain. Elucidation of the mechanism by which massive information is processed is a fundamental problem in neuroscience. In the course of processing, neurons communicate neural information through synaptic transmissions. To gain insight into information processing, it is important to delineate each information flow communicated through synaptic transmissions, whereby modulating behavioral outputs. Although studies in vertebrates have provided a wealth of data on mechanisms of synaptic transmissions, dissection of information flow conveyed between neurons has been hindered by difficulty of the identification of neural codes, because of the complexity of the vertebrate nervous system (Di Maio, 2008).

The nematode *C. elegans* is well suited for the analysis of information processing in neural circuits because of its simple nervous system consisting of only 302 neurons with entirely known synaptic connections and gap junctions (White et al, 1986). In addition, because of the accessible genetics of *C. elegans* (Brenner, 1974), we can isolate and analyze mutants of genes required for information processing. Recent technical advances also improve the analysis of information processing in *C. elegans*. A growing number of promoters which drive gene expression in specific neurons allow us to label specific neurons and neuronal

components with fluorescence protein tags in live worms (Chalfie et al, 1994; Nonet, 1999), and define the neurons in which particular gene functions (Kuhara et al, 2002). Further, physiological analysis of neurons in live worms has become possible by cameleon, a genetically encoded calcium indicator used to monitor calcium influx (Kerr et al, 2000; Miyawaki et al, 1997).

As a model organism with many advantages for analyzing information processing, *C. elegans* exhibits various behavioral outputs in response to many different environmental stimuli (de Bono & Maricq, 2005). Thermotaxis is one of the most plastic behaviors in *C. elegans*, in which the animals remember the ambient temperature in association with the past feeding state and migrates to and move isothermally around the previous experienced temperature when placed on a temperature gradient (Hedgecock & Russell, 1975; Mohri et al, 2005; Mori & Ohshima, 1995). To analyze details of thermotaxis, two types of thermotaxis assay systems have established; the individual TTX assay that is suitable for scoring isothermal tracking (IT) behavior (Figure 1; Gomez et al, 2001; Mori & Ohshima, 1995) and the population TTX assay that is suitable for quantitatively assessing the migration ability to the cultivation temperature (Figure 2; Ito et al, 2006). The neural circuit model for thermotaxis has been proposed. Temperature is sensed and remembered by AFD and AWC sensory neurons, thermal information from AFD and AWC is transmitted to AIY interneuron, and the subsequent information from AIY is further transmitted to AIZ and RIA interneurons for further neural information processing (Figure 3. ; Biron et al, 2008; Clark et al, 2006; Kuhara et al, 2008; Mori & Ohshima, 1995).

Previous attempts to dissect thermotaxis successfully identified several molecular components related to temperature sensing signal transduction in AFD and AWC thermosensory neurons. In AFD neurons, three guanylyl cyclases, GCY-8, GCY-18 and GCY-23 appear to redundantly produce cGMP as a second messenger, and cGMP-dependent cation channel composed of TAX-2 and TAX-4 increase internal Ca^{2+} concentration upon reception of temperature change (Figure 4; Inada et al, 2006; Kimura et al, 2004; Komatsu et al, 1996). Additional molecules such as TAX-6 (Calcineurin) and TTX-4 (PKC-1; nPKC-epsilon/eta), both of which likely adjust the temperature input in AFD, have been also reported (Kuhara et al, 2002; Okochi et al, 2005). In AWC thermosensory neurons, ODR-1 (guanylyl cyclase) produces cGMP through activation of ODR-3 (G-alpha), and TAX-4 (cGMP-dependent cation channel) increases internal Ca^{2+} concentration upon reception of temperature stimuli (Figure 4; Kuhara et al, 2008). It have been also reported that EAT-16 RGS (regulator of G protein signaling) suppressed the G protein coupled signaling in AWC (Figure 4; Kuhara et al, 2008). However, how critical neurons for the thermotaxis neural circuit communicate with each other to modulate the neural activity still remains to be understood.

Although the nervous system of *C. elegans* is less complex than those of vertebrates, *C. elegans* contains many of neurotransmitters found in vertebrates, including acetylcholine, serotonin, dopamine, gamma-aminobutyric acid (GABA), and glutamate (de Bono & Maricq, 2005). Many genes involved in

synaptic transmission through these transmitters have been identified. EAT-4 is one of the key components of glutamate-mediated neurotransmission; a *C. elegans* homolog of mammalian vesicular glutamate transporter (VGLUT) that concentrates glutamate into synaptic vesicles (Figure 9; Bellocchio et al, 2000; Lee et al, 1999; Takamori et al, 2000). Past study of EAT-4 revealed that EAT-4 is involved in chemotaxis, habituation of the tap-withdrawal response, local search, and migration toward colder temperature (Chalasani et al, 2007; Clark et al, 2007; Hills et al, 2004; Rankin & Wicks, 2000). In this study, molecular, genetic and calcium imaging analysis revealed that coordinated functions of EAT-4 dependent glutamate signals from AFD and AWC thermosensory neurons and RIA interneurons are essential for thermotaxis. EAT-4 dependent glutamate signals from AFD inhibit AIY through activation of GLC-3/GluCl inhibitory glutamate receptor (Horoszok et al, 2001), whereas EAT-4 dependent glutamate signals from AWC stimulate AIY. Alteration of the strength of EAT-4 dependent AFD and AWC glutamate signals onto AIY leads to significant changes of AIY activity, which, through EAT-4 dependent glutamate signals from RIA, results in drastic modulation of thermotaxis. Our results provide an important insight on information processing, where two glutamatergic synaptic transmissions encoding opposite information flows regulate neural activities to generate various behavioral outputs.

Results

Thermotaxis defects of *nj2* and *nj6* mutants are caused by defective function of *eat-4* gene

In our attempt to analyze thermotaxis defective mutants through forward genetic approach, we isolated *nj2* and *nj6* mutants that exhibit abnormal migration in a radial temperature gradient (Figure 5 and 6). Both *nj2* and *nj6* mutations mapped to the region including *eat-4* gene and the thermotaxis defects of *nj2* and *nj6* mutants are restored by introducing the genomic fragment including *eat-4* gene (Figure 7). Our sequencing of *nj2* and *nj6* genomes revealed that *nj2* is associated with two missense mutations of the conserved amino acid (G16E) and the putative transmembrane domain (G355R), and that *nj6* is a missense mutation of the conserved amino acid (G494R) (Figure 8; Ni et al, 1994). These results suggest that thermotaxis defects of *nj2* and *nj6* mutants are caused by defective function of *eat-4* gene.

VGLUT homolog EAT-4 is essential for thermotaxis behavior

To ensure the importance of EAT-4 for thermotaxis, we performed two types of thermotaxis (TTX) assays of *eat-4(ky5)* mutants carrying loss-of-function mutation in *eat-4* gene. The individual TTX assay is suitable for scoring isothermal tracking (IT) behavior (Figure 1; Gomez et al, 2001). Although many wild-type animals (55% ± 8%) exhibited IT behavior in a radial thermal gradient

form 17°C to 25°C after cultivation at 20°C in well-fed conditions, no *eat-4(ky5)* mutants (0%) exhibited IT behavior (Figure 5B and 6). In addition to IT behavior defect, *eat-4(ky5)* mutants showed severe impairment in the population TTX assay that is suitable for quantitatively assessing the migration ability to the cultivation temperature (Figure 2; Ito et al, 2006). After cultivation at 23°C, 20°C, and 17°C in well-fed conditions, most of wild-type animals migrated up or down the linear temperature gradient (0.45°C/cm) until they reached the region nearly corresponding to the previous cultivation temperature (Figure 10A). However, *eat-4(ky5)* animals had little tendencies to migrate toward cultivation temperature and mostly dispersed in a wide area (Figure 10A). TTX indices, quantified as shown in Figure 1C, of 23°C-cultivated and 17°C-cultivated *eat-4(ky5)* mutants (0.47 ± 0.14 at 23°C, -1.46 ± 0.08 at 17°C) differed significantly from those of wild-type animals (2.27 ± 0.19 at 23°C, -2.96 ± 0.22 at 17°C; Figure 10B). Although *eat-4(ky5)* mutants did not show locomotion defect, they dispersed less than wild-type animals in the absence of a temperature gradient (Figure 11; Ségalat et al, 1995), indicating the possibility that abnormal TTX indices of *eat-4(ky5)* mutants could result from other defects, such as local search defect. Nevertheless, *eat-4(ky5)* mutants dispersed more broadly from cultivation temperature than wild type animals on a thermal gradient (Figure 10A). This tendency was also observed in the population TTX assay of 20°C-cultivated *eat-4(ky5)* mutants after placed at the higher and lower temperature positions in the gradient (Figure 12). These results suggest that *eat-4(ky5)* mutants indeed exhibit thermotaxis defect, implicating the behavioral regulation by EAT-4/VGLUT dependent glutamatergic neurotransmission

(Figure 9).

EAT-4/VGLUT is expressed in subsets of neurons including constituents of the thermotaxis neural circuit

Previous report showed that *eat-4::lacZ* and *eat-4::gfp* fusion genes, which contain the 2.4kb fragment upstream of *eat-4* as a promoter, did not appear to express in neurons critical for the thermotaxis neural circuit (Lee et al, 1999). These results are consistent with our result that *eat-4* genomic fragment driven by this 2.4kb promoter did not restore the normal thermotaxis in *eat-4* mutants (Figure 13). Hence, the 2.4kb promoter region does not drive sufficient expression of *eat-4* gene for thermotaxis behavior. Given that another gene resides 5.5 kb upstream of *eat-4* gene, we constructed *full length eat-4::gfp* fusion gene that contains the whole *eat-4* genomic fragment and the 5.5kb upstream fragment (Figure 14A). We found that this *full length eat-4::gfp* fusion gene rescued the abnormal thermotaxis of *eat-4* mutants (Figure 13).

To identify cells expressing EAT-4, we observed the expression pattern of *full length eat-4::gfp* in wild-type animals (Figure 14B), and also in *unc-104(e1265)* mutants defective in UNC-104/KIF1A kinesin-like motor protein in order to prevent the EAT-4::GFP-caused strong fluorescence of the nerve ring (Figure 14C; Hall & Hedgecock, 1991; Otsuka et al, 1991). Based on cell-body positions and morphologies (Sulston & Horvitz, 1977; Sulston et al, 1983), we observed consistent expression of EAT-4::GFP in many head neurons including AFD

thermosensory neurons, AWC thermosensory neurons, AIZ interneurons and RIA interneurons, which are constituents of the thermotaxis neural circuit (Figure 14B, C, and D; Kuhara et al, 2008; Mori & Ohshima, 1995).

EAT-4/VGLUT dependent glutamatergic neurotransmissions from AFD and RIA neurons are essential for isothermal tracking

Expression of *eat-4* cDNA from its own promoter (5.5kb) and in all neurons strongly rescued the IT behavior defect of *eat-4(ky5)* mutants ($50\% \pm 2\%$ and $31\% \pm 2\%$, respectively) in the individual TTX assay (Figure 15), suggesting that neuronal expression of EAT-4 is essential for thermotaxis. To identify neurons that require EAT-4 mediated glutamatergic neurotransmission for thermotaxis, we conducted cell-specific rescue experiments by introducing *eat-4* cDNA under the control of various cell-specific promoters into *eat-4(ky5)* mutants. In the individual TTX assay, expression of EAT-4 in AFD, AWC or AIZ neurons did not induce any changes in IT behavior defect of *eat-4(ky5)* mutants (0%), whereas expression of EAT-4 in RIA interneurons weakly rescued IT behavior defect ($10\% \pm 2\%$) (Figure 15). Remarkably, simultaneous expression of EAT-4 in AFD and RIA rescued IT behavior defect ($39\% \pm 1\%$), as expression of EAT-4 under the control of *eat-4* promoter did ($p > 0.05$) (Figure 15). The rescue efficiency of the transgenic animals expressing EAT-4 in AFD and RIA was not increased by additional expression of EAT-4 in other neurons (Figure 15). These results suggest that EAT-4 dependent glutamate transmissions from AFD thermosensory neurons and RIA interneurons are crucial steps that process and

convey thermal information in the thermotaxis neural circuit.

EAT-4/VGLUT dependent glutamatergic neurotransmissions from AFD, AWC, and RIA neurons regulate migration toward cultivation temperature

Since expression of *eat-4* cDNA from its own promoter and in all neurons also strongly restored normal migrations to cultivation temperature ($p > 0.05$ compared with wild-type animals at every cultivation temperature) in the population TTX assay (Figure 16), we performed the population TTX assay for cell-specific rescue experiments (Figure 16). Similar to the results of individual TTX assay, expression of EAT-4 in AIZ interneurons did not induce any changes in thermotaxis behavior of *eat-4(ky5)* mutants (Figure 16). In contrast, expression of EAT-4 in AFD thermosensory neurons, in AWC thermosensory neurons, or in RIA interneurons induced different migration pattern from that of *eat-4(ky5)* mutants (Figure 16). Further, each expression had different effects on migration; the transgenic animals expressing EAT-4 in AFD migrated toward colder temperature than *eat-4(ky5)* mutants, the transgenic animals expressing EAT-4 in AWC migrated toward warmer temperature after cultivated at 20°C and 23°C, and the transgenic animals expressing EAT-4 in RIA neurons migrated toward warmer temperature only after cultivated at 23°C (Figure 16). These results imply that EAT-4 dependent glutamate transmissions from AFD, AWC, and RIA to their postsynaptic neurons are involved in regulation of thermotaxis and each transmission induces different behavioral outputs.

Because of the importance of EAT-4 in RIA interneurons that lie downstream of AFD and AWC thermosensory neurons (Figure 3 and 15), it was difficult to detect the effect of EAT-4 solely in AFD or AWC for thermotaxis behavior without expression of EAT-4 in RIA. To reconcile this problem, we introduced several doses of *AFDp::eat-4 cDNA* and *AWCp::eat-4 cDNA* with constant 5ng/ul dose of *RIAp::eat-4 cDNA* into *eat-4(ky5)* mutants, and performed the population TTX assay (Figure 17). Through the analysis, we found that simultaneous expression of EAT-4 in AFD, AWC, and RIA with 0.2ng-5ng-5ng/ul of *AFDp::eat-4 cDNA*, *AWCp::eat-4 cDNA*, and *RIAp::eat-4 cDNA*, respectively, almost rescued the defective migration to cultivation temperature of *eat-4(ky5)* mutants (Figure 17). Although the rescue was not complete in these transgenic animals, they showed similar behavior to that of *eat-4(ky5)* mutants expressing EAT-4 in all neurons (Figure 17). As shown in Figure 16, these results further suggest that EAT-4 dependent glutamate transmission from AFD, AWC, and RIA to their postsynaptic neurons regulates thermotaxis behavior.

Glutamatergic neurotransmissions from AFD and AWC neurons induce opposite migration

In the population TTX assay for cell-specific rescue experiments with simultaneous expression of EAT-4 in AFD, AWC, and RIA, alteration in introducing doses of *AFDp::eat-4 cDNA* and *AWCp::eat-4 cDNA* induced various migration (Figure 17). Simultaneous expression of EAT-4 in AFD and RIA with both 5ng/ul doses enhanced migration toward colder temperature than

wild-type animals after cultivation at 20°C and 23°C (cryophilic movement) (Figure 17). Although tracks of these transgenic animals in the individual TTX assay did not indicate cryophilic phenotype (Figure 18), cryophilic phenotype would not be detectable on the assay plate, where the steepness of thermal gradient differs in different areas (Figure 1A). Simultaneous expression of EAT-4 in AFD, AWC and RIA with introduction of 5ng-5ng-5ng/ul, 2ng-5ng-5ng/ul, and 0.5ng-5ng-5ng/ul of *AFDp::eat-4 cDNA*, *AWCp::eat-4 cDNA*, and *RIAp::eat-4 cDNA*, respectively, into *eat-4(ky5)* mutants still drove tendency to migrate toward cold temperature, while the cryophilic movement of these transgenic animals weakened as compared with the animals expressing EAT-4 in AFD and RIA at 20°C and 23°C (Figure 17). In contrast, simultaneous expression of EAT-4 in AWC and RIA neurons with 5ng/ul enhanced migration toward warmer temperature than wild-type animals after cultivation at 17°C and 20°C (thermophilic movement) (Figure 17). These results suggest that glutamate transmission from AFD induces cryophilic movement and that glutamate transmission from AWC induces thermophilic movement. Considering that EAT-4 in AWC was not necessary for IT behavior (Figure 15) and simultaneous expression of EAT-4 in AFD, AWC, and RIA induced migration toward cold temperature until the introduction dose of *AFDp::eat-4 cDNA* to the *eat-4* mutants was reduced up to less than one tenth of *AWCp::eat-4 cDNA* (Figure 17), AFD-mediated glutamate transmission is likely to be more influential than AWC-mediated glutamate transmission for thermotaxis behavior.

RIA neurons have multiple neurotransmitter outputs

As already described in Figure 15, EAT-4 dependent glutamate transmission from RIA is crucial for thermotaxis. Nevertheless, *eat-4* transgenic mutants expressing EAT-4 only in AFD but not in RIA (*eat-4 (AFD+)*) could migrate to colder temperature as opposed to *eat-4(ky5)* mutants that dispersed in broader area in the population TTX assay (Figure 16). In addition, the phenotype of *eat-4 (AFD+)* did not change with additional expression of EAT-4 in RIA (Figure 17). These results raise two possibilities: signals from AFD flow either through RIA independent pathway or through RIA dependent pathway coupled with both EAT-4 dependent and independent synaptic transmissions. To distinguish these possibilities, we examined the effect of genetically impaired RIA on *eat-4 (AFD+)* (Figure 19). Correct localization of synapses in RIA requires TTX-7/IMPase, and *ttx-7(nj50)* mutants showed abnormal thermotactic phenotype, similar to that of wild-type animals in which RIA was ablated (Tanizawa et al, 2006). Thermotactic defect of *ttx-7(nj50)* mutants completely masked the defect of *eat-4 (AFD+)* (Figure 19), suggesting the essential role of RIA dependent pathway involving both EAT-4 dependent and independent transmissions.

EAT-4/VGLUT dependent glutamatergic neurotransmissions is influential for the thermal response of AIY

As an attempt to analyze molecular physiology of the EAT-4 dependent

glutamate transmission for thermotaxis, we conducted calcium imaging of AIY interneurons that are postsynaptic to both AFD and AWC neurons in *eat-4(ky5)* mutants after being cultivated at 20°C (Figure 3 and 21). We monitored the temperature-dependent neural activity changes of intact AIY interneurons by measuring temperature stimulus-evoked calcium concentration changes using cameleon, a genetically encoded calcium indicator (Figure 20; Miyawaki et al, 1997; Nagai et al, 2004). We verified that expression of cameleon (*yc3.60*) in AIY itself did not affect thermotaxis, since wild type animals transgenic with cameleon construct (*AIYp::yc3.60*) showed normal thermotaxis (Figure 18B). Consistent with our previous results representing the responsiveness of AIY neurons to temperature change, the calcium concentration in AIY neurons of wild-type animals increased with warming (Figure 21A; Kuhara et al, 2008). The fluorescence resonance energy transfer (FRET) ratios did not return to baseline, which might be caused by faster degradation of YFP fluorescence than CFP fluorescence in *yc3.60* (Figure 21A and Figure 22). Upon warming, fluorescence resonance energy transfer (FRET) ratios in AIY neurons of *eat-4(ky5)* mutants changed more than those of wild-type animals ($11\% \pm 1\%$ for *eat-4(ky5)* mutants and $8\% \pm 0.6\%$ for wild-type animals at 90S after starting temperature change) (Figure 21A and C), which likely represents the hyper-responsiveness of AIY neurons in *eat-4(ky5)* mutants. These results suggest that EAT-4 dependent glutamate transmission is quite influential for the response of AIY neurons to temperature change.

Glutamatergic neurotransmissions from AFD and AWC neurons oppositely modulate activity of postsynaptic neuron AIY in response to warming

Previous reports showed that activation of AIY interneurons mediates movement toward warmer temperature (Hobert et al, 1997; Kuhara et al, 2008; Mori & Ohshima, 1995) indicate the correlation between the activity of AIY neurons and behavioral output. Hence, we hypothesized that glutamate transmission from AFD and AWC neurons have opposite effects on the activity of AIY neurons, similar to the opposite effect of AFD and AWC on behavioral outputs. To test this hypothesis, we monitored the activity of AIY on warming in transgenic *eat-4(ky5)* animals expressing EAT-4 in either AFD or AWC after being cultivated at 20°C by calcium imaging (Figure 21). Since EAT-4 dependent glutamate transmission from RIA is absolutely important for connecting neural activity and behavioral output (Figure 15), we also expressed EAT-4 in RIA of transgenic animals as shown in Figure 4. The FRET ratio changes in AIY of *eat-4(ky5)* mutants expressing EAT-4 in RIA (*eat-4 (RIA+)*) were larger than those of wild-type animals and similar to those of naïve *eat-4(ky5)* mutants (*eat-4*) ($10\% \pm 0.6\%$ at 90S) (Figure 21A and C), suggesting that expression of EAT-4 in RIA has almost no effect on the AIY activity. Additional expression of EAT-4 in AFD neurons of *eat-4(ky5)* mutants (*eat-4 (AFD+,RIA+)*) induced smaller change of FRET ratios in AIY neurons ($3.9\% \pm 1.5\%$ at 90S) as compared with *eat-4(ky5)* mutants and wild-type animals (Figure 21A and C). Notably, smaller change in FRET ratio observed in *eat-4 (AFD+,RIA+)* were similarly observed in *eat-4(ky5)* mutants expressing EAT-4 only in AFD (*eat-4 (AFD+)*), which argues against any feedback pathway to AIY through RIA (Figure 23). Expression of EAT-4 in AWC

and RIA (*eat-4* (*AWC+*,*RIA+*)) caused not only the larger change like *eat-4(ky5)* mutants (*eat-4*) ($10.6\% \pm 1\%$ at 90s), but also faster change of FRET ratios in AIY ($5.1\% \pm 0.7\%$ at 45s) (Figure 21). Consistent to our hypothesis, our results on calcium imaging of AIY in 20°C-cultivated animals suggest that glutamate transmissions from AFD or from AWC thermosensory neurons inhibit or stimulate AIY interneurons, respectively.

We also monitored activity of AIY on warming in 17°C- and 23°C-cultivated wild-type animals and the *eat-4(ky5)* transgenic animals by calcium imaging (Figure 24). After being cultivated at 17°C, whereas the FRET ratio changes in AIY of *eat-4* (*AFD+*,*RIA+*) were similar to those of wild-type animals, the ratio changes of *eat-4* (*AWC+*,*RIA+*) were larger than those of wild-type animals (Figure 24A and B). In contrast, whereas the ratio changes of *eat-4* (*AWC+*,*RIA+*) were similar to those of wild-type animals, the ratio changes of *eat-4* (*AFD+*,*RIA+*) were smaller than those of wild-type animals after being cultivated at 23°C (Figure 24C and D). Thus, our results on response of AIY upon warming closely correlated with behavioral data shown in Figure 17 in every cultivation temperature, further suggesting the opposite modulation of AIY activity by glutamate transmissions from AFD and AWC.

Glutamatergic neurotransmissions from AFD neurons inhibit activity of AIY neurons in response to cooling

To verify the effect of glutamate signals on the response of AIY, we also

conducted calcium imaging of AIY on cooling in 20°C-cultivated wild-type animals and the *eat-4(ky5)* transgenic animals (Figure 25). Upon cooling, FRET ratios in AIY of *eat-4(ky5)* mutants did not decrease as much as those of wild-type animals ($-14\% \pm 0.7\%$ for *eat-4(ky5)* mutants and $-19\% \pm 1.0\%$ for wild-type animals at 120S) (Figure 25). Consistent with the results on warming shown in Figure 21, this result likely represents hyperactive state of AIY in *eat-4(ky5)* mutants (Figure 25). The FRET ratios in AIY of *eat-4 (RIA+)* and *eat-4 (AWC+,RIA+)* showed the similar change to those of *eat-4(ky5)* mutants ($-14\% \pm 0.8\%$ for *eat-4 (RIA+)* and for *eat-4 (AWC+,RIA+)* at 120S) (Figure 25), implicating no effects of glutamate signals from AWC and RIA on the response of AIY to cooling. By contrast, the FRET ratios in AIY of *eat-4 (AFD+,RIA+)* decreased to the similar level to those of wild-type animals ($-18\% \pm 0.9\%$ for *eat-4 (AFD+,RIA+)* at 120S) (Figure 25), suggesting that inhibition through glutamate transmission from AFD regulates the response of AIY to cooling. Altogether, our results on calcium imaging of AIY demonstrate that glutamate transmission from AFD inhibits AIY activity in response to both warming and cooling, and glutamate transmission from AWC stimulates AIY activity in response to only warming, thereby implying that AFD-mediated glutamate transmission more effectively contributes to thermotaxis behavior than AWC-mediated glutamate transmission.

Opposite glutamate signals from AFD and AWC neurons induce drastic changes in behavioral output

To elucidate whether various behavioral outputs shown in Figure 17 are caused by modulation of AIY activity through EAT-4 dependent glutamate transmission from AFD and AWC neurons, we investigated dose-dependency of AIY activity in transgenic *eat-4(ky5)* mutants expressing relative different doses of EAT-4 in AFD, AWC and RIA neurons with calcium imaging (Figure 26). Intriguingly, FRET ratios showed similar small changes when introducing 5ng, 2ng or 0.5ng of *AFDp::eat-4 cDNA* to the *eat-4* mutants, whereas FRET ratios dramatically increased when introducing 0.2ng or 0ng of *AFDp::eat-4 cDNA* (Figure 26), which is consistent with the results of cell-specific rescue experiments shown in Figure 17. Thus, our results demonstrated that modulation of the AIY activity by opposite glutamate signals from AFD neurons and from AWC neurons induces drastic change of behavioral output.

Identification of glutamate receptors involved in thermotaxis

Our analysis of EAT-4/VGLUT in thermotaxis suggested that glutamate receptors receive EAT-4 dependent glutamate signals in AIY interneurons and motorneurons postsynaptic to RIA interneurons. In *C. elegans*, four classes of glutamate receptors are predicted (Table 1); AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)-type glutamate-gated cation channels (GluR) encoded by eight *glr* genes, NMDA (N-methyl-D-aspartic acid)-type glutamate-gated cation channels (NR) encoded by two *nmr* genes, glutamate-gated

chloride channels (GluCl) encoded by four *glc* genes and two *avr* genes, and metabotropic G-protein-coupled glutamate receptors (mGluR) encoded by three *mgl* genes (Brockie & Maricq, 2003; Dillon et al, 2006; Yates et al, 2003). Of those, MGL-1/mGluR and GLC-3/GluCl were reported to be expressed in AIY interneurons (Horoszok et al, 2001; Wenick & Hobert, 2004), and GLR-1/GluR and AVR-14/GluCl were reported to be expressed in many motorneurons (Dent et al, 2000; Hart et al, 1995). In addition, the previous research of AIY interneurons showed the similarity between AIY interneurons and vertebrate bipolar cells in which mGluR is indicated to receive glutamate from presynaptic photo receptor neurons (Hobert et al, 1997; Nakajima et al, 1993; Nakanishi, 1994; Satterlee et al, 2001). Because these reports imply the possible involvement of GLC-3/GluCl, GLR-1/GluR, AVR-14/GluCl, and three mGluRs (MGL-1, MGL-2, and MGL-3) in thermotaxis, we performed the population TTX assay of mutants containing mutations in each gene encoding these glutamate receptors (Figure 27 and 28). *glr-1(n2461)* mutants migrated to their cultivation temperature normally in the population TTX assay (Figure 27). In contrast, *glc-3(ok321)* mutants migrated to warmer temperature than the cultivation temperature after cultivation at 17°C and 20°C (thermophilic movement), and *avr-14(ad1302)* mutants migrated to colder temperature than the cultivation temperature after cultivation at 20°C and 23°C (cryophilic movement) (Figure 27). Additionally, although single mutants of mGluRs (*mgl-1(tm1811)*, *mgl-2(tm355)*, and *mgl-3(tm1766)*) migrated to their cultivation temperature normally, *mgl-3(tm1766); mgl-1(tm1811)* double mutants and *mgl-2(tm355); mgl-3(tm1766); mgl-1(tm1811)* triple mutants migrated to colder temperature

than the cultivation temperature after cultivation at 23°C and after cultivation at 20°C and 23°C, respectively (Figure 28). These results imply that GLC-3/GluCl, AVR-14/GluCl, MGL-1/mGluR, and MGL-3/mGluR are involved in thermotaxis.

Cell-specific rescue experiments of thermotaxis-defective *avr-14(ad1302)* mutants

To identify neurons that require AVR-14/GluCl for thermotaxis, we tried to conduct cell-specific rescue experiments by introducing *avr-14* cDNA under the control of various cell-specific promoters into *avr-14(ad1302)* mutants. Because two types of *avr-14* cDNAs were predicted (Dent et al, 2000), we constructed both of *avr-14* cDNAs and introduced them simultaneously. First, to elucidate that *avr-14* cDNAs are functional and AVR-14 functions in neural circuit, we performed the population TTX assay of transgenic *avr-14(ad1302)* animals expressing *avr-14* cDNAs from its own promoter or in all neurons (Figure 29). Contrary to my expectation, neither of these expressions induced any changes in thermotaxis defect of *avr-14(ad1302)* mutants after being cultivated at 23°C (Figure 29).

MGL-3/mGluR functions in sensory neurons for thermotaxis

Expression of *mgl-3* cDNA in all neurons fully rescued the thermotaxis defect of *mgl-2(tm355); mgl-3(tm1766); mgl-1(tm1811)* triple mutants cultivated at 23°C in the population TTX assay (Figure 30), suggesting that neuronal expression of

MGL-3/mGluR is important for thermotaxis. To identify neurons that require MGL-3/mGluR for thermotaxis, we introduced *mgl-3* cDNA under the control of various cell-specific promoters into *mgl-2(tm355); mgl-3(tm1766); mgl-1(tm1811)* triple mutants and performed the population TTX assay of them after being cultivated at 23°C (Figure 30). Interestingly, expression of *mgl-3* cDNA in sensory neurons rescued thermotaxis defect of the triple mutants, suggesting the requirement of MGL-3/mGluR in several sensory neurons for thermotaxis (Figure 30). These results imply the regulation of thermotaxis through glutamatergic neurotransmission not being detected by our analysis of *eat-4*.

GluCl homolog GLC-3 inhibits the activity of AIY interneurons in thermotaxis

We also conducted cell-specific rescue experiments of *glc-3(ok321)* mutants by introducing *glc-3* cDNA under the control of various cell-specific promoters and found that the thermotaxis defect of *glc-3(ok321)* mutants was fully rescued by expressing *glc-3* cDNA in AIY interneurons but not in any other neurons (Figure 31). These results suggest that GLC-3/GluCl is required in AIY interneurons for thermotaxis.

Given that the activation of AIY interneuron mediates movement toward warmer temperature (Hobert et al, 1997; Kuhara et al, 2008; Mori & Ohshima, 1995) and glutamate-gated chloride channels (GluCls) mediate inhibitory neurotransmission (Dent et al, 1997), we hypothesized that GLC-3/GluCl

regulates thermotaxis through inhibition of the AIY activity. Consistent with this hypothesis, FRET ratios in AIY neurons of *glc-3(ok321)* mutants changed more than that of wild-type animals ($10\% \pm 0.5\%$ for *glc-3(ok321)* mutants and $6.6\% \pm 0.6\%$ for wild-type animals at 90S) (Figure 32), implicating the hyper-responsiveness of AIY neurons in *glc-3(ok321)* mutants. The increased change of FRET ratios was rescued by expressing *glc-3* cDNA in AIY interneurons ($6.2\% \pm 0.4\%$ at 90s) (Figure 32), suggesting that GLC-3 cell autonomously inhibits the activity of AIY.

GLC-3/GluCl receives glutamatergic signals from AFD thermosensory neurons

We addressed whether GLC-3 glutamate receptors in AIY interneuron perceive EAT-4 dependent glutamate signals from AFD or AWC thermosensory neurons. Thermosensory signal transduction in AFD neurons is thought to be mediated by the change in intracellular concentration of cGMP through GCY-23, GCY-8, and GCY-18 guanylyl cyclases (Figure 4A; Inada et al, 2006), and *gcy-23(nj37)* *gcy-8(oy44)* *gcy-18(nj38)* triple mutants showed abnormal thermotactic phenotype, similar to that of wild-type animals in which AFD neurons were ablated (Figure 33; Inada et al, 2006; Kuhara et al, 2008). *gcy-23 gcy-8 gcy-18; glc-3* quadruple mutants showed quite similar thermotactic abnormality to that of *gcy-23 gcy-8 gcy-18* triple mutants in the population TTX assay, suggesting that the abolishment of thermosensory signaling in AFD by *gcy-23 gcy-8 gcy-18* mutation entirely suppressed *glc-3(ok321)* mutation (Figure 33). Likewise,

thermotactic defect of the *eat-4* mutants expressing EAT-4 in AWC and RIA but not in AFD neurons (*eat-4 (AWC+,RIA+)*) completely masked the defect of *glc-3* mutants (Figure 33). These results suggest that GLC-3 glutamate receptors in AIY neurons receive EAT-4 mediated glutamate signals from AFD neurons.

Recently, ODR-3 G-alpha dependent heterotrimeric G protein coupled signaling was found to mediate thermosensory signal transduction in AWC neurons, and EAT-16 RGS (regulator of G protein signaling) suppressed the G protein coupled signaling in AWC (Figure 4B; Kuhara et al, 2008). *odr-3(n1605)* mutants and *eat-16(nj8)* mutants migrated toward warmer and colder temperature than the cultivation temperature, respectively, in the population TTX assay (Figure 34; Kuhara et al, 2008). *glc-3 odr-3* double mutants migrated much warmer temperature than both *glc-3* and *odr-3* single mutants after cultivation at 17°C and 20°C, implicating the additive effect of *odr-3* and *glc-3* mutation (Figure 34). Similarly, thermotactic defect of *eat-16(nj8)* mutants and the *eat-4(ky5)* mutants expressing EAT-4 in AFD and RIA (*eat-4 (AFD+,RIA+)*) influenced additively on the defect of *glc-3* mutants after cultivation at 17°C, 20°C and 23°C (Figure 34). These results implicate GLC-3 independent EAT-4 mediated glutamatergic neurotransmission in AWC. Altogether, our genetic analyses suggest that GLC-3/GluCl is required for reception of EAT-4/VGLUT dependent glutamate signals from AFD neurons.

We conducted calcium imaging to verify that EAT-4 dependent glutamate transmission from AFD thermosensory neurons is mediated by GLC-3 in AIY

interneurons (Figure 35). FRET ratios in AIY neurons of the double mutants (*eat-4 (AWC+,RIA+); glc-3*) constructed from the *eat-4* mutants expressing EAT-4 in AWC and RIA (*eat-4 (AWC+,RIA+)*) and *glc-3* mutants changed more than that of wild-type animals ($9.8\% \pm 0.8\%$ for double mutants and $7\% \pm 0.7\%$ for wild-type animals at 90S), similar to those in both single mutants ($10\% \pm 0.6\%$ for transgenic *eat-4* mutants and $10\% \pm 0.8\%$ for *glc-3* mutants at 90S) (Figure 35 A and B), thereby indicating the same hyper-responsiveness of double mutants to thermal stimuli as both single mutants. By contrast, FRET ratios in AIY neurons of the double mutants (*eat-4 (AFD+,RIA+); glc-3*) constructed from the *eat-4* mutants expressing EAT-4 in AFD and RIA (*eat-4 (AFD+,RIA+)*) and *glc-3* mutants showed the change in-between those in both single mutants ($7.6\% \pm 0.4\%$ for double mutants, $3.3\% \pm 0.9\%$ for transgenic *eat-4* mutants, and $10\% \pm 0.8\%$ for *glc-3* mutants at 90S) (Figure 35 C and D). These physiological results are consistent with the results obtained by genetic analysis, further implicating the molecular model that GLC-3 inhibits AIY interneurons upon receiving EAT-4 dependent glutamate signals from AFD neurons.

Discussion

EAT-4/VGLUT dependent glutamatergic neurotransmission in the thermotaxis neural circuit

In the current model for temperature sensing, thermal stimuli are transduced in AFD through three functionally redundant guanylyl cyclases and cGMP-gated channels (Figure 4A; Inada et al, 2006; Komatsu et al, 1996) and in AWC through heterotrimeric G protein signaling and cGMP-gated channels (Figure 4B; Kuhara et al, 2008). In this study, our results suggest a model for signaling pathways downstream of temperature sensing (Figure 36). After temperature sensing in AFD and AWC sensory neurons, thermosensory information is transmitted to postsynaptic interneuron AIY through EAT-4/VGLUT dependent glutamatergic neurotransmission. EAT-4 dependent glutamate signals from AFD inhibit AIY through activation of inhibitory glutamate receptor GLC-3/GluCl, thereby inducing migration toward colder temperature. By contrast, EAT-4 dependent glutamate signals from AWC stimulate AIY to induce migration toward warmer temperature. Hence, we demonstrated that the bidirectional regulation of AIY through EAT-4 dependent glutamate transmissions from AFD and AWC is essential for terminal behavioral phenotypes.

Previous reports showed that AFD and AWC differ in contribution for thermotaxis; AFD-ablated animals exhibited severe defect (Kuhara et al, 2008;

Mori & Ohshima, 1995), whereas AWC-ablated animals exhibited slight defect in thermotaxis (Biron et al, 2008; Kuhara et al, 2008). These results suggested that thermotaxis is regulated by the orchestrated information in major thermosensory neuron AFD and supportive thermosensory neuron AWC. Consistent with these previous results, our behavioral and calcium imaging results demonstrated that contributions of inhibitory glutamate signals from AFD and excitatory glutamate signals from AWC are not equivalent. For IT (isothermal tracking) behavior, EAT-4 dependent glutamate transmission from AFD was indispensable, while that from AWC was not necessary (Figure 15). In addition, transgenic *eat-4* mutants expressing EAT-4 in AFD, AWC, and RIA showed strong tendency to migrate toward colder temperature except for the case where the introduction dose of *AFDp::eat-4 cDNA* to the *eat-4* mutants was reduced to less than one tenth of *AWCp::eat-4 cDNA* (Figure 17). Further, glutamate transmission from AFD inhibits AIY activity in response to both warming and cooling and that from AWC stimulates AIY activity in response to only warming (Figure 21 and 25), suggesting that the contribution of inhibitory glutamate signals from AFD is larger than that of excitatory glutamate signals from AWC. Our study also showed that well-balanced glutamate signals from AFD and AWC could drive nearly normal responsiveness of AIY to temperature changes and eventual normal thermotaxis (Figure 17 and 26). Taken together, glutamate signals from AFD and AWC are implied to be the critical factor for mediating the orchestration of major information in AFD and supportive information in AWC. Also, our study demonstrated that GLC-3 receives EAT-4 dependent glutamate signals from AFD, although glutamate receptors that

receive glutamate signals from AWC remained to be identified. The failure of finding excitatory glutamate receptors may possibly be caused by the difference in contribution between AFD and AWC for thermotaxis, thus reflecting the biased detection ability of thermotaxis defect toward AFD over AWC.

Signal transduction from RIA interneurons to postsynaptic motor neurons

The RIA interneuron, known as one of the most pivotal interneurons in *C. elegans*, integrates signals processed in the thermotaxis neural circuit and emits outputs to downstream neurons (Figure 3; Mori & Ohshima, 1995; Tanizawa et al, 2006). Given those previous reports, our work revealed that multiple transmissions include EAT-4 dependent glutamate from RIA are involved in communicating processed information in the circuit to downstream neurons, thereby generating ultimate thermotactic outputs (Figure 15 and 36). Although there are no solid evidences as to which neurons downstream of RIA are main component neurons in the circuit, RIA is heavily connected with numerous presynapses to SMD and RMD head motoneurons that regulate turning behavior (Figure 37; Gray et al, 2005; White et al, 1986). Considering that temperature regulates turn frequency and run duration (Zariwala et al, 2003), it is quite likely that SMD and RMD control turn frequency, depending on thermal information transmitted by RIA. We expect that inspection of glutamate transmission from RIA to SMD or RMD, including identification of glutamate receptors functioning in SMD or RMD for thermotaxis, reveal the information processing that consequently generates a variety of thermotactic behavioral

outputs.

Information processing in the simple circuit composed of AFD, AWC, and AIY neurons

Mori and Ohshima (1995) proposed the neural circuit for thermotaxis, in which AFD thermosensory neurons transmit excitatory signal to AIY interneurons. Consistent with this proposal, Clark et al, (2006) showed with calcium imaging that AIY of animals lacking AFD did not respond to temperature changes. Given these results and our results on EAT-4, one can propose that the neural signals transmitted from AFD to AIY are of at least two kinds, EAT-4 dependent inhibitory signals and EAT-4 independent excitatory signals. In the present study, we further showed that the thermotactic abnormality of *gcy-23 gcy-8 gcy-18* triple mutants with defective AFD function is considerably different from that of the *eat-4* mutants expressing EAT-4 in AWC and RIA but not in AFD (*eat-4 (AWC+,RIA+)*) (Figure 33), and that AIY of *eat-4* mutants are hyper-responsive to temperature change than wild-type animals (Figure 21), thereby undoubtedly implicating EAT-4 independent excitatory signals from AFD to AIY. Recent electrophysiological study showing that AFD responds to both cooling and warming (Ramot et al, 2008) is consistent with these results.

Our results also suggest that AWC transmit excitatory signals to AIY through EAT-4 dependent glutamatergic neurotransmission (Figure 21), although we previously reported that hyper-responsiveness of AWC to temperature change

induced lower-responsiveness of AIY (Kuhara et al, 2008). It is probable that AWC transmits EAT-4 independent inhibitory signals in addition to EAT-4 dependent excitatory glutamate signals to AIY, which is reminiscent of the regulation of behavioral response to odorants, where AWC releases both glutamate and neuropeptide NLP-1 to postsynaptic AIA interneurons (Chalasani et al, 2010).

AWC senses odorants as well as temperature (Bargmann et al, 1993; Kuhara et al, 2008). How does *C. elegans* distinguish these qualitatively different signals within a sensory neuron? Similar to thermal signal, olfactory signal is transduced through G protein signaling and cGMP-gated channels in AWC, and is further transmitted to AIY through EAT-4 dependent glutamatergic neurotransmission (Chalasani et al, 2007; Coburn & Bargmann, 1996; L'Etoile & Bargmann, 2000; Roayaie et al, 1998). Intriguingly, olfactory signal from AWC inhibits AIY upon reception of glutamate signal by GLC-3/GluCl (Chalasani et al, 2007), whereas, as shown in this study, thermal signal from AWC stimulates AIY through putative glutamate receptors other than GLC-3/GluCl. Hence, our results demonstrated that segregation of different sensory signals such as olfactory and thermosensory signals is mediated through not only distinct signal transductions in a sensory neuron, but also discrete neurotransmissions in downstream neural circuits.

We demonstrated one of the simplest neural circuits consisting of two different sensory neurons and a single postsynaptic interneuron, where AFD and AWC

sensory neurons utilize the same EAT-4/VGLUT dependent glutamatergic neurotransmission to inhibit or stimulate the postsynaptic neuron AIY, respectively. The balance between inhibition and stimulation of the AIY activity significantly affected thermotactic behavior. Strong EAT-4 dependent glutamatergic transmission from AFD enhanced the tendency for animals to migrate toward colder temperature than the cultivation temperature, while weak EAT-4 dependent transmission from AFD relative to AWC weakened the tendency to migrate colder temperature and rather strengthened the tendency to migrate toward warmer temperature than the cultivation temperature. Thus, two synaptic transmissions encoding opposite information flows regulate neural activities to generate various behavioral outputs. Recently, from the network theory perspective, it was proposed that elucidation of information processing in simple network motifs reveal information processing in neural network of vertebrates (Milo et al, 2002). Studies on synaptic transmission in the simple neural circuit consisting of AFD, AWC, and AIY should shed light onto information processing underlying vertebrate intertwined neural networks.

Materials and methods

Strains and genetics

The techniques used for culturing and handling *C. elegans* were essentially as described by Brenner (1974). We used the following strains: wild-type *C. elegans* variety Bristol strain (N2), IK604 *eat-4(ky5)* III, IK600 *eat-4(nj2)* III, IK602 *eat-4(nj6)* III, KP4 *glr-1(n2461)* III, IK703 *avr-14(ad1302)* I, IK708 *glc-3(ok321)* V, IK732 *mgl-1(tm1811)* X, IK712 *mgl-2(tm355)* I, IK706 *mgl-3(tm1766)* IV, IK726 *mgl-2(tm355)* I; *mgl-1(tm1811)* X, IK728 *mgl-3(tm1766)* IV; *mgl-1(tm1811)* X, IK724 *mgl-2(tm355)* I; *mgl-3(tm1766)* IV, IK736 *mgl-2(tm355)* I; *mgl-3(tm1766)* IV; *mgl-1(tm1811)* X, CB1265 *unc-104(e1265)* II, IK589 *ttx-7(nj50)* I, IK597 *gcy-23(nj37)* *gcy-8(oy44)* *gcy-18(nj38)* IV, MT3644 *odr-3(n1605)* V, IK839 *eat-16(nj8)* I, IK813 *gcy-23(nj37)* *gcy-8(oy44)* *gcy-18(nj38)* IV; *glc-3(ok321)* V, IK815 *glc-3(ok321)* *odr-3(n1605)* V, IK840 *eat-16(nj8)* I; *glc-3(ok321)* V, IK818 *eat-4(ky5)*; *Ex[gcy-8p::eat-4* cDNA, *glr-3p::eat-4* cDNA, *ges-1p::NLS-GFP*] designated as *eat-4* (AFD+,RIA+), IK819 *eat-4(ky5)*; *Ex[odr-3p::eat-4* cDNA, *glr-3p::eat-4* cDNA, *ges-1p::NLS-GFP*] designated as *eat-4* (AWC+,RIA+), IK820 *eat-4* (AFD+,RIA+) III; *glc-3(ok321)* V, IK822 *eat-4* (AWC+, RIA+) III; *glc-3(ok321)* V, and many transgenic strains derived from them. *eat-4(nj2)* and *eat-4(nj6)* were isolated in genetic screens as described by Okochi et al, (2005). The isolated *eat-4* mutants were outcrossed to wild-type animals more than six times before the analyses.

The individual TTX assay

The individual thermotaxis (TTX) assay was performed using a 9-cm agar plate and a vial containing frozen acetic acid, as described by Mori and Ohshima (1995) and Mohri et al, (2005). The L4 larvae (12-24 animals per 6-cm plate) were cultured with food (*E. coli* strain OP50) at 20°C for 8-16 hours under uncrowded condition. In this study, we used a 45-ml glass vial (Wheaton) for creating the radial temperature gradient on the assay plate, where fully matured, actively egg-laying adult animals were allowed to move freely for 60 minutes. The results of the assays were calculated as the percentages of animals showing isothermal tracking (IT).

The thermotaxis assay on a radial thermal gradient shown in Figure 5A and 13 was performed as the individual TTX assay with some modification. The L4 larvae were cultured with food (*E. coli* strain OP50) at the designated temperature (17°C, 20°C, or 23°C). The results of thermotaxis assays were calculated as the percentages of animals that migrated to their cultivation temperature.

The population TTX assay

The population TTX assay was performed as previously reported (Ito et al, 2006). Equipment for establishing the linear thermal gradient was used as essentially described by Hedgecock and Russell (1975). A stable, linear thermal gradient was established on a 60-cm long aluminum platform, one end of which

was placed in a water bath at 5°C and the opposite end in a water bath at 35°C. TTX plate (13.5 cm×6 cm, 1.8 cm height) containing 10 ml of TTX medium (3g/l NaCl, 20g/l Bacto Agar, 25mM KPO₄) with 2% agar was placed on the aluminum platform such that temperature gradient could be established along the agar surface of 13.5cm long. The extra space between the bottom of the TTX plate and the aluminum platform was filled with water to increase thermal conductivity as much as possible. The center of the 13.5 cm long-agar surface in TTX plate was adjusted at 20°C and the TTX plate was maintained for 15 min before a linear thermal gradient ranging from approximately 17°C to 23°C was established on the agar surface. Uncrowded and well-fed animals were used for the TTX assay. A single adult animal was placed on a 6-cm plate containing 14 ml of nematode growth medium (NGM) with 2% agar, on which *E. coli* OP50 was seeded; the animal and its progeny were cultured at respective temperature. The animals were collected with 1ml of NG buffer (0.3% NaCl, 1 mM CaCl₂, 1 mM MgSO₄, 25 mM potassium phosphate, pH 6.0) kept at 20°C and were washed twice with NG buffer at 20°C. Approximately 50–200 animals were placed at the center of the TTX plate. Excess water surrounding the animals was removed with tissue paper. After 60 minutes, the animals were immobilized by placing TTX plates at 4°C over night, and the animals in each of the eight regions were scored (Figure 2A). The TTX index was calculated as shown in Figure 2C.

Counting bends

Counting bends was performed as described by Segalat et al. (1995). Animals cultured with OP50 at 20°C were analyzed at room temperature (about 22 to 24°C) on 6cm agar plates (2% agar, 1mM CaCl₂, 1mM MgSO₄, 25mM pH 6.0 potassium phosphate) containing OP50. Two minutes after transfer to agar plates, the number of body bends was counted for one minute.

Molecular Biology

The *eat-4* genomic fragment including 2.4 kb or 5.5 kb of the promoter region and 2 kb of the downstream region was amplified by PCR from N2 genome. The *full length eat-4::gfp* translational fusion gene fragment, which was amplified by PCR from N2 genome and pPD95.75, contains 5.5 kb fragment upstream of *eat-4* gene, *eat-4* gene (4.5 kb) including all exons and introns, *gfp* (0.9 kb), and 2 kb fragment downstream of *eat-4* gene.

For cell-specific rescue experiments, *cell-specific promoter::eat-4* cDNA construct, *cell-specific promoter::avr-14* cDNA constructs, *cell-specific promoter::mgl-3* cDNA construct, and *cell-specific promoter::glc-3* cDNA construct were generated. *eat-4* cDNA was PCR-amplified by using tagged primers from yk32h2 EST clone that contains *eat-4* cDNA lacking initial 7 bp, and was cloned into pPD49.26 to generate pNR8. One type of *avr-14* cDNAs (*avr-14a* cDNA) were PCR-amplified by using tagged primers from yk1522g07 EST clone that contains full-length *avr-14a* cDNA with putative sixth intron, and was cloned into pPD49.26 to

generate pNR57. The other type of *avr-14* cDNAs (*avr-14b* cDNA) were PCR-amplified by using tagged primers from yk1522g07 EST clone and yk1592d04 EST clone that contains *avr-14b* cDNA lacking three exons, and was cloned into pPD49.26 to generate pNR59. *mgl-3* cDNA was made artificially in accordance with putative *mgl-3* cDNA shown in the *C. elegans* genome database by GenScript Corporation, USA, and was cloned into pPD49.26 to generate pNR94. *glc-3* cDNA was PCR-amplified from yk1649c11 EST clone that contains full-length *glc-3* cDNA, and was cloned into pPD49.26 to generate pNR56. The resultant plasmids were confirmed to contain the intact cDNA by sequencing. All plasmids used for cell-specific rescue experiments were generated by inserting cell-specific promoter fragments into pNR8, pNR57, pNR59, pNR94 or pNR56. All cell-specific promoter constructs were previously generated by PCR to contain only non-coding region. Before conducting the rescue experiments, expression patterns of *cell-specific promoter::gfp* constructs were verified by examining GFP fluorescence. Cell-specific promoters are 0.7 kb of *gcy-8p* for AFD, 2.3 kb of *odr-3p* for AWC which also induces expression slightly in AWB, 0.8 kb of *ttx-3p* for AIY, 1.9 kb of *glr-3p* for RIA, 1.4 kb of *lin-11p* for AIZ which also induces expression in ADF, AVJ, AVH, AVG, ADL and RIC, 2.1 kb of *osm-6p* for sensory neurons, 5.3 kb of *glr-1p* for motor and command neurons, and 1.4 kb of *unc-14p* for all neurons.

For calcium imaging of AIY, *ttx-3p::yc3.60* construct was generated. yellow cameleon 3.60 was amplified from YC3.60/pcDNA3 (Nagai et al, 2004) and cloned into pPD49.26 to generate pNR46. *ttx-3p* fragment was inserted into

pNR46 to generate *ttx-3p::yc3.60* (pNR86). The resultant plasmid was confirmed to contain the intact *ttx-3p::yc3.60* by sequencing.

Transgenic animals

Germline transformation was performed by co-injecting experimental DNA (0.2-100 ng/ul) and an injection marker pKDK66 *ges-1p::NLS-GFP* (50 ng/ul) or pNAS88 *ges-1p::NLS TagRFP* (50 ng/ul) into the gonad (Mello et al, 1991). Multiple independent transgenic lines were established for each experimental DNA. For comparison of phenotypes on different genetic backgrounds, transgenic arrays were transferred by intercrossing.

***In vivo* calcium imaging and data analysis**

In vivo calcium imaging was performed essentially according to previous reports with some modifications (Kimura et al, 2004; Kuhara et al, 2008). After cultivated at 20°C, well-fed animals expressing *ttx-3p::yc3.60* were glued onto a 1.5% agar pad on glass, immersed in M9 buffer, and covered by cover glass. The agar pad and M9 buffer were kept at the initial imaging temperature. Sample preparation was completed within 2 min. The sample was then placed onto a peltier-based thermocontroller (Tokai Hit, Japan) on the stage of an Olympus BX61WI at the initial imaging temperature for 2 min, and fluorescence was introduced into a Dual-View (Molecular devices, USA) optics systems. Cyan fluorescent protein (CFP; F480) and yellow fluorescent protein (YFP; F535)

images were simultaneously captured by an EM-CCD camera C9100-13 ImagEM (Hamamatsu Photonics). Images were taken with a 500-ms exposure time with 2×2 binning. The temperature on the agar pad was monitored by a thermometer system, DCM-20 (Tokai Hit and Hamamatsu Photonics). For each imaging experiment, fluorescence intensities of F535 and F480 were measured using MetaMorph imaging analysis system (Molecular Device). Since a computer regulates all the recorded images and the outcome of the analysis, any intention of a researcher should be excluded. Relative increases or decreases in the intracellular calcium concentration were measured as increases or decreases in the YFP/CFP fluorescence ratio of the cameleon protein (Ratio Change).

Oligonucleotide primers and usage of them

For detection of *eat-4(nj2)* mutation by PCR

nri005: GCTTGTCAGAAGACAAGTGC

nri017: ACAAAAATCTCAAAGAACTCACTTGGCGAGTGGAGCT

PCR extension time: 40sec

To detect *nj2* mutation, digest PCR fragments with SacI.

For detection of *eat-4(nj6)* mutation by PCR

nri019: TCACTTTACTGGAGTCACTTTTTATGCTGTCTATGCCTGC

nri021: TTTACCTGGAGCCGCCTGAG

PCR extension time: 40sec

To detect *nj6* mutation, digest PCR fragments with PstI.

For detection of *eat-4(ky5)* mutation by PCR

nri005: GCTTGTCAGAAGACAAGTGC

nri006: CGATGCAATTTCCGGTGCAGC

nri014: GTTATCTTGACCGGAGAACC

PCR extension time: 60sec

Two amount of nri014 (10uM), one amount of nri005 and nri006 (5uM) are mixed and used for PCR.

For detection of *avr-14(ad1302)* mutation by PCR

nri099: TTCGAGAAGTTGGCAAGTCG

nri100: TGAATCGGCAGGTTTCAGGAG

PCR extension time: 40sec

To detect *ad1302* mutation, digest PCR fragments with XbaI.

For detection of *glc-3(ok321)* mutation by PCR

nri097: TCAAATACAGGGGTAGGCG

nri098: ACAATTCCTGGAACACTCACGG

PCR extension time: 180sec

For detection of *mgl-1(tm1811)* mutation by PCR

nri107: CATCATCTGGACATATCGCT

nri108: AATGCACCGAGGCTGAATTA

PCR extension time: 150sec

For detection of *mgl-2(tm355)* mutation by PCR

nri105: ACATAGCCTTCAAGCCGAGA

nri106: GGATTGGATCTGATGGATGG

PCR extension time: 40sec

For detection of *mgl-3(tm1766)* mutation by PCR

nri091: TGCTGGAAGTCAAGTCAGTG

nri092: CCACCAGACAACCTGCAAGC

nri093: CCACAACAGTGGAAATGCGAC

PCR extension time: 180sec

Two amount of nri093 (10uM), one amount of nri091 and nri092 (5uM) are mixed and used for PCR.

Statistics

All error bars in the Figures indicate standard error of mean (SEM). Results of behavioral experiments were treated as parametric data. The statistical analysis for all behavioral experiments was performed by one-way analysis of variance (ANOVA) for multiple comparisons. Comparisons between each result were followed by post hoc Tukey-Kramer test, except for data sets including results of "0 %". Results of calcium imaging were treated as nonparametric data. The statistical analysis for all calcium imaging was performed by Steel-Dwass tests.

A single asterisk (*), double asterisk (**), and nonsignificant (n.s.) in the Figures indicate $p < 0.05$, $p < 0.01$, and $p > 0.05$, respectively.

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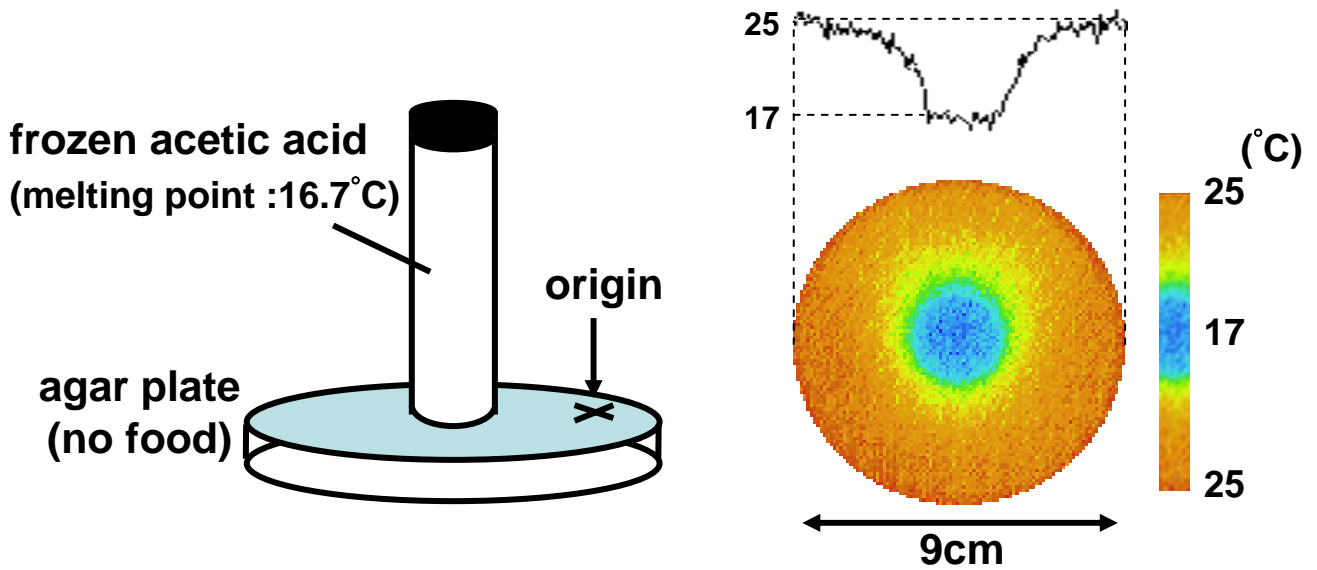
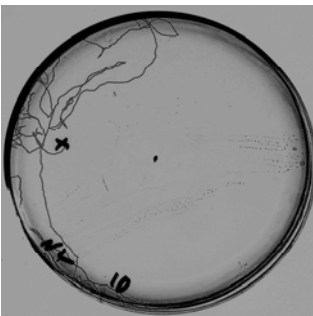
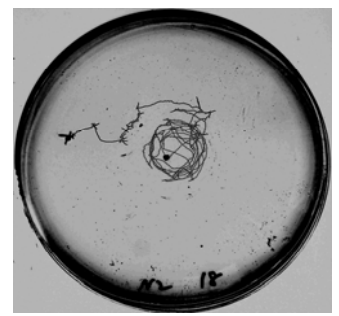
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A**B** **23°C -cultivated** **20°C -cultivated** **17°C -cultivated****Figure 1. Individual TTX assay**

(A) Procedures for individual TTX assay. A stable radial thermal gradient with 17°C at the center and 25°C at the periphery (9cm-diameter) was established by using a glass vial containing frozen acetic acid and keeping room temperature at 25°C . Adult animals were individually placed on a radial thermal gradient (origin) and were allowed to move freely for 60 minutes.

(B) Thermotaxis tracks of wild-type animals after cultivation at 23°C , 20°C , and 17°C on a radial thermal gradient. Most wild-type animals migrated to their cultivation temperature and moved isothermally on a radial thermal gradient.

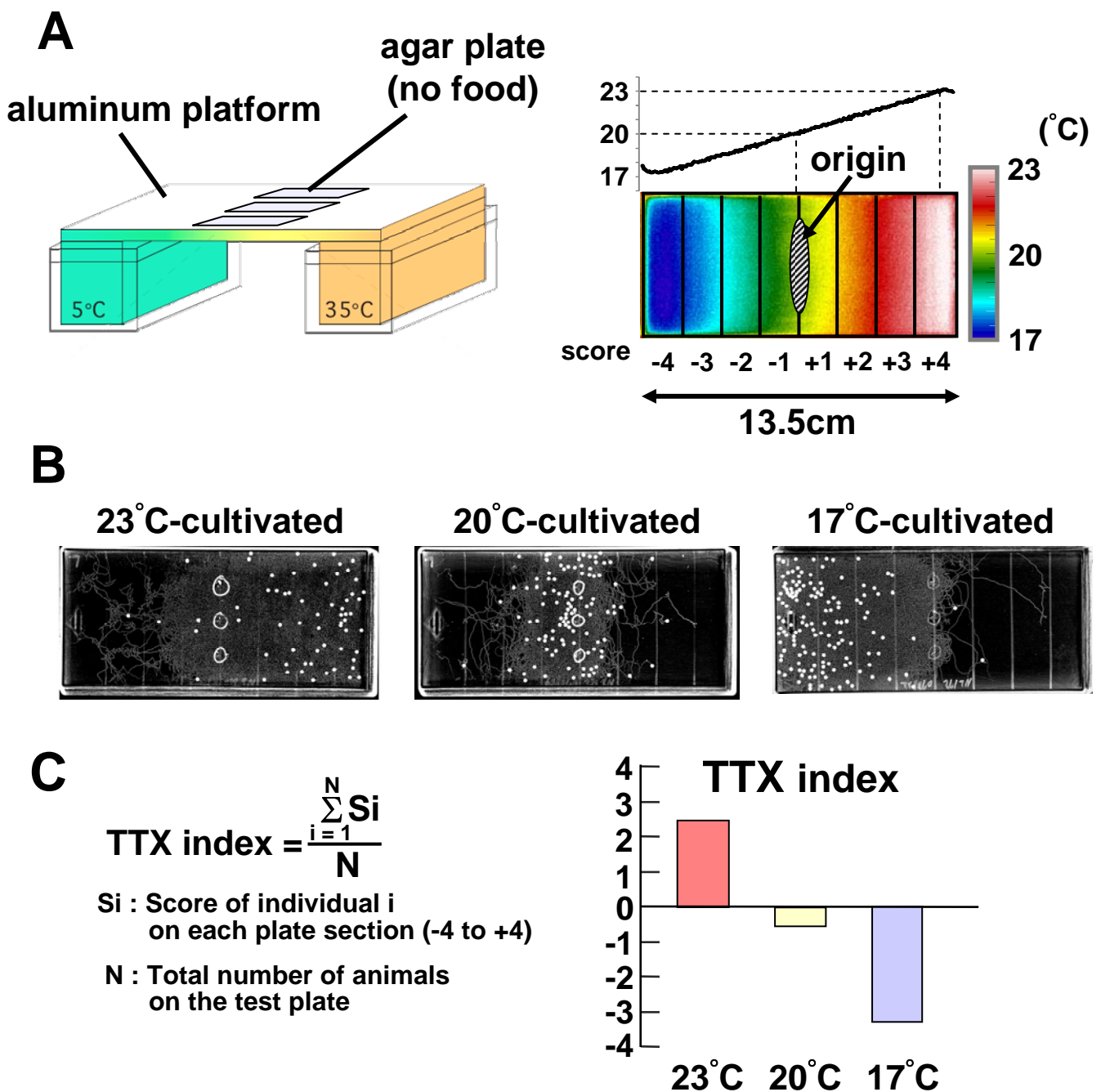


Figure 2. Population TTX assay

(A) Procedures for population TTX assay. A stable linear thermal gradient from 17°C to 23°C (13.5cm) was established on a 60-cm long aluminum platform, one end of which was placed in a water bath at 5°C and the opposite end in a water bath at 35°C. About 50-200 animals were placed on the agar surface of 20°C (origin) and allowed to move freely for 60 minutes. The steepness of the temperature gradient was stably kept at 0.45°C/cm.

(B) Thermotaxis tracks of wild-type animals after cultivation at 23°C, 20°C, and 17°C on a linear thermal gradient. Most wild-type animals migrated to their cultivation temperature (white dots).

(C) The thermotactic behavior was quantified as TTX index. Red bars, yellow bars, and blue bars represent TTX indices of animals cultivated at 23°C, 20°C, and 17°C, respectively.

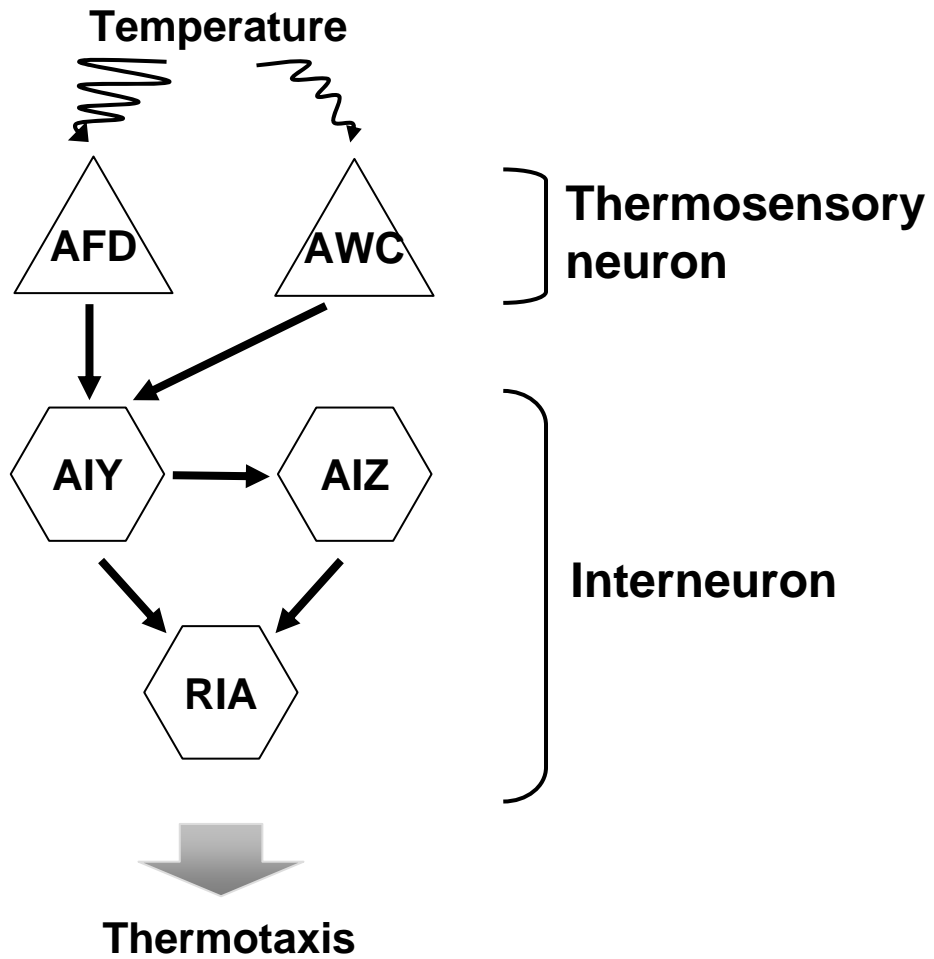


Figure 3. The neural circuit model for thermotaxis

Temperature is sensed by AFD and AWC sensory neurons, thermal information from AFD and AWC is conveyed to AIY interneuron, and the subsequent neural information from AIY is further conveyed to AIZ and RIA interneuron. Black arrows between neurons represent chemical synapses.

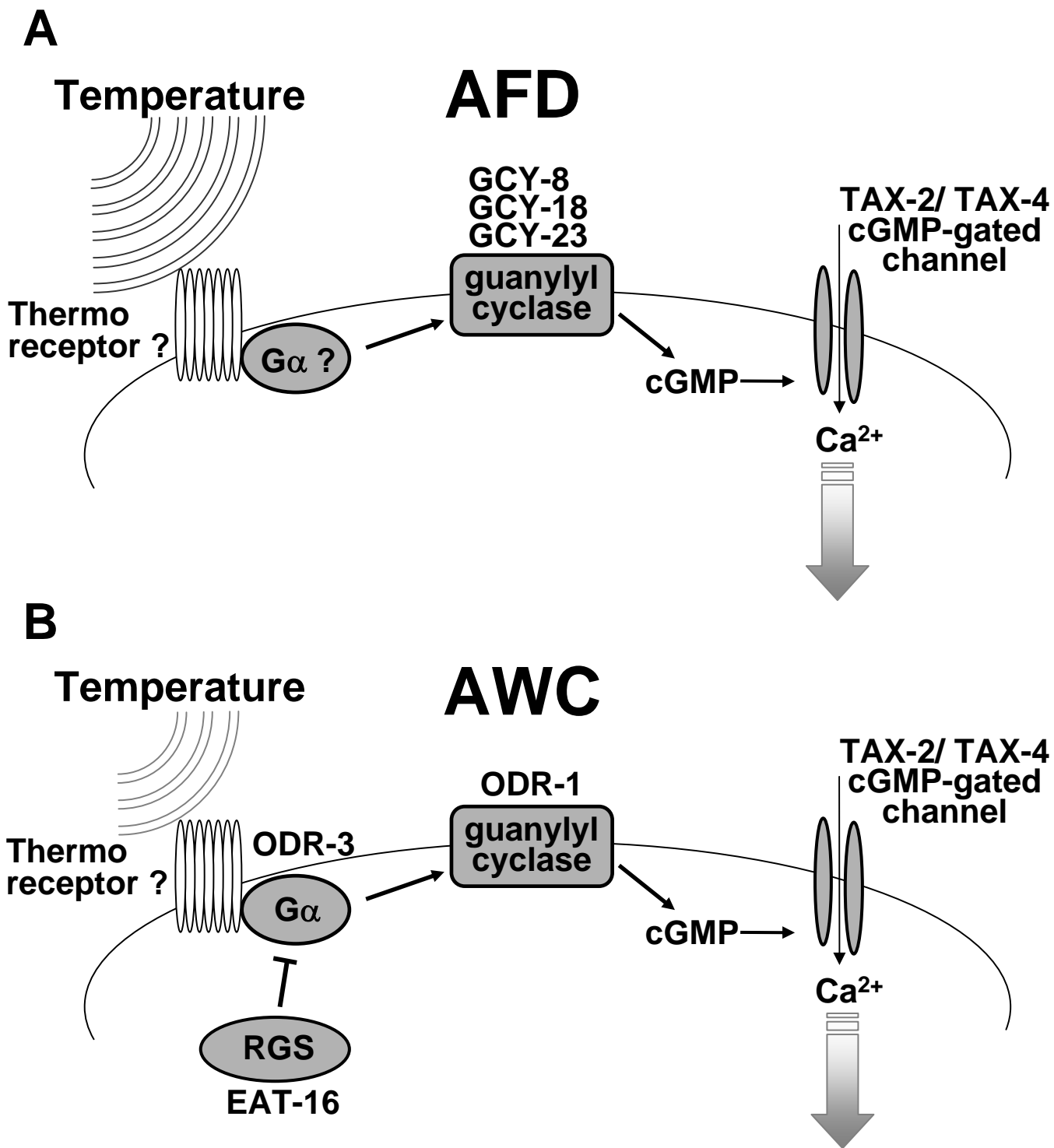


Figure 4. Temperature sensing signal transduction in thermosensory neurons

(A) The model of signal transduction in AFD. Three guanylyl cyclases, GCY-8, GCY-18 and GCY-23 redundantly produce cGMP, and TAX-2/TAX-4 cGMP-dependent cation channel increase internal calcium concentration.

(B) The model of signal transduction in AWC. ODR-1 guanylyl cyclase produces cGMP through activation of ODR-3 G-alpha, and TAX-2/TAX-4 increases internal calcium concentration. This G protein coupled signaling is suppressed by EAT-16 RGS.

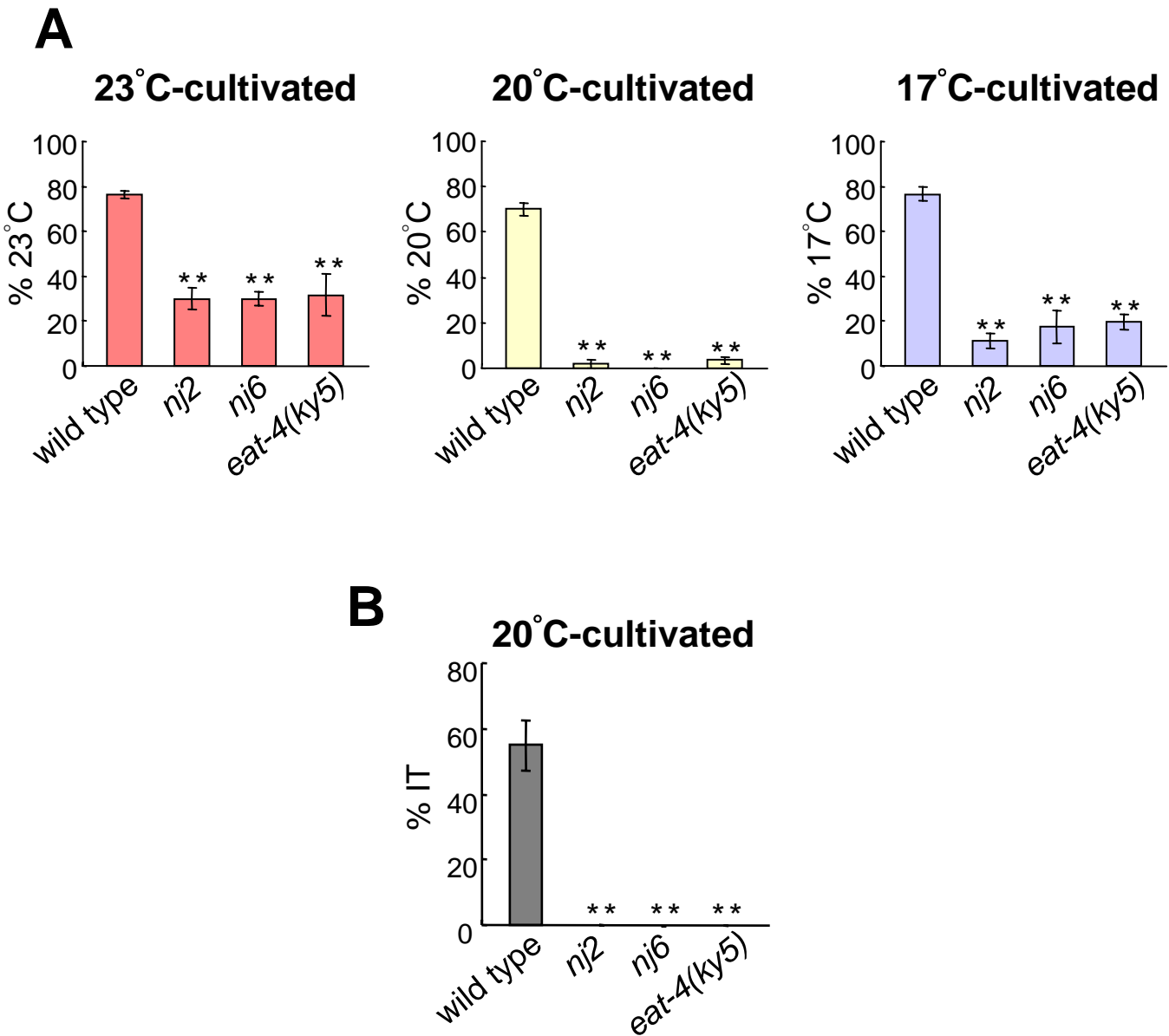


Figure 5. Thermotaxis defects of *nj2*, *nj6*, and *eat-4(ky5)* mutants in the individual TTX assay.

(A) Fraction of animals that migrated to their cultivation temperature after cultivated at 23°C (red), 20°C (yellow), or 17°C (blue). $n > 60$ animals for each genotype. Error bar indicates SEM. Double asterisk at 23°C-cultivated and 17°C-cultivated both indicate $p < 0.01$ in post hoc Tukey-Kramer test for a comparison with wild-type animals. Double asterisk at 20°C-cultivated indicates $p < 0.01$ in ANOVA for a comparison with wild-type animals.

(B) Fraction of animals that moved isothermally after cultivated at 20°C. $n = 60$ animals for both strains. Error bar indicates SEM. Double asterisk indicates $p < 0.01$ in ANOVA for a comparison with wild-type animals.

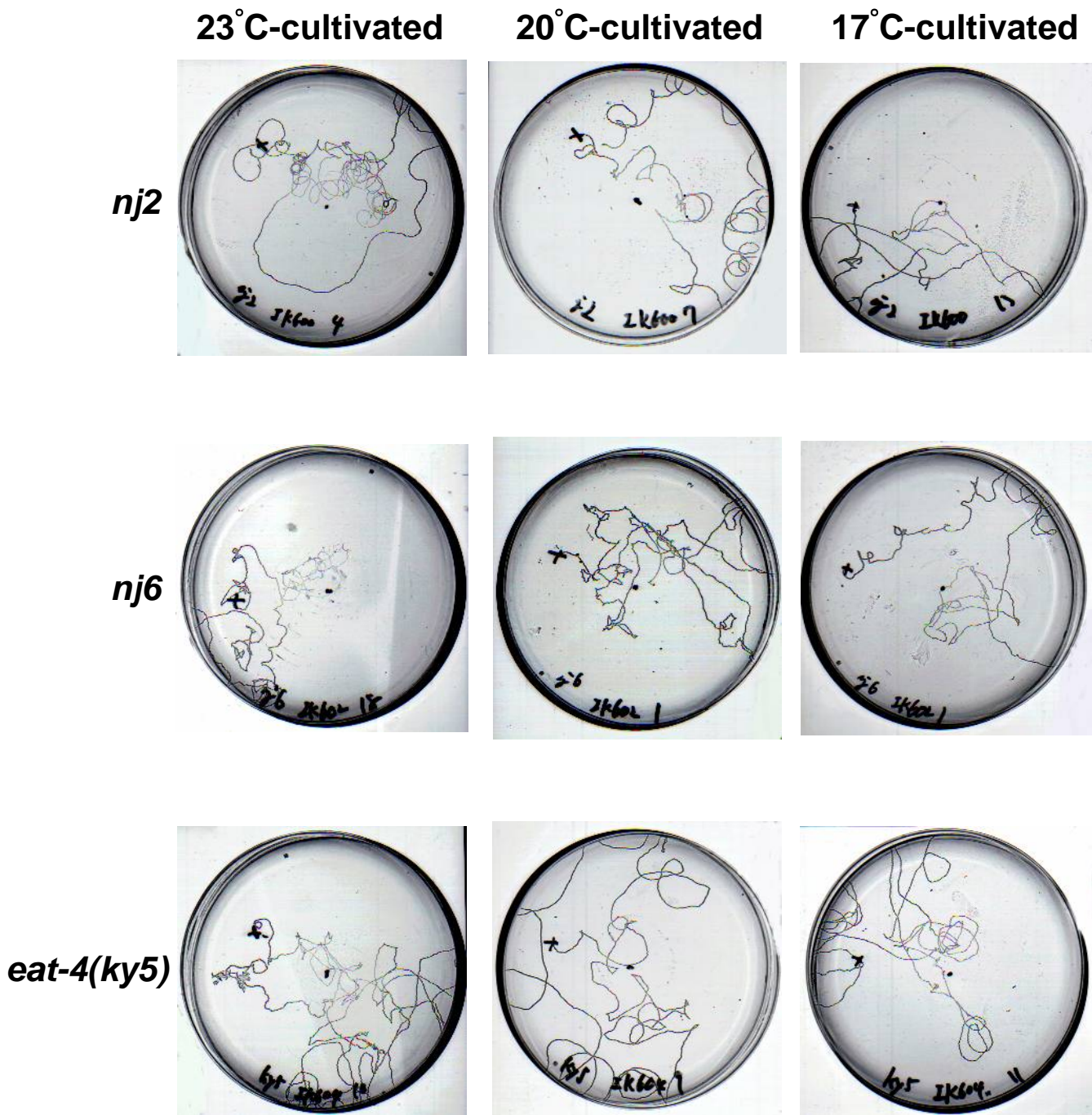


Figure 6. Thermotaxis tracks of *nj2*, *nj6*, and *eat-4(ky5)* mutants on a radial thermal gradient.

All mutants moved randomly after cultivated at 23°C, 20°C, or 17°C.

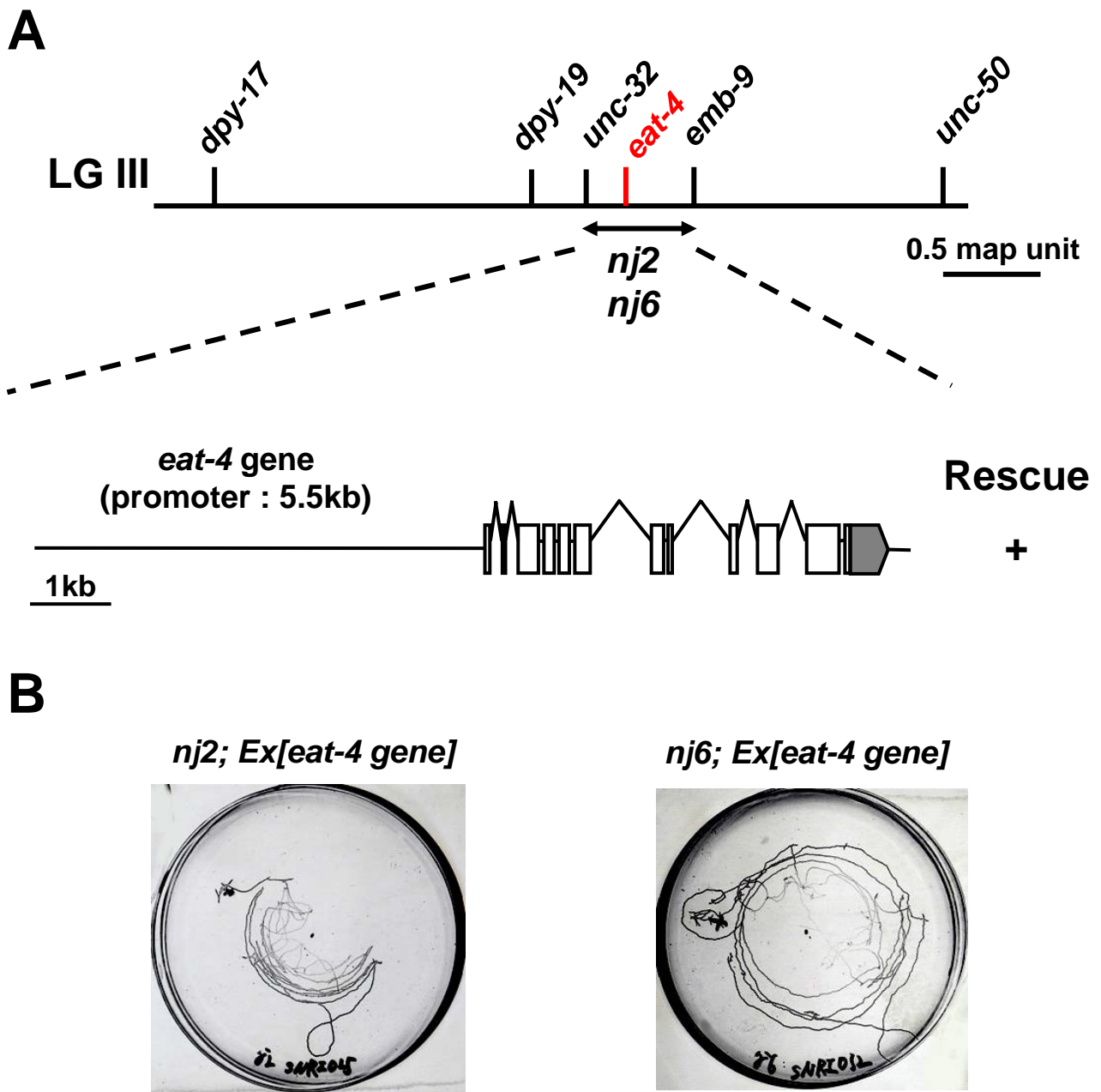


Figure 7. The result of genetic mapping of *nj2* and *nj6* mutations.

(A) *nj2* and *nj6* mutations were mapped to the region including *eat-4* gene by multi-factor crosses. The thermotaxis defects of *nj2* and *nj6* mutants were restored by introducing *eat-4* gene.

(B) Thermotaxis tracks of *nj2* and *nj6* transgenic mutants carrying *eat-4* gene fragment after cultivated at 20°C. These transgenic mutants moved isothermally on a radial thermal gradient.

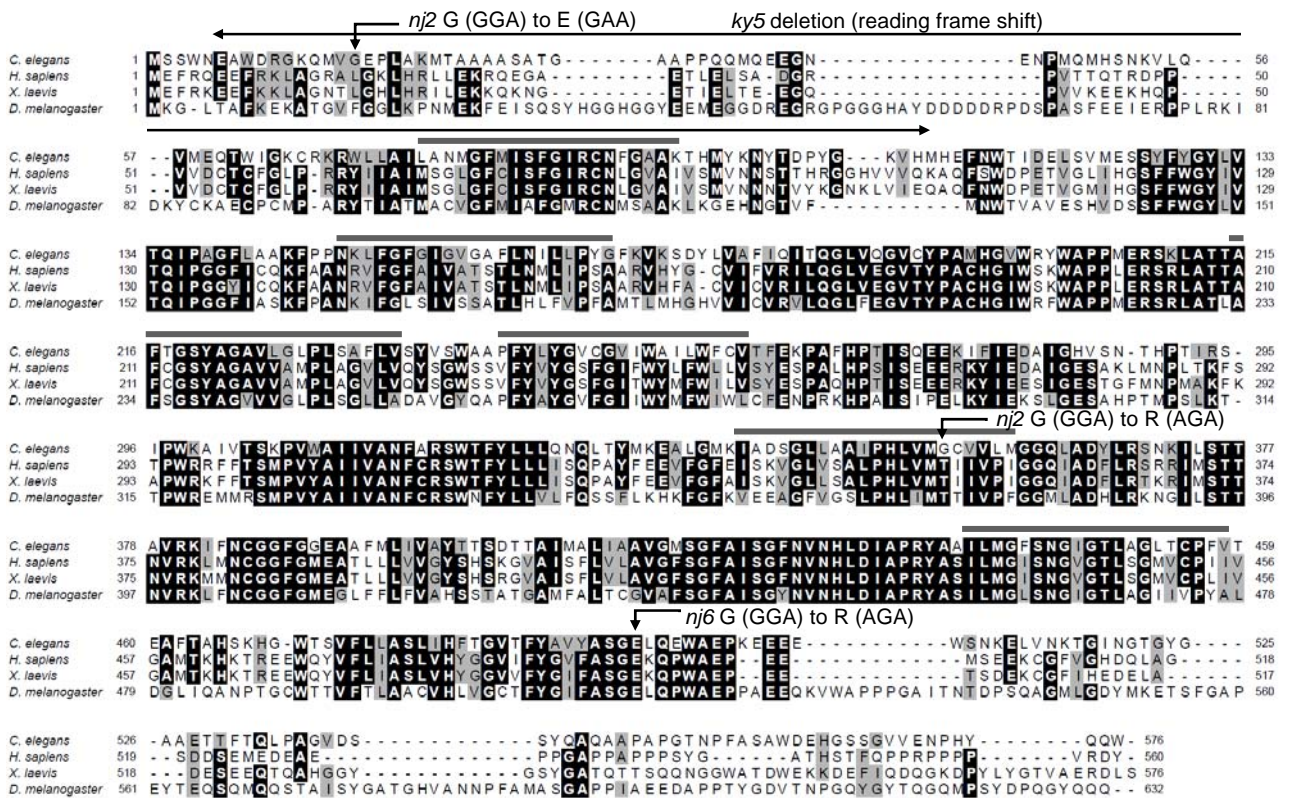


Figure 8. Comparison of sequences of EAT-4 and VGLUTs in other species.
 Black and gray boxes highlight identical and similar residues, respectively. Gray bars are putative transmembrane segments predicted in the previous report (Ni et al, 1994). Single arrows indicate the mutation sites of *nj2* and *nj6*. Deletion of *ky5* is indicated by a double-headed arrow.

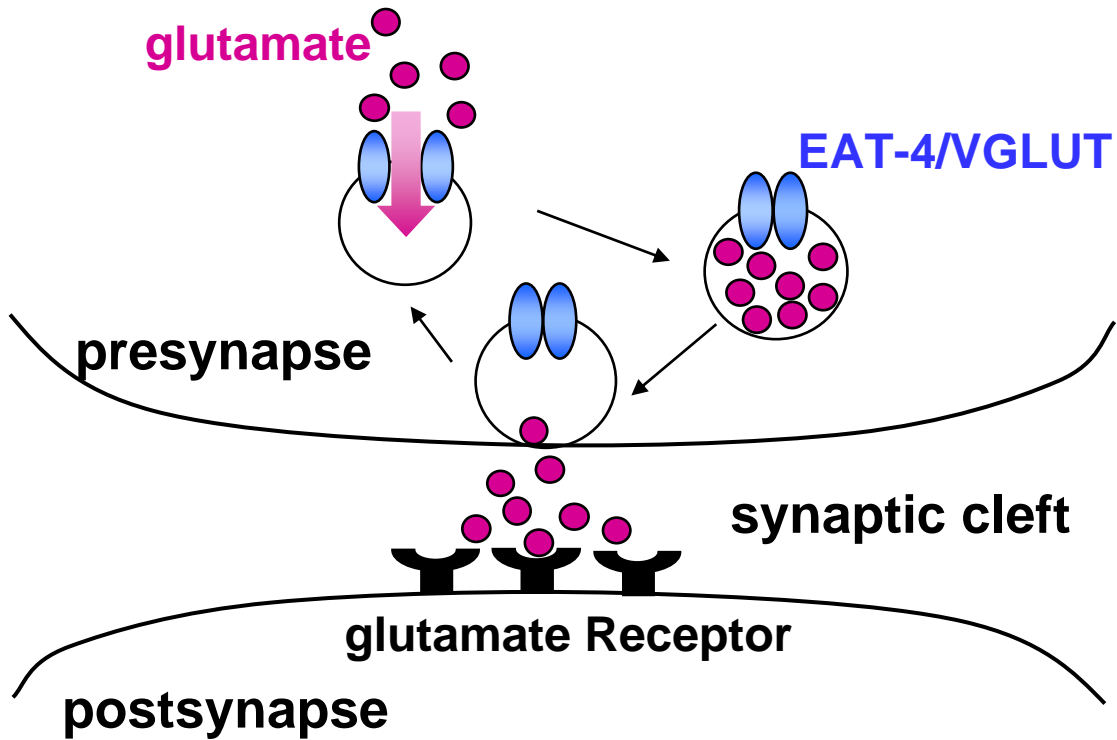


Figure 9. Function of EAT-4 .

EAT-4 is a *C. elegans* homolog of mammalian vesicular glutamate transporter (VGLUT) that concentrates glutamate into synaptic vesicles.

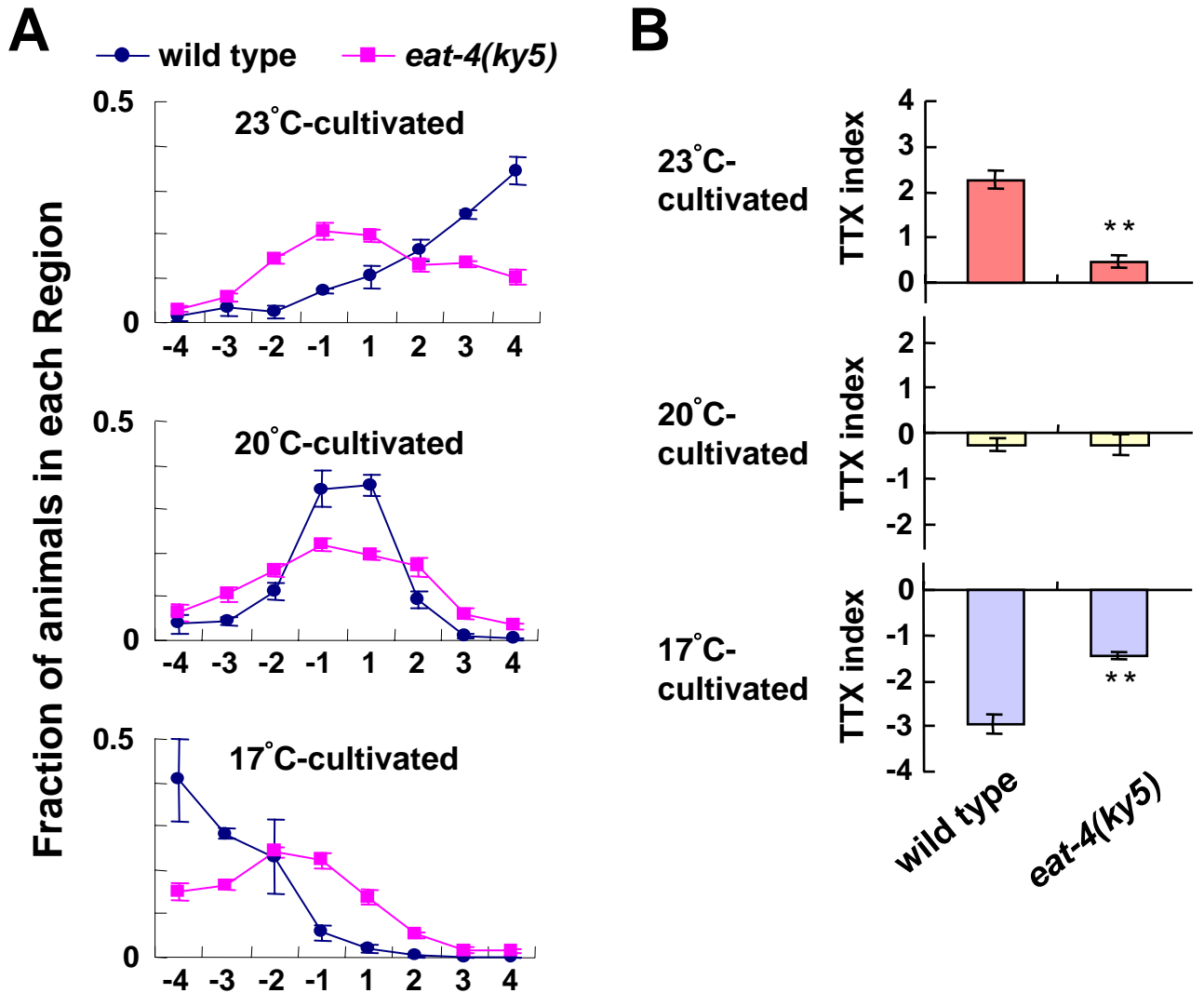


Figure 10. Thermotaxis defects of *eat-4(ky5)* mutants in the population TTX assay. (A) Distributions of wild-type animals and *eat-4(ky5)* mutants cultivated at 23°C, 20°C, and 17°C in the population thermotaxis assay. (B) TTX indices of wild-type animals and *eat-4(ky5)* mutants. Red bars, yellow bars, and blue bars represent TTX indices of animals cultivated at 23°C, 20°C, and 17°C, respectively. $n = 3$ or more assays. Error bar indicates SEM. Double asterisk indicates $p < 0.01$ in post hoc Tukey-Kramer tests for a comparison with wild-type animals.

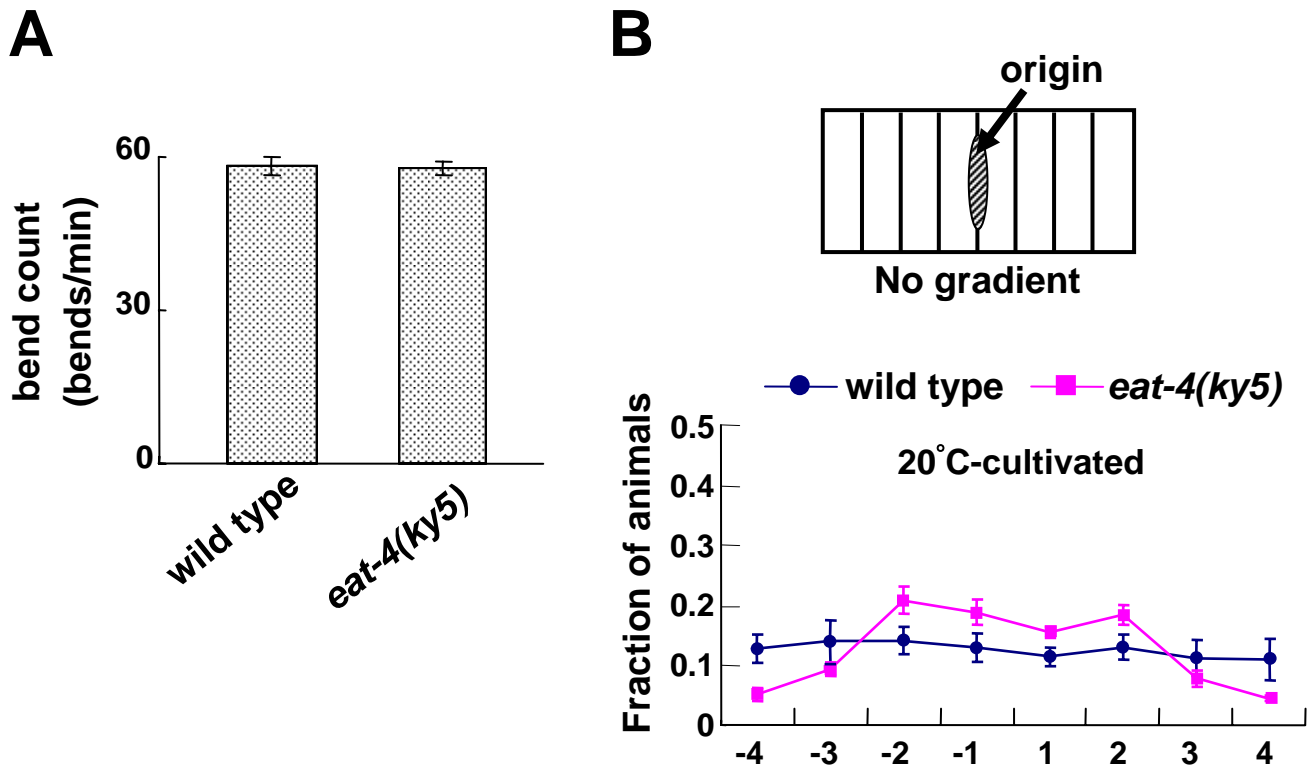


Figure 11. Assay for motor activity of *eat-4(ky5)* mutants.

(A) Bend counts per minute of wild-type animals and *eat-4(ky5)* mutants. $n = 10$ animals for each genotype. Error bar indicates SEM. $p > 0.05$ in post hoc Tukey-Kramer test.

(B) Distribution of wild-type animals and *eat-4(ky5)* mutants on TTX plates without the temperature gradient. Animals cultivated at 20°C were placed at the center line of the plate and left for 60 min (top). $n = 3$ or more assays. Error bar indicates SEM.

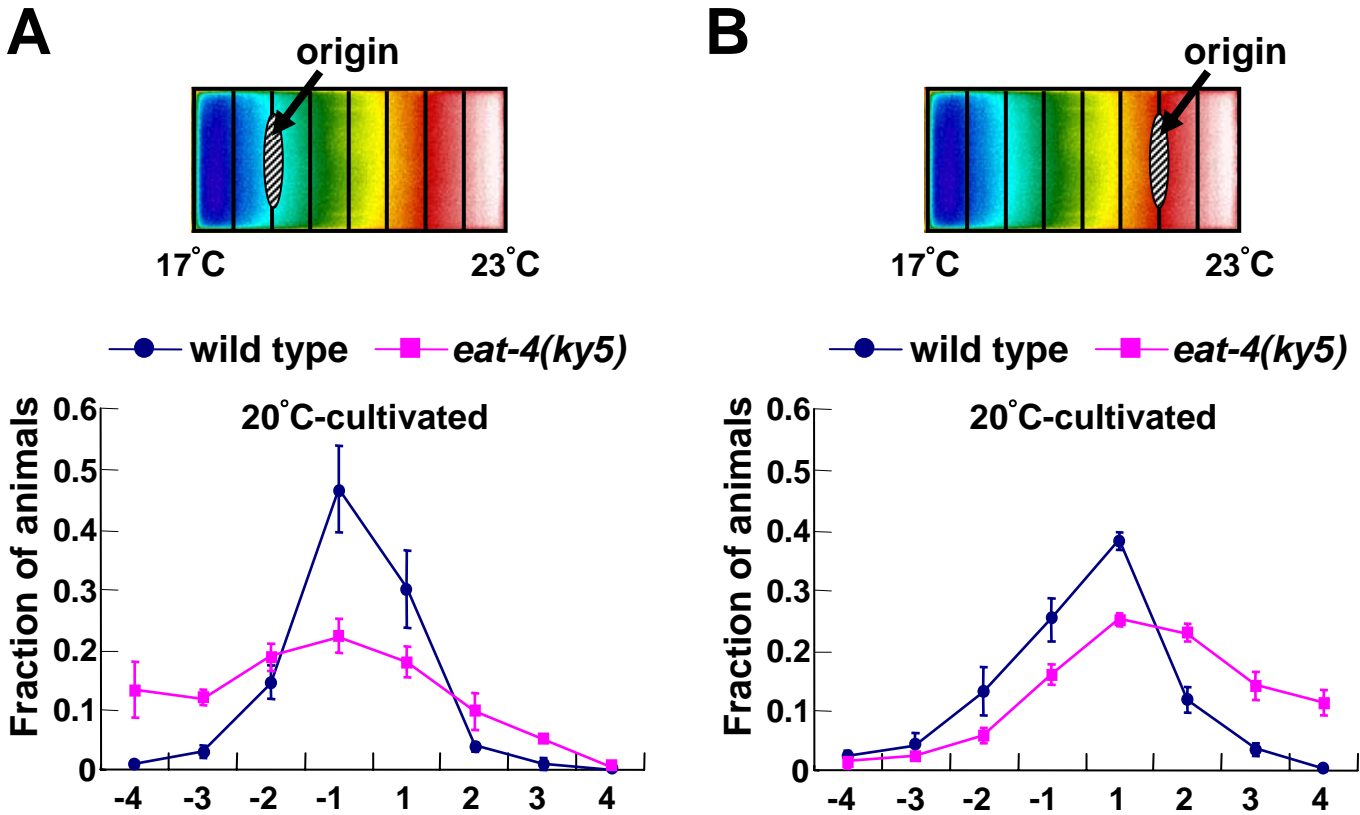


Figure 12. Distribution of wild-type animals and *eat-4(ky5)* mutants on the linear temperature gradient after placed at different points from 20°C. Animals cultivated at 20°C were placed at the lower temperature point (A) or higher temperature point (B) than 20°C and left for 60 min (top). Most of wild-type migrated around 20°C. *eat-4(ky5)* showed a greater tendency to move in the opposing direction from 20°C than wild-type. n = 3 or more assays. Error bar indicates SEM.

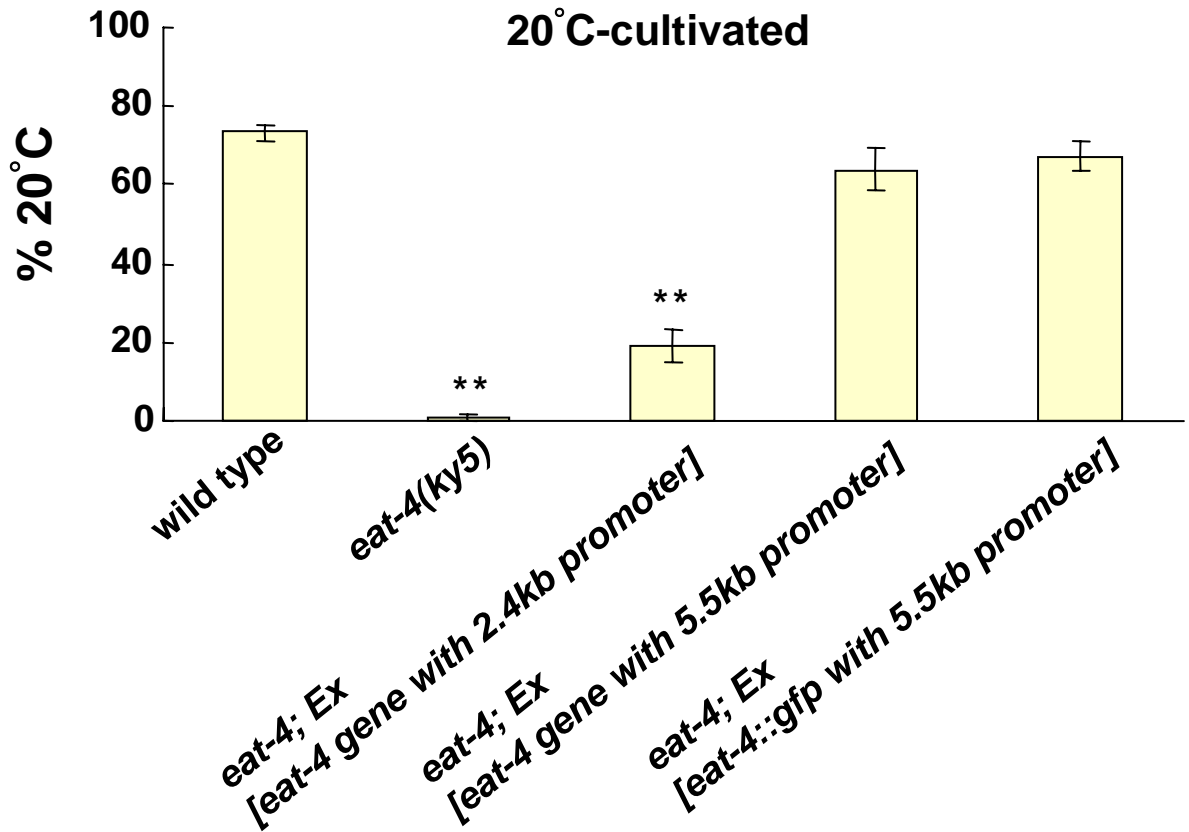


Figure 13. Analysis for activities of two *eat-4* promoters by the individual TTX assay. Fraction of wild-type animals and *eat-4* transgenic mutants containing genomic *eat-4* gene or *eat-4::gfp* that migrated to their cultivation temperature after cultivated at 20°C in the individual TTX assay. $n > 60$ animals for each genotype. Error bar indicates SEM. Double asterisk indicates $p < 0.01$ in post hoc Tukey-Kramer tests for a comparison with wild-type animals.

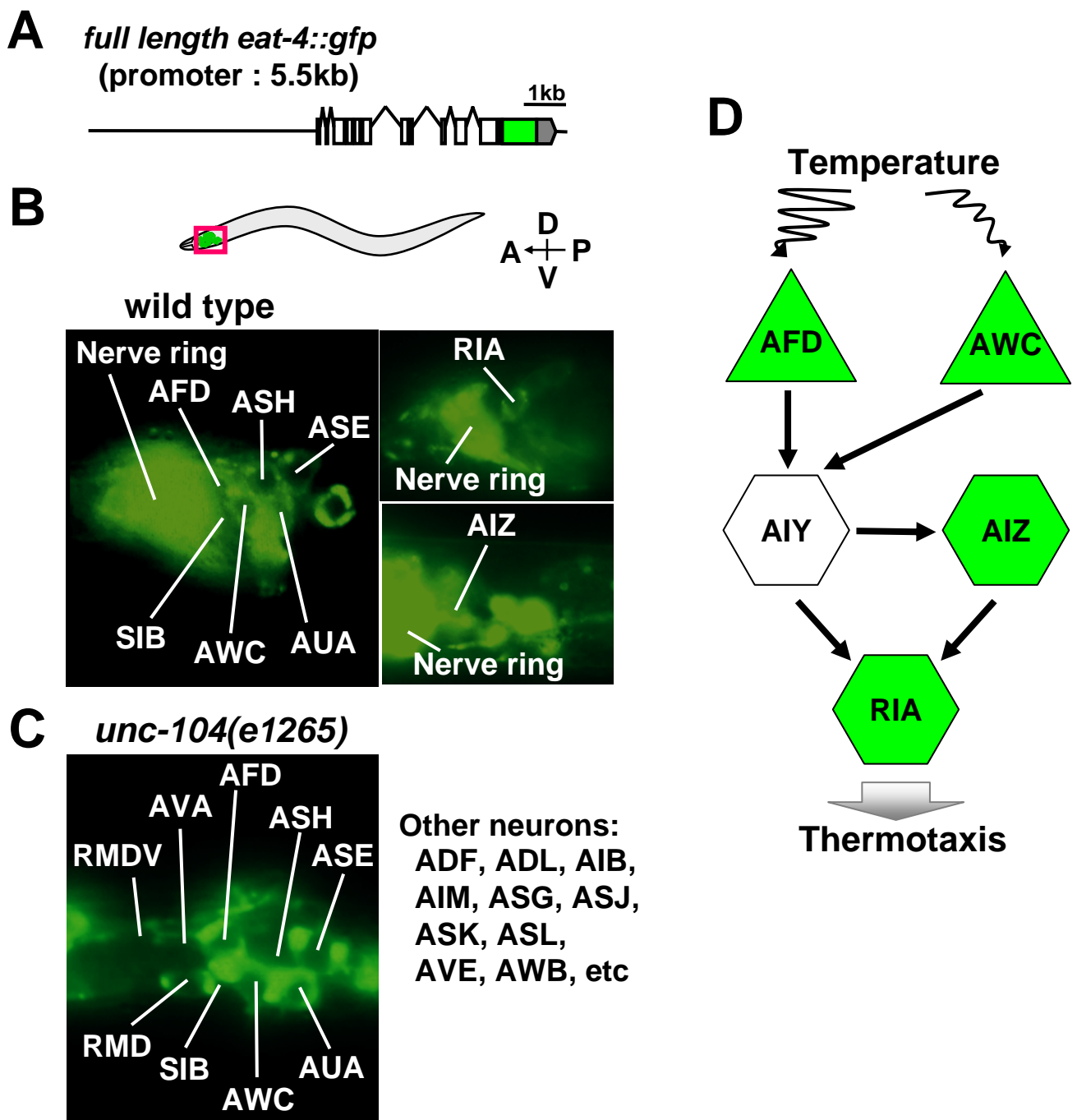


Figure 14. Expression pattern of EAT-4.

(A) The *full length eat-4::gfp* fusion gene with 5.5kb of promoter region.

(B, C) The expression of *full length eat-4::gfp* in the head of wild-type animals (B), and of *unc-104(e1265)* mutants (C). Anterior is to the left and dorsal is up (top). (B) Fluorescence was observed in nerve ring and cell body of many neurons including AFD, AWC (left panel), RIA (top right panel), and AIZ neurons (bottom right panel) in wild-type. (C) Fluorescence was not observed in nerve ring but in cell body of many neurons including AFD, AWC in *unc-104(e1265)* mutants.

(D) Neurons expressing the *full length eat-4::gfp* in the thermotaxis neural circuit are colored green.

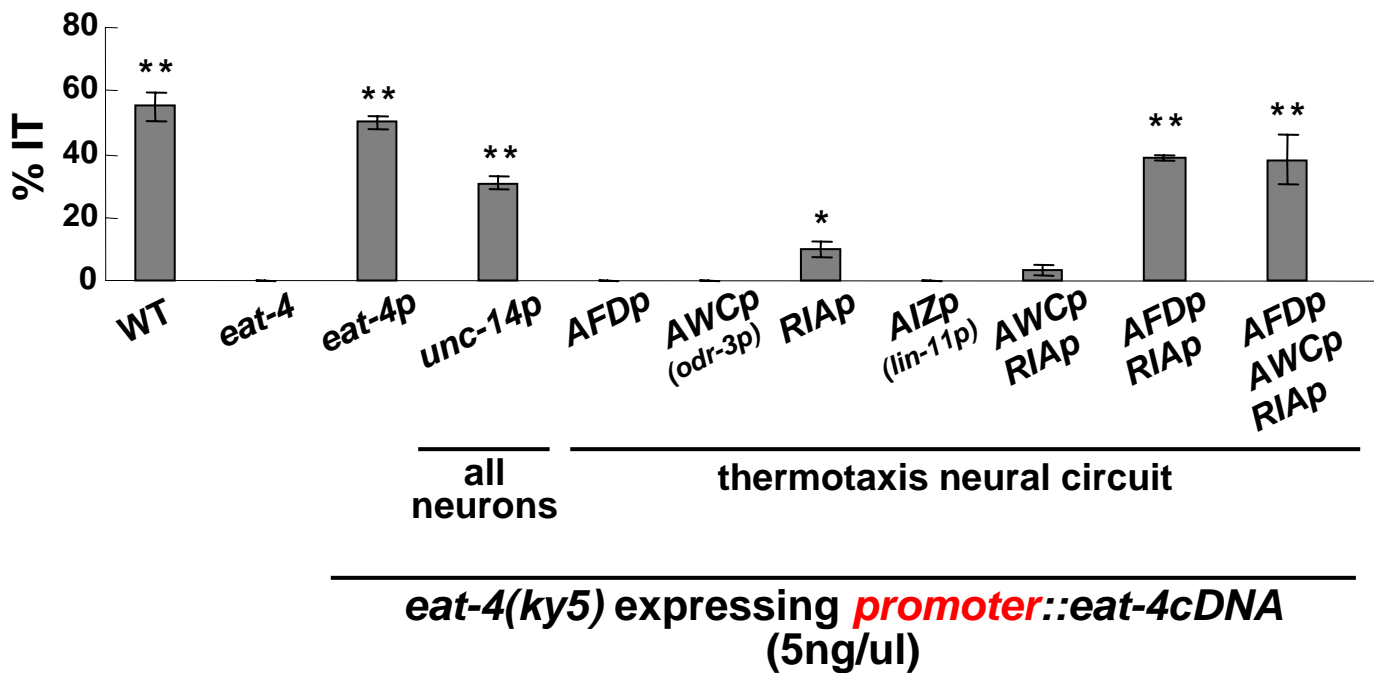


Figure 15. Cell-specific rescue experiments of *eat-4(ky5)* mutants in the individual TTX assay.

Rescue experiments for defective isothermal tracking (IT behavior) of *eat-4(ky5)* mutants by introducing cell-specific *promoters::eat-4 cDNA* at 5ng/ul. Gray bars represent fraction of animals that moved isothermally. n = 58 or more animals. Error bar indicates SEM. Single and double asterisk indicate $p < 0.05$ and 0.01 , respectively, in ANOVA for a comparison with *eat-4(ky5)* mutants.

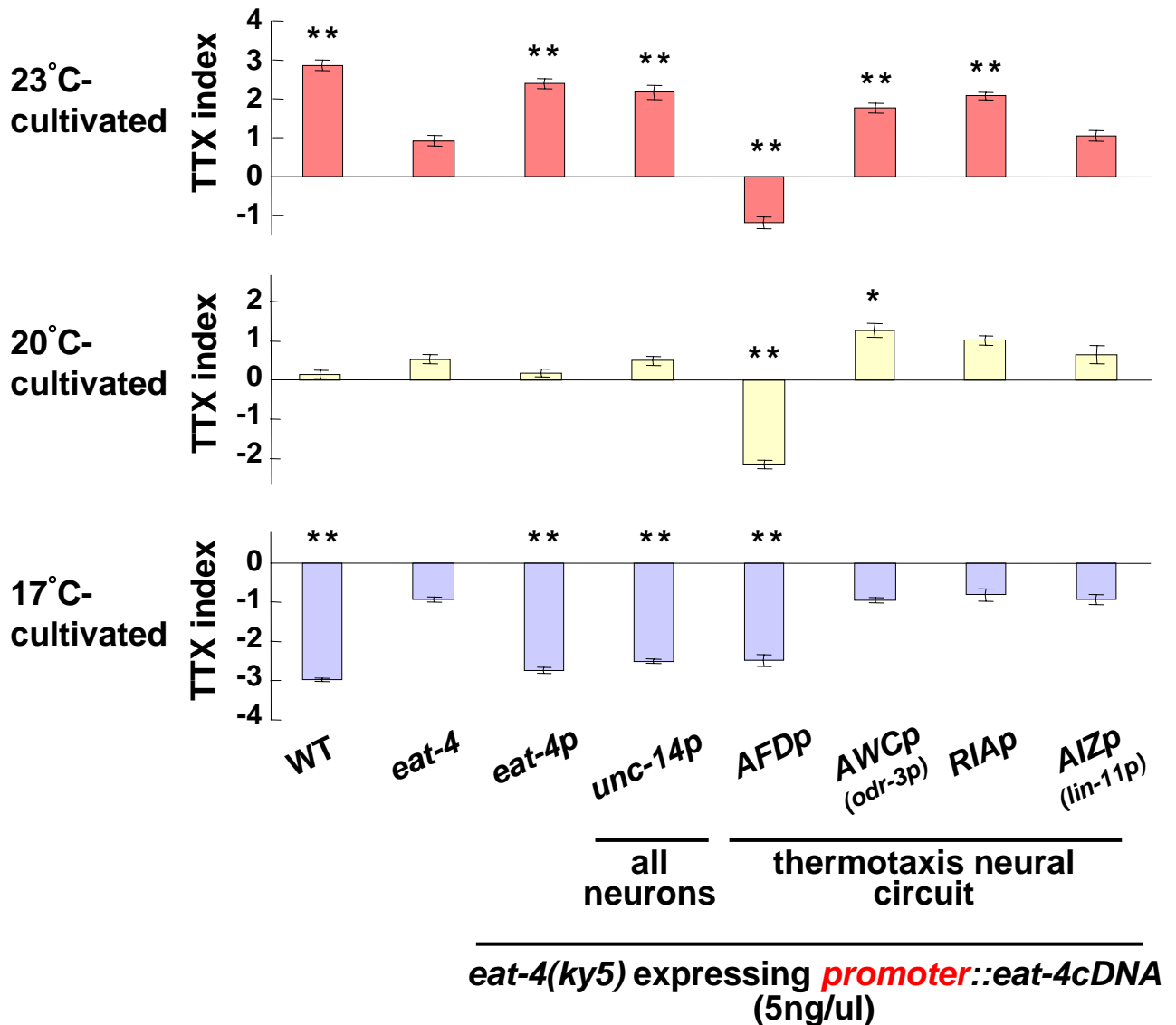


Figure 16. Cell-specific rescue experiments of *eat-4(ky5)* mutants in the population TTX assay.

Rescue experiments for defective migration to cultivation temperature of *eat-4(ky5)* mutants by introducing individual cell-specific *promoters::eat-4 cDNA* at 5ng/ul. Red bars, yellow bars, and blue bars represent TTX indices of animals cultivated at 23°C, 20°C, and 17°C, respectively. n = 3 or more assays. Error bar indicates SEM. Single asterisk and double asteris indicate p < 0.05 and p < 0.01 respectively in post hoc Tukey-Kramer tests for a comparison with *eat-4(ky5)* mutants.

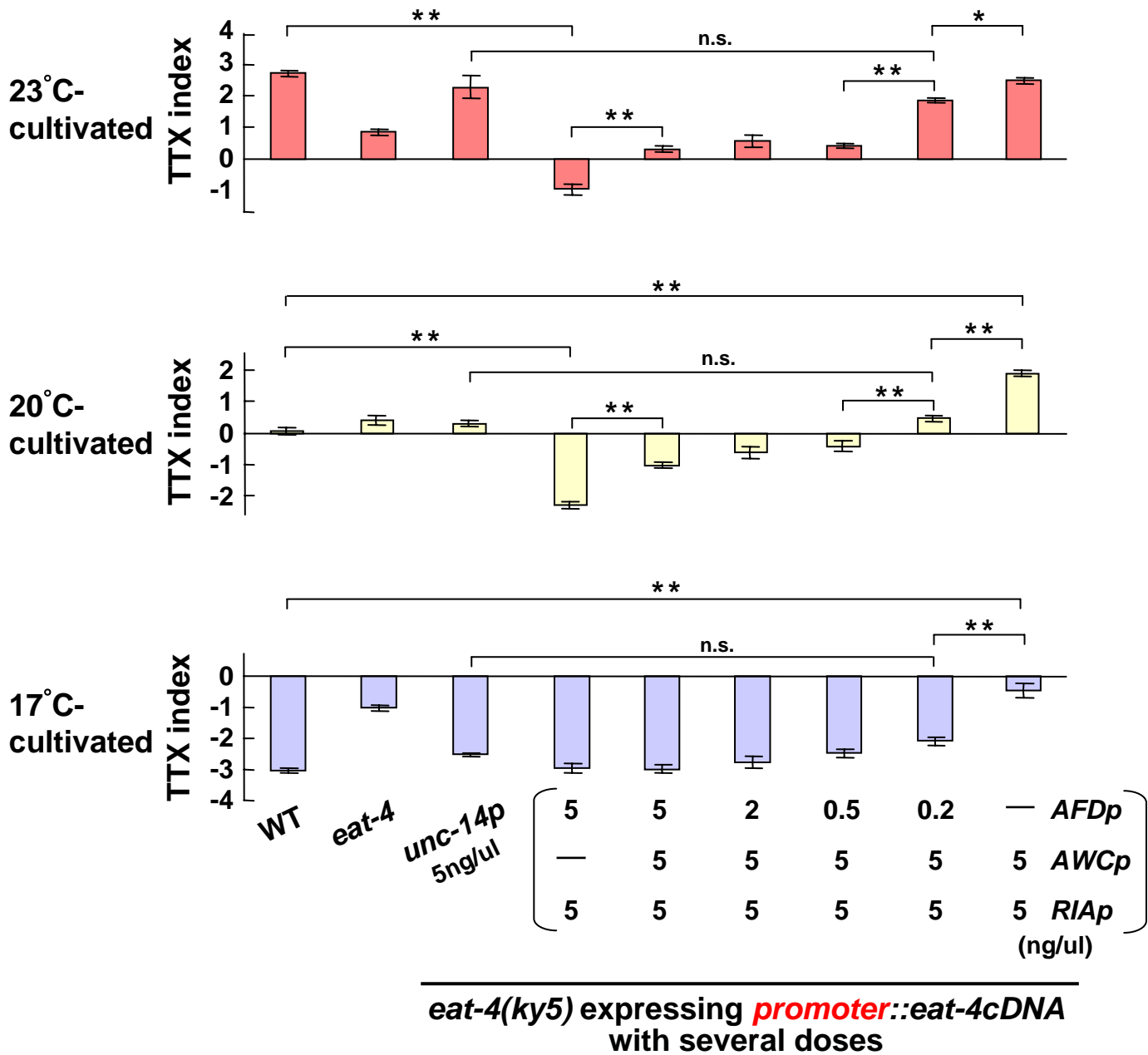


Figure 17. Rescue experiments of *eat-4(ky5)* mutants with simultaneous expression of EAT-4 in AFD, AWC, and RIA in the population TTX assay. Rescue experiments for defective migration to cultivation temperature of *eat-4(ky5)* mutants by introducing *AFDp::eat-4 cDNA*, *AWCp::eat-4 cDNA*, and *RIA p::eat-4 cDNA* simultaneously at several doses. n = 3 or more assays. Error bar indicates SEM. Single asterisk, double asterisk, and n.s. indicate $p < 0.05$, $p < 0.01$, and $p > 0.05$ respectively, in post hoc Tukey-Kramer tests for a comparison of each genotype.

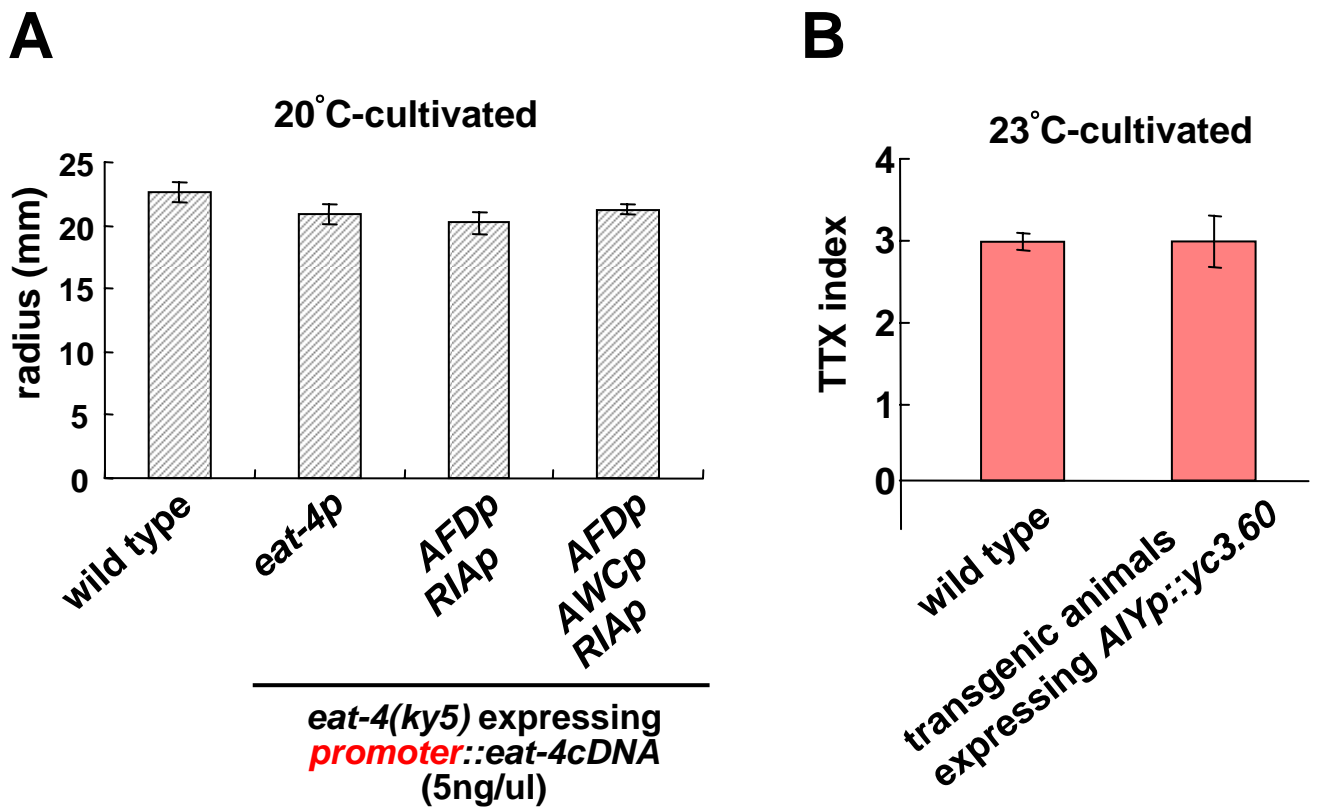


Figure 18. Radius of isothermal tracks of *eat-4* transgenic animals on the individual TTX assay plates and the population thermotaxis assays of transgenic animals containing *AIYp::yc3.60* after cultivated at 23°C.

(A) We measured radius of isothermal tracks of animals that moved isothermally as shown in Figure 15. Radius of all *eat-4* transgenic mutants showing normal fraction of isothermal tracks did not differ from that of wild-type animals ($p > 0.05$ in post hoc Tukey-Kramer test). $n = 3$ assays. Error bar indicates SEM.

(B) *AIYp::yc3.60* did not affect the normal thermotaxis of wild-type ($p > 0.05$ in post hoc Tukey-Kramer test). $n = 3$ assays. Error bar indicates SEM.

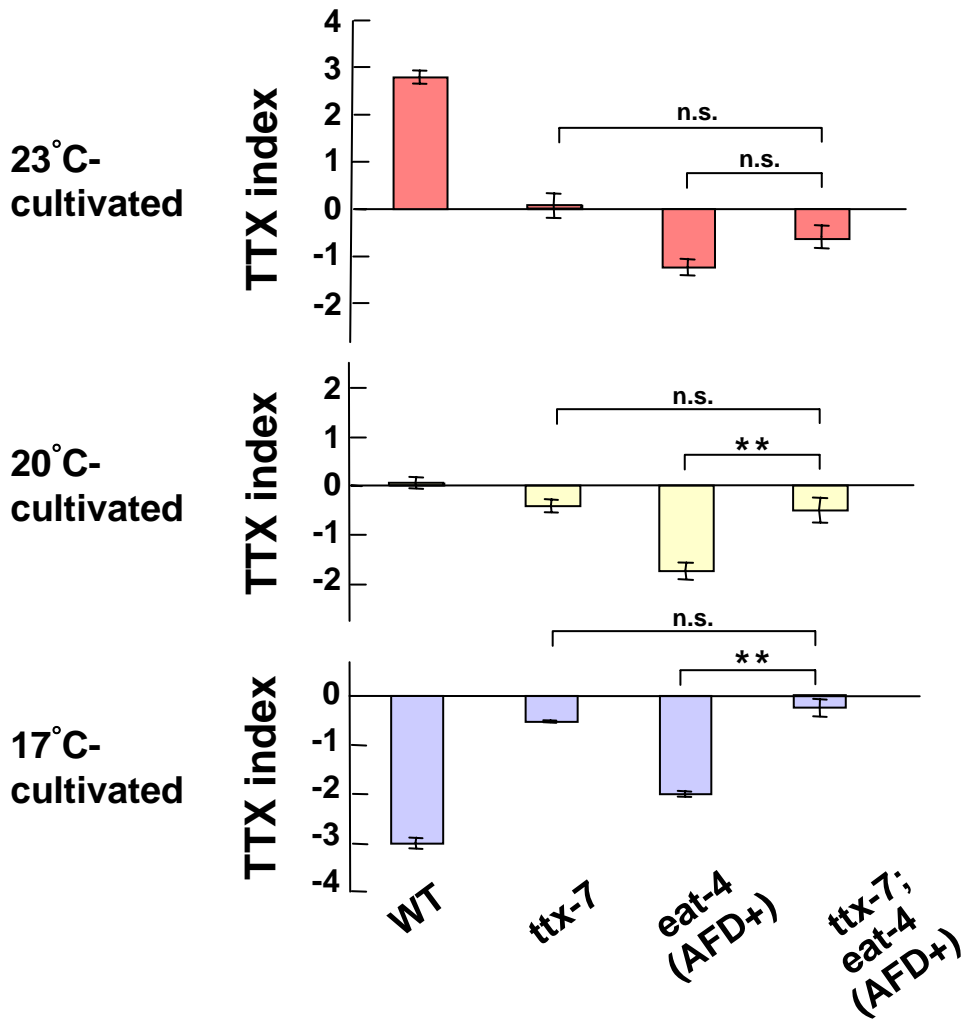


Figure 19. The population thermotaxis assay of *eat-4* transgenic mutants carrying genetically abnormal function in RIA.

eat-4 (AFD+) represents the transgenic *eat-4(ky5)* mutants expressing EAT-4 in AFD. Thermotactic defect of *ttx-7(nj50)* mutants completely masked the defect of *eat-4* (AFD+). n = 3 or more assays. Error bar indicates SEM. Double asterisk and n.s. indicate p < 0.01 and p > 0.05, respectively, in post hoc Tukey-Kramer tests for a comparison of each genotype.

cameleon (YC3.60)

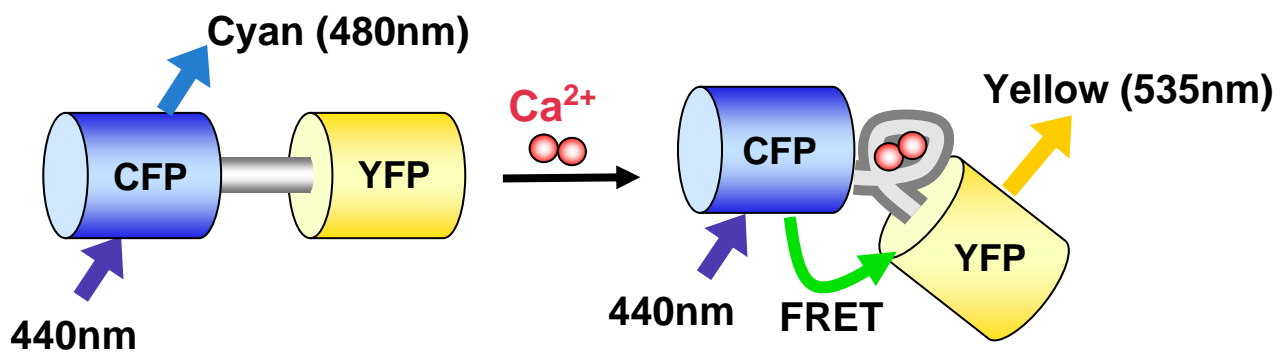


Figure 20. Schematic structure of cameleon YC3.60.

The color of fluorescence emitted by cameleon changes from cyan to yellow with fluorescence resonance energy transfer (FRET) which is mediated by the binding of Ca²⁺ to cameleon.

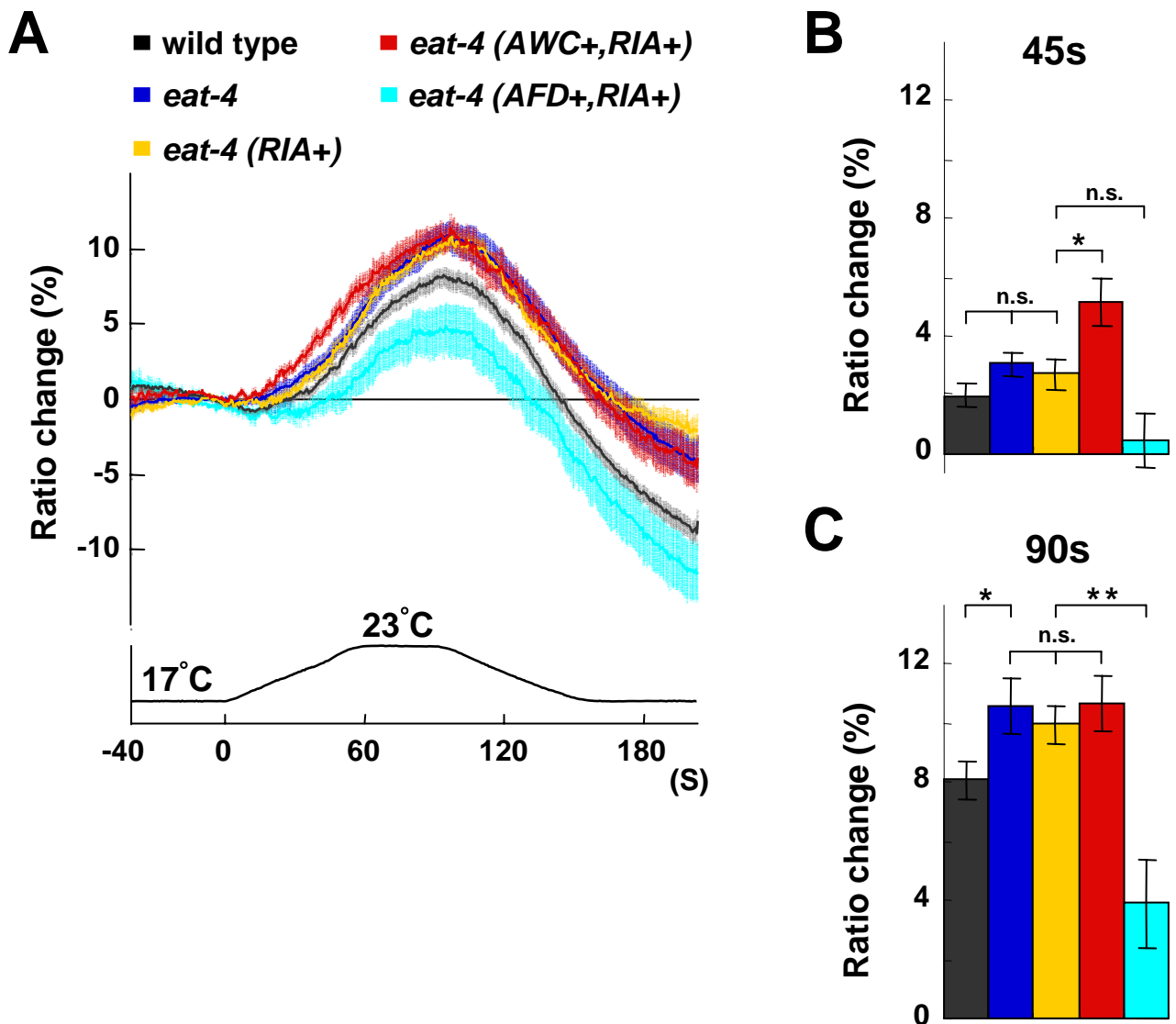


Figure 21. *in vivo* calcium imaging of AIY according to warming in *eat-4(ky5)* mutants and the *eat-4(ky5)* transgenic animals after cultivated at 20°C.

(A) The intracellular calcium concentration change was measured as the YFP/CFP fluorescence change (Ratio change) of cameleon (*yc3.6*). *eat-4* (*AWC+*, *RIA+*), *eat-4* (*AFD+*, *RIA+*), and *eat-4* (*RIA+*) represent the transgenic *eat-4(ky5)* mutants expressing EAT-4 in AWC and RIA but not in AFD, in AFD and RIA but not in AWC, and in RIA but not in AFD and AWC, respectively (top). Response curves represent the average of Ratio change according to temperature change. Temperature change along time is shown at the bottom of the response curve. 0s is the time starting temperature change from 17°C to 23°C (bottom). (B, C) The average of Ratio changes to temperature stimuli at 45s (B) and at 90s (C) regarding the results shown in Figure 21A. The color of bars in the graphs corresponds to the color of response curves described in Figure 21A. n = 18 or more animals. Error bar indicates SEM. Single asterisk, double asterisk, and n.s. indicate $p < 0.05$, $p < 0.01$, and $p > 0.05$, respectively, in Steel-Dwass tests for a comparison of each genotype.

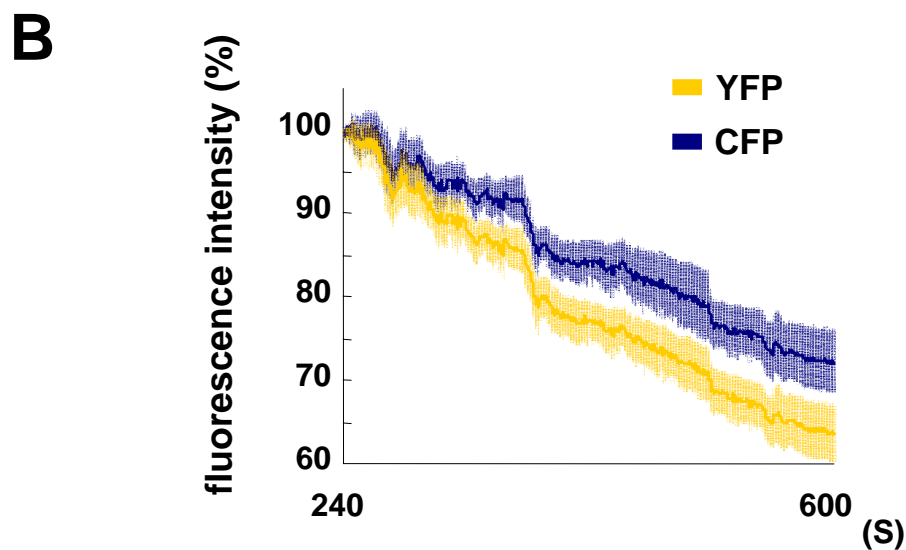
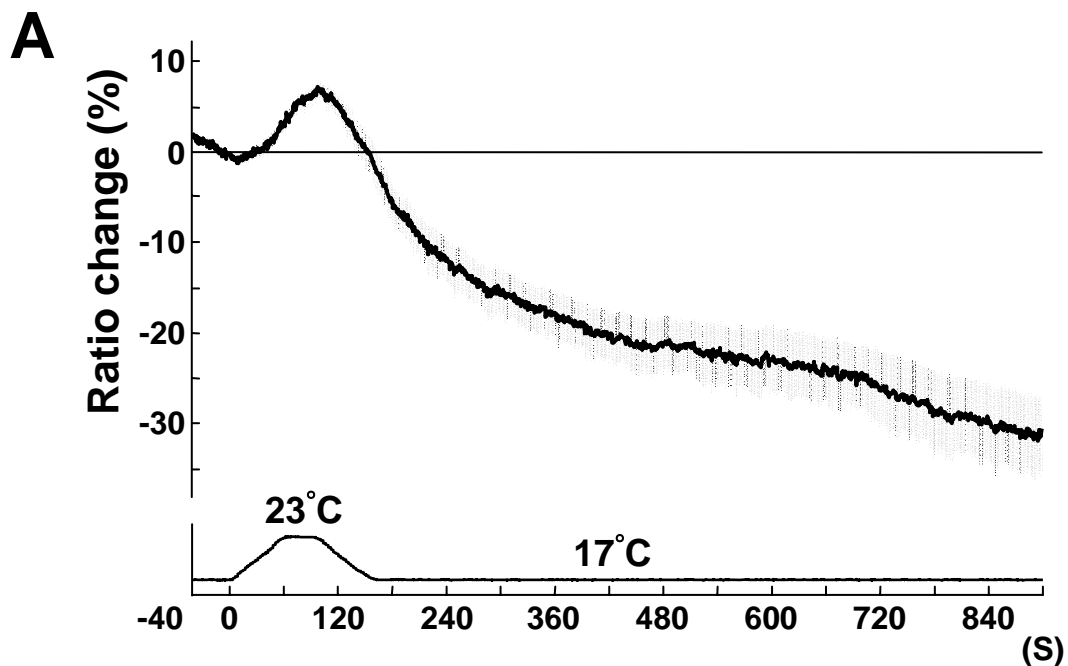


Figure 22. long-term *in vivo* calcium imaging of AIY in 20°C-cultivated wild-type animals
 (A) Ratio change of *yc3.6* in AIY of wild-type animals in response to warming. By providing same temperature change as shown in Figure 21, we monitored ratio change until 900s. After the temperature change from 17°C to 23°C, FRET ratios continued to decrease gradually. $n = 11$ animals. Error bar indicates SEM.

(B) Change of fluorescence intensities of YFP (yellow) and CFP (blue) in *yc3.6* from 240s to 600s in Figure 22A. We set the average intensities of YFP and CFP at 240s as 100%. Fluorescence intensities of YFP decrease faster than that of CFP. $n = 7$ animals. Error bar indicates SEM.

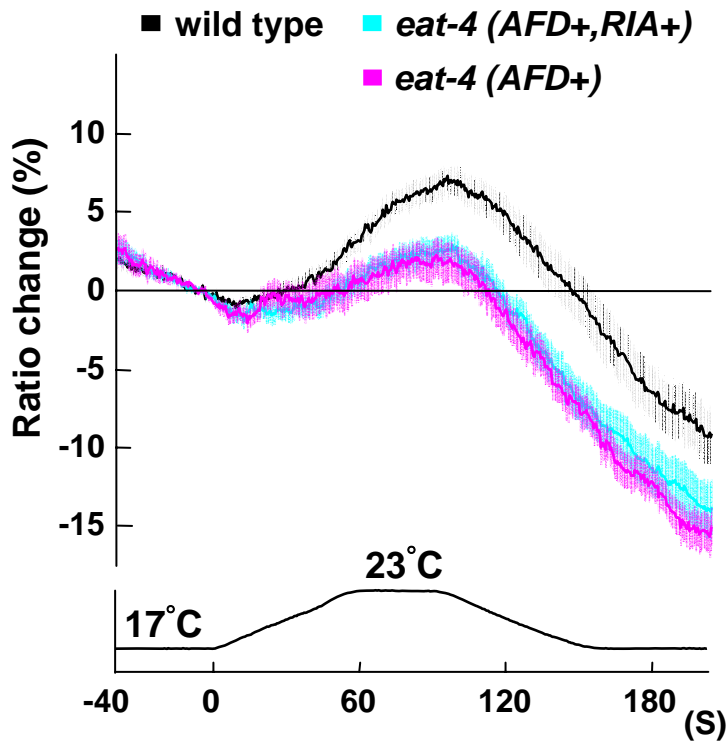
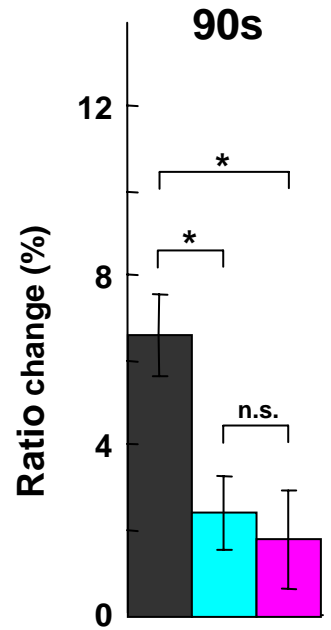
A**B**

Figure 23. *in vivo* calcium imaging of AIY in 20°C-cultivated *eat-4(ky5)* transgenic animals
 (A) Ratio change of *yc3.6* in AIY of *eat-4* transgenic animals in response to temperature change. *eat-4 (AFD+, RIA+)* and *eat-4 (AFD+)* represent the transgenic *eat-4(ky5)* mutants expressing EAT-4 in both AFD and RIA, and in AFD but not in RIA, respectively (top).
 (B) The average of Ratio changes to temperature stimuli at 90s concerning the results shown in Figure 23A. The color of bars in the graphs corresponds to the color of response curves described in Figure 23A. $n = 15$ animals. Error bar indicates SEM. Single asterisk and n.s. indicate $p < 0.05$ and $p > 0.05$, respectively, in Steel-Dwass tests for a comparison of each genotype.

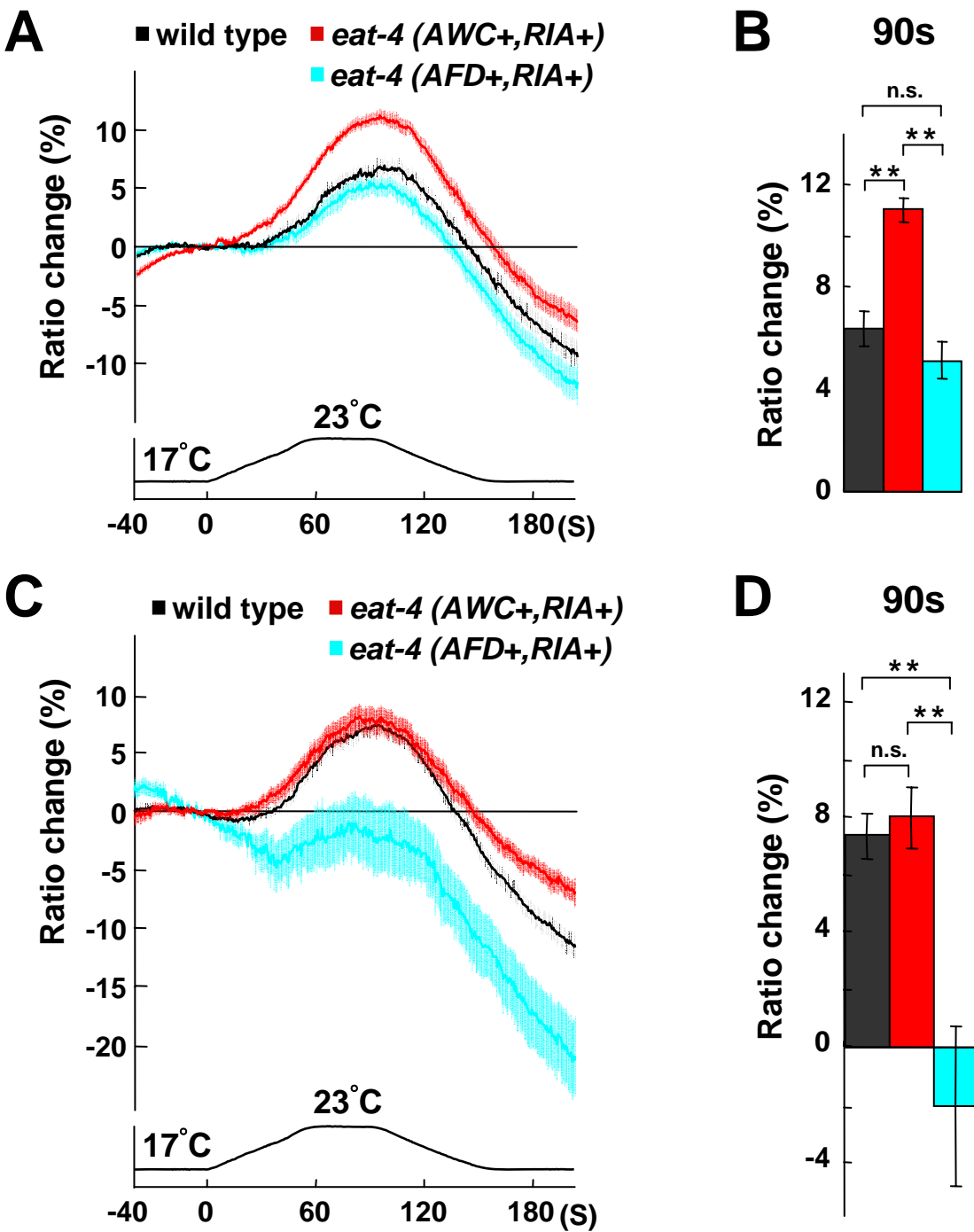


Figure 24. *in vivo* calcium imaging of AIY in 17°C- and 23°C-cultivated *eat-4(ky5)* transgenic animals shown in Figure 21.

(A) Ratio change of *yc3.6* in AIY of 17°C-cultivated animals in response to warming.

(B) The average of Ratio changes to temperature stimuli at 90s concerning the results shown in Figure 23A.

(C) Ratio change of *yc3.6* in AIY of 23°C-cultivated animals in response to warming.

(D) The average of Ratio changes to temperature stimuli at 90s concerning the results shown in Figure 24C.

(A, B, C, D) $n = 21$ or more animals. Error bar indicates SEM. Double asterisk and n.s. indicate $p < 0.01$ and $p > 0.05$, respectively, in Steel-Dwass tests for a comparison of each genotype.

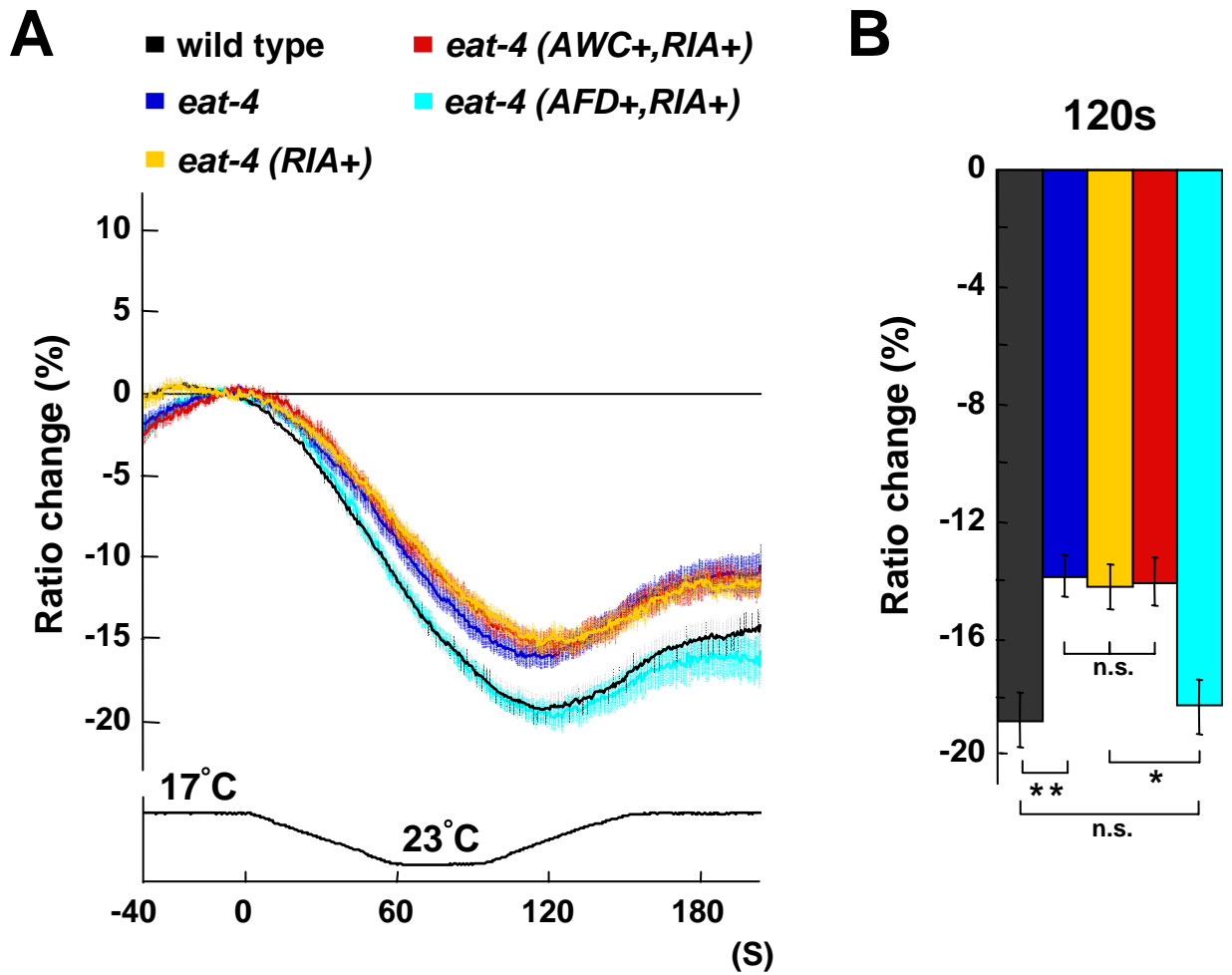


Figure 25. *in vivo* calcium imaging of AIY according to cooling in *eat-4(ky5)* mutants and the *eat-4(ky5)* transgenic animals after cultivated at 20°C.

(A) Ratio change of *yc3.6* in AIY in response to temperature change from 23°C to 17°C.

(B) The average of Ratio changes to temperature stimuli at 120s for the results shown in Figure 23. $n = 18$ or more animals. Error bar indicates SEM. Single asterisk, double asterisk, and n.s. indicate $p < 0.05$, $p < 0.01$, and $p > 0.05$, respectively, in Steel-Dwass tests for a comparison of each genotype.

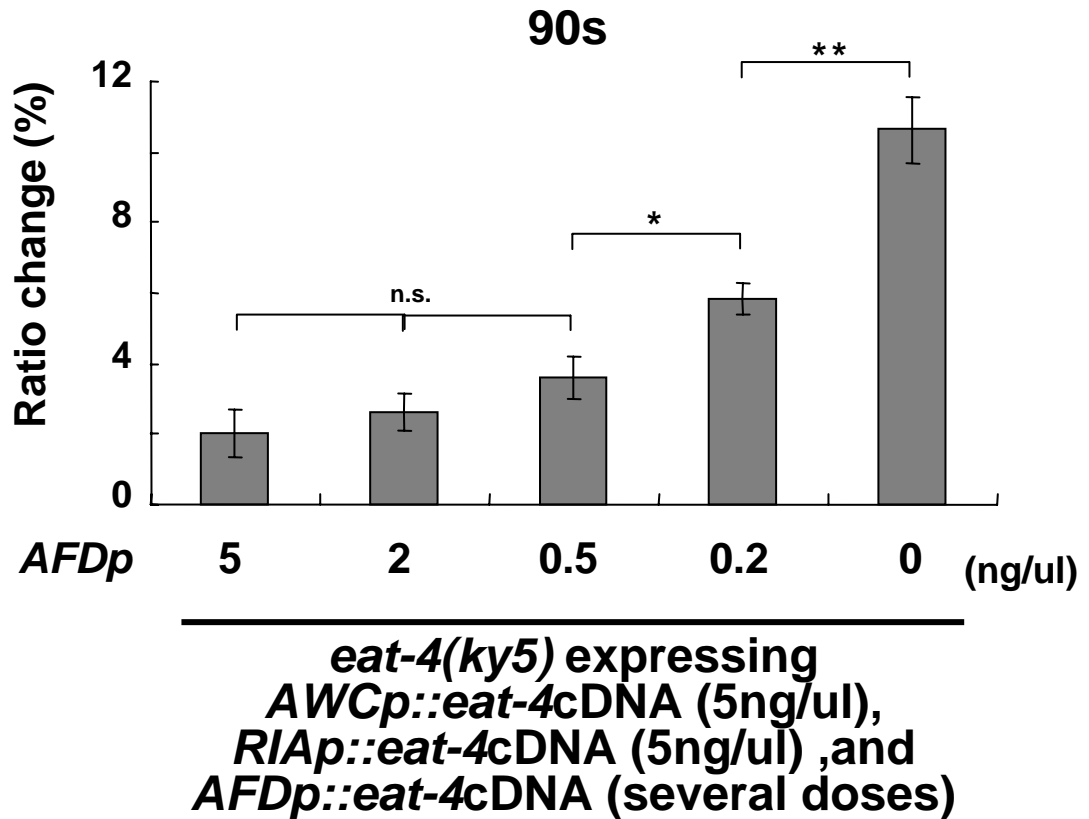


Figure 26. *in vivo* calcium imaging of AIY according to warming in 20°C-cultivated *eat-4(ky5)* transgenic animals expressing EAT-4 in AFD, AWC and RIA.

The average of Ratio changes of *eat-4(ky5)* expressing EAT-4 in AFD, AWC and RIA simultaneously by introducing several doses of *AFDp::eat-4 cDNA* with 5ng/ul of *AWCp::eat-4 cDNA* and *RIAp::eat-4 cDNA* at 90s. n = 18 or more animals. Error bar indicates SEM. Single asterisk, double asterisk, and n.s. indicate $p < 0.05$, $p < 0.01$, and $p > 0.05$, respectively, in Steel-Dwass tests for a comparison of each genotype.

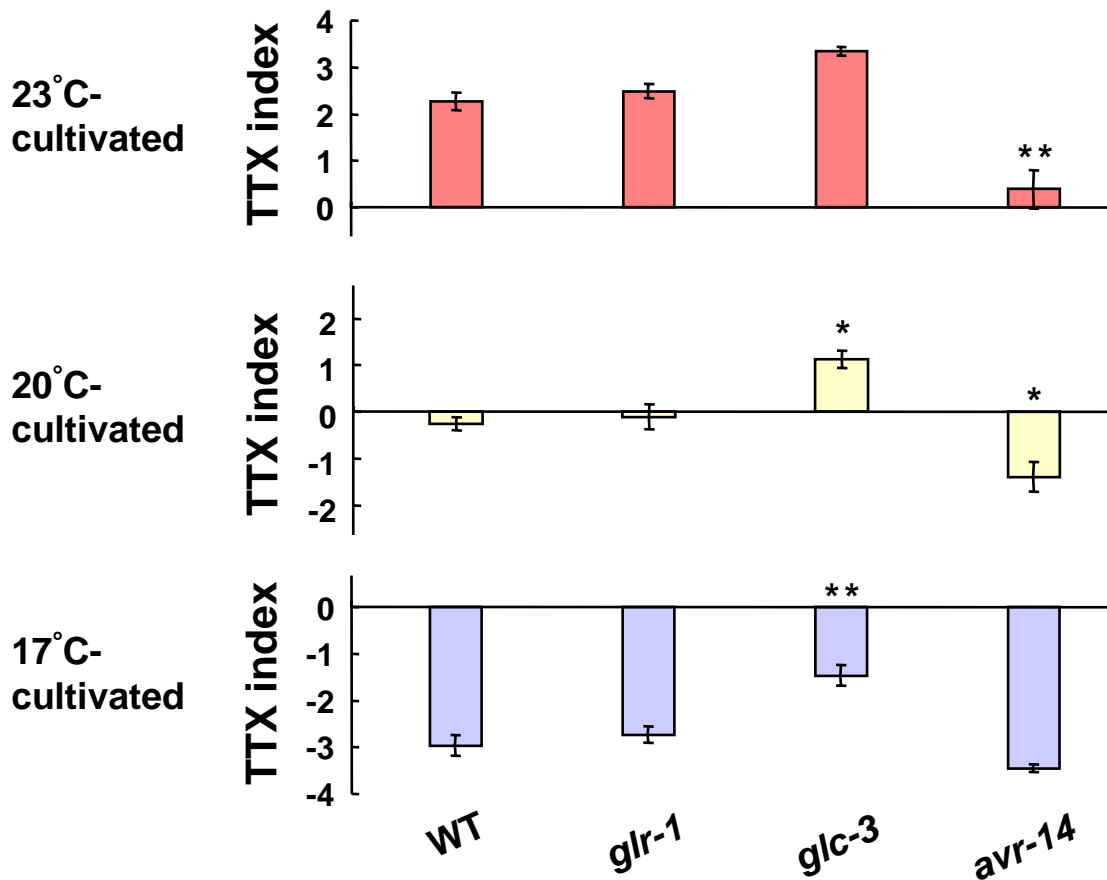


Figure 27. The population TTX assay of *glr-1*, *glc-3*, and *avr-14* mutants.

Red bars, yellow bars, and blue bars represent TTX indices of animals cultivated at 23°C, 20°C, and 17°C, respectively. n = 3 or more assays. Error bar indicates SEM. Single asterisk and double asteris indicate p < 0.05 and p < 0.01 respectively in post hoc Tukey-Kramer tests for a comparison with wild type animals.

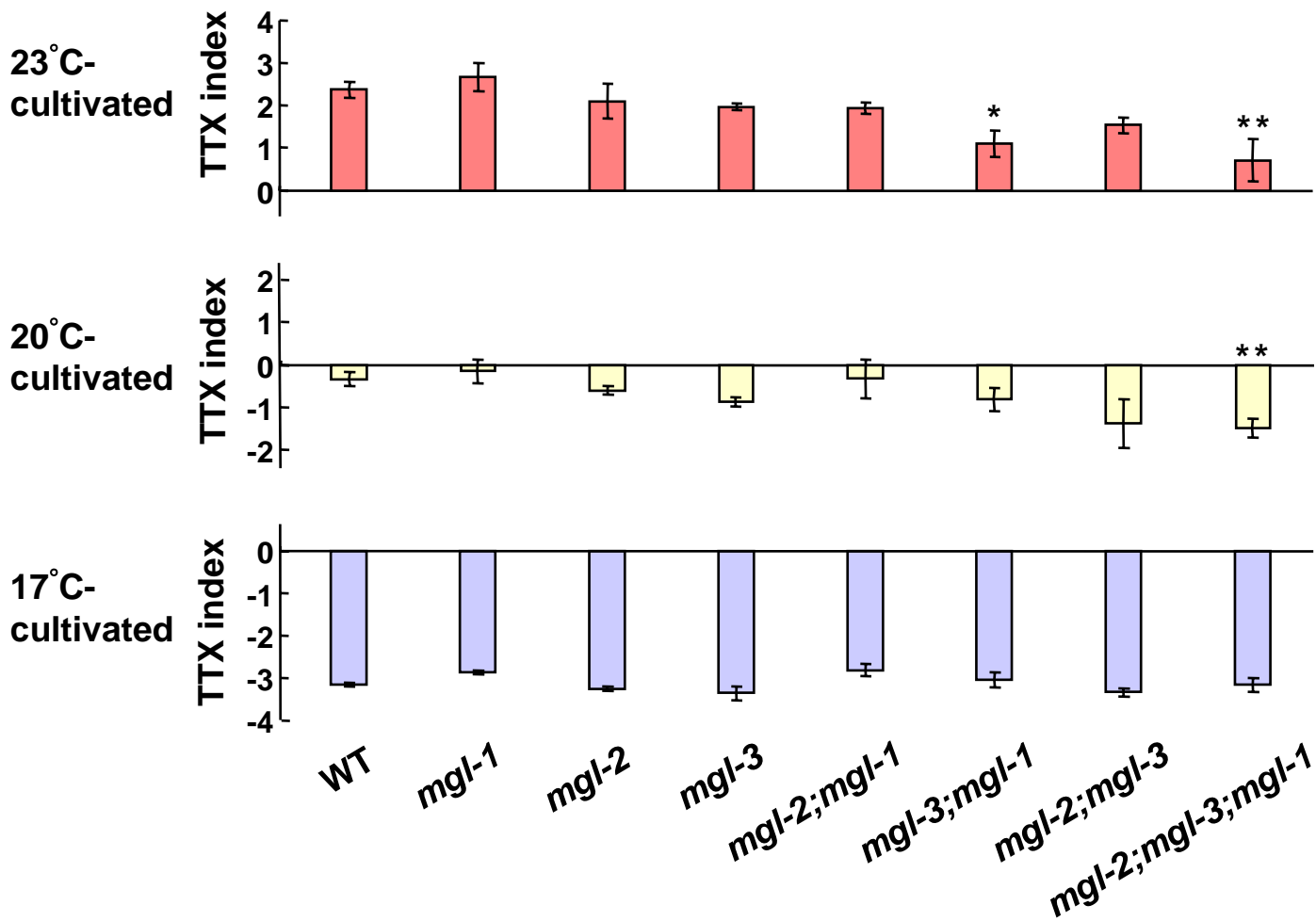


Figure 28. The population TTX assay of mGluR mutants.

TTX indices of *mgl-1*, *mgl-2*, and *mgl-3* mutants, and these double or triple mutants cultivated at 23°C, 20°C, and 17°C. n = 3 or more assays. Error bar indicates SEM. Single asterisk and double asteris indicate p < 0.05 and p < 0.01 respectively in post hoc Tukey-Kramer tests for a comparison with wild type animals.

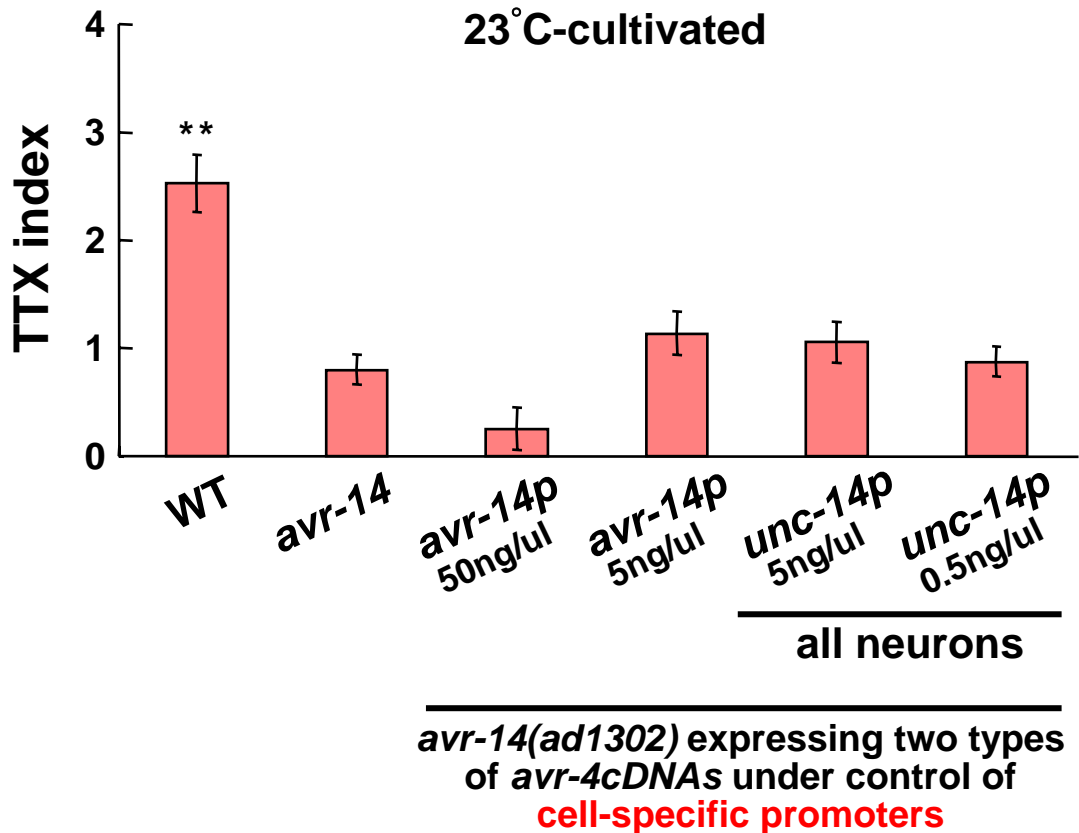


Figure 29. Cell-specific rescue experiments of *avr-14(ad1302)* mutants in the population TTX assay.

Rescue experiments for cryophilic movement of 23° C-cultivated *avr-14(ad1302)* mutants by introducing cell-specific promoters::*eat-4* cDNAs at several doses. Red bars represent TTX indices of animals. n = 3 or more assays. Error bar indicates SEM. Double asteris indicate p < 0.01 in post hoc Tukey-Kramer tests for a comparison with *avr-14(ad1302)* mutants.

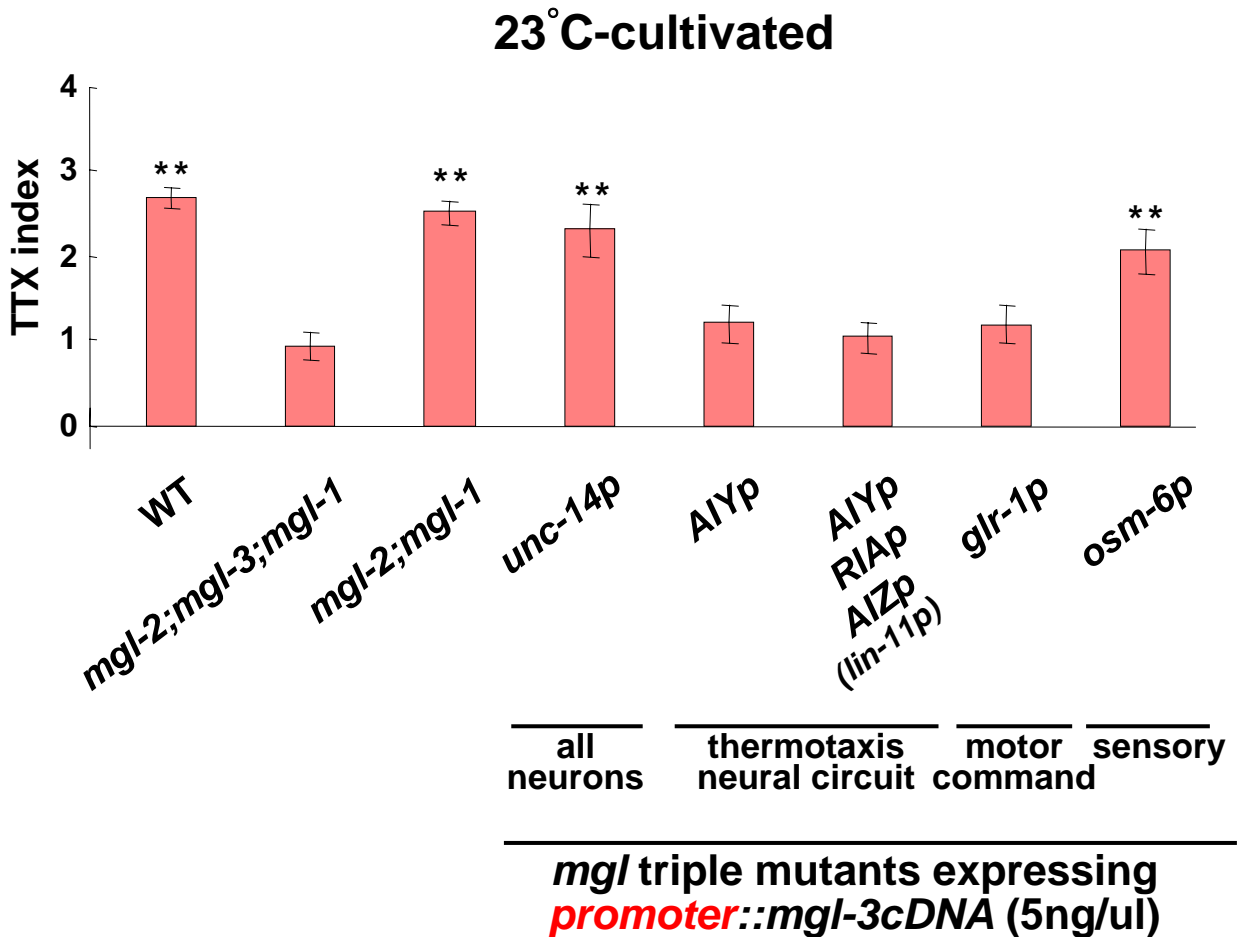


Figure 30. Cell-specific rescue experiments of *mgl-2(tm355); mgl-3(tm1766); mgl-1(tm1811)* triple mutants in the population TTX assay. Rescue experiments for cryophilic movement of 23° C-cultivated *mgl* triple mutants by introducing cell-specific *promoters::mgl-3 cDNAs* at 5ng/ul. Red bars represent TTX indices of animals. n = 3 or more assays. Error bar indicates SEM. Double asteris indicate p < 0.01 in post hoc Tukey-Kramer tests for a comparison with *mgl-2(tm355); mgl-3(tm1766); mgl-1(tm1811)* triple mutants.

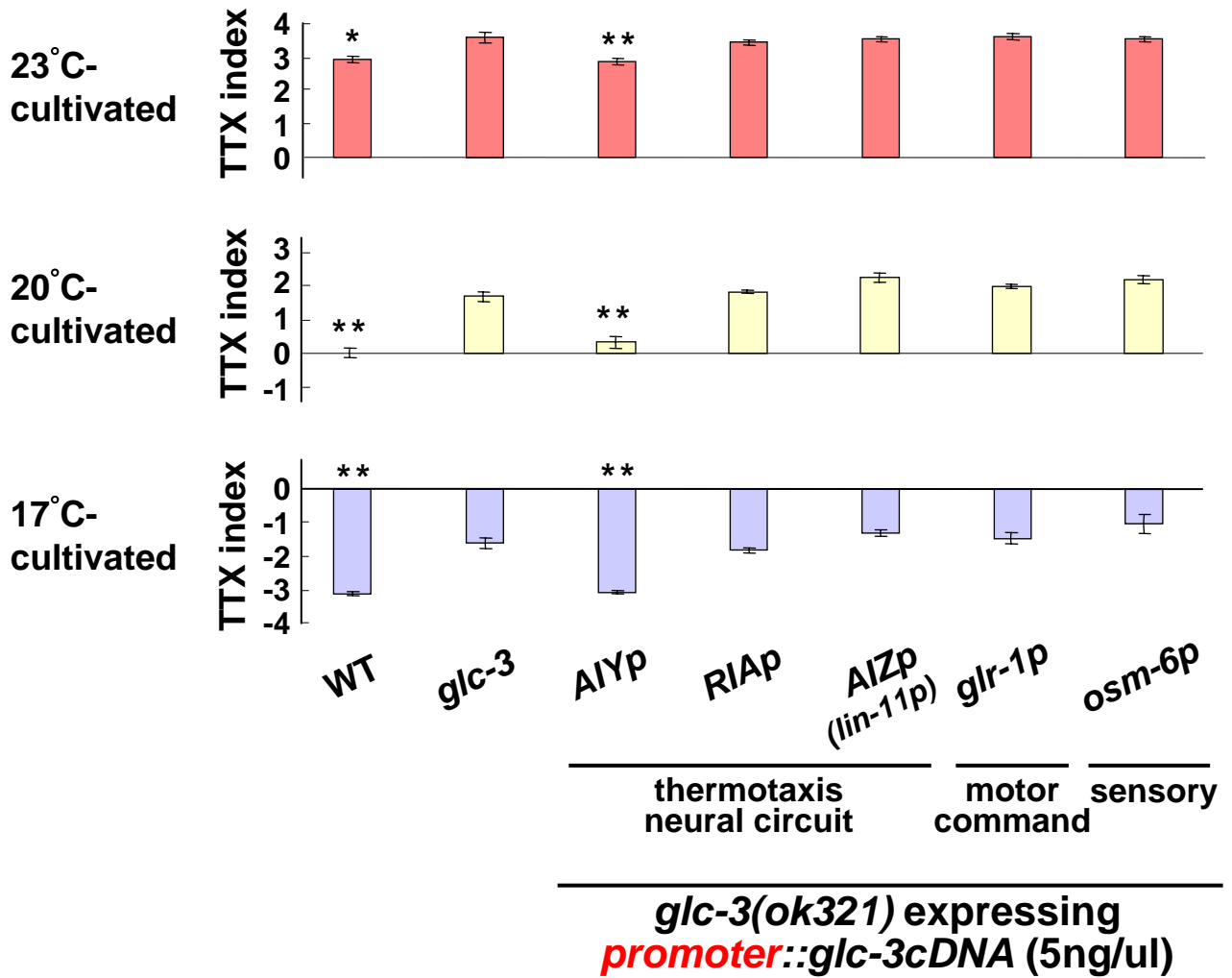


Figure 31. Cell-specific rescue experiments of *glc-3(ok321)* mutants in the population TTX assay.
 Rescue experiments for thermophilic movement of *glc-3(ok321)* mutants by introducing cell-specific *promoters::glc-3 cDNAs* at 5ng/ul. Red bars, yellow bars, and blue bars represent TTX indices of animals cultivated at 23°C, 20°C, and 17°C, respectively. n = 3 or more assays. Error bar indicates SEM. Double asteris indicate p < 0.01 in post hoc Tukey-Kramer tests for a comparison with *glc-3(ok321)* mutants.

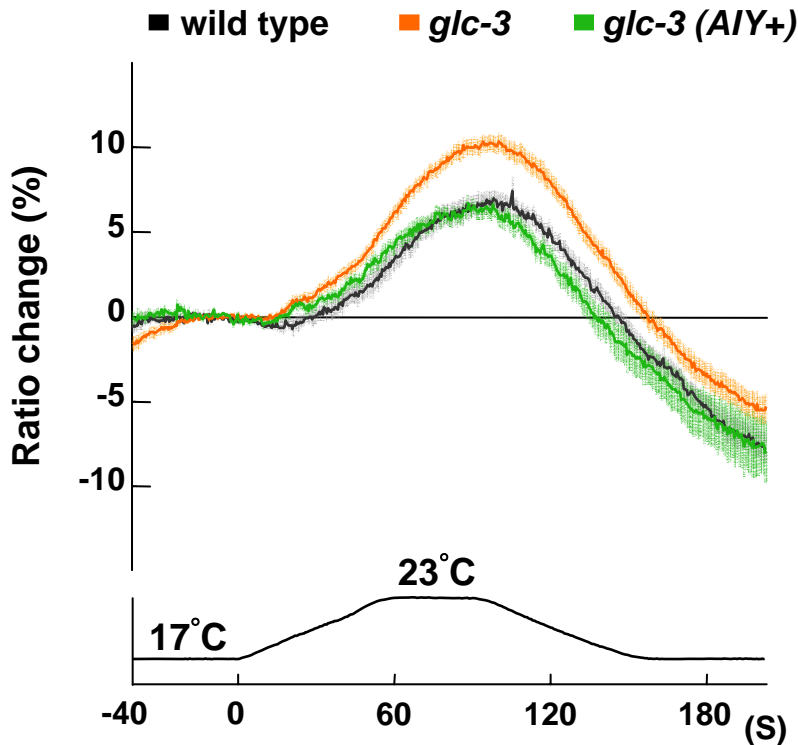
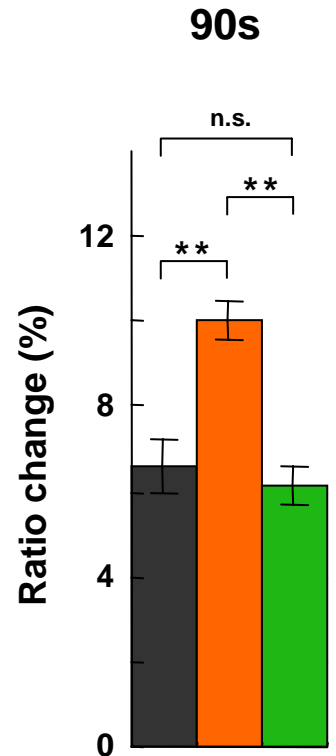
A**B**

Figure 32. *in vivo* calcium imaging of AIY in 20°C-cultivated *glc-3(ok321)* mutants and the *glc-3(ok321)* transgenic animals.

(A) Ratio change of *yc3.6* in AIY according to temperature change. *glc-3 (AIY+)* represents the transgenic *glc-3(ok321)* mutants expressing GCL-3 in AIY. (B) The average of Ratio changes to temperature stimuli at 90s regarding the results shown in Figure 31A. $n = 24$ or more animals. Error bar indicates SEM. Double asterisk and n.s. indicates $p < 0.01$ and $p > 0.05$, respectively, in Steel-Dwass tests for a comparison with wild-type animals.

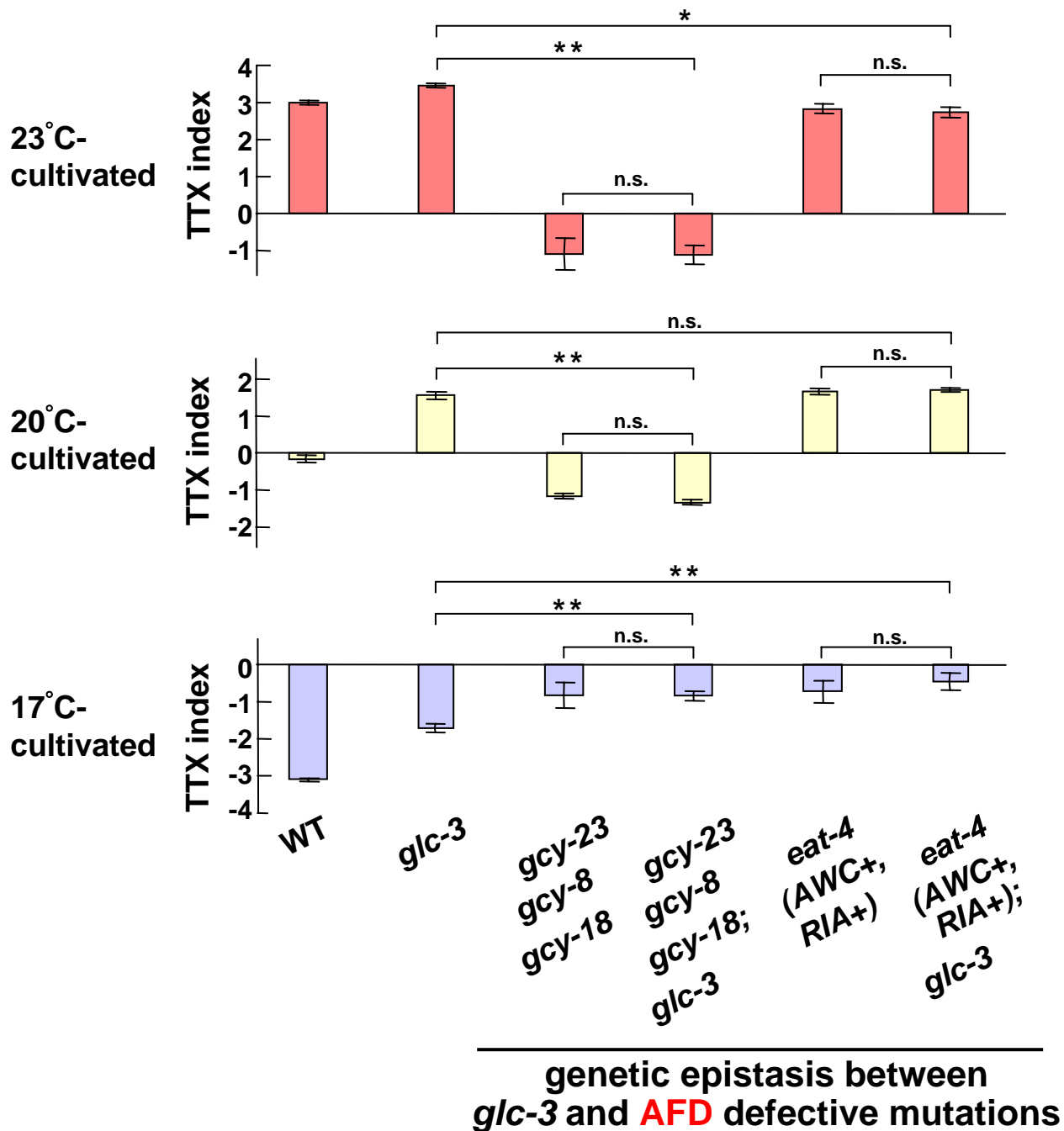
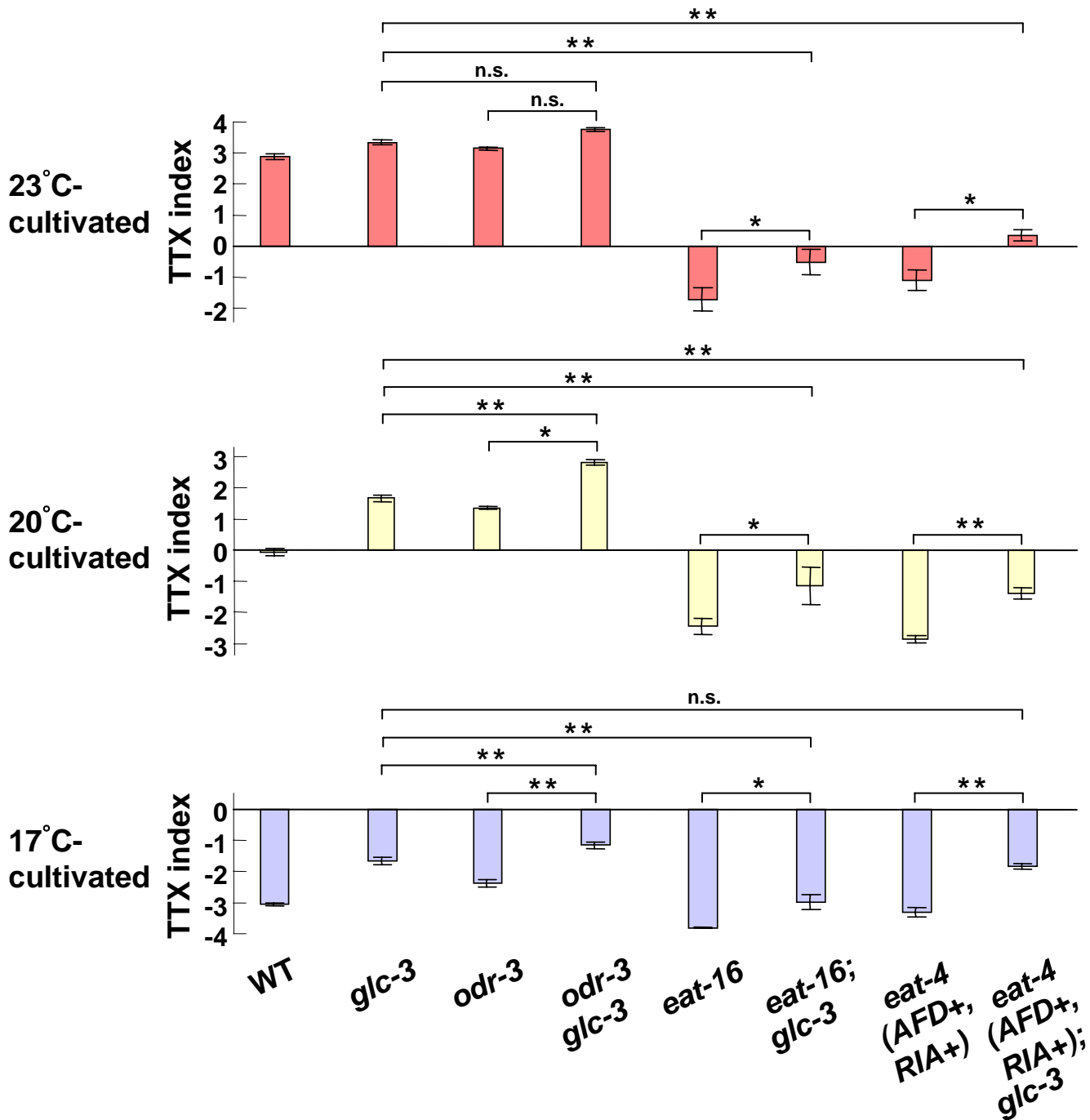


Figure 33. Analysis of genetic epistasis between *glc-3* and AFD-defective mutants. The population thermotaxis assays of AFD defective mutants, and *glc-3* mutants in the background of abnormal neural signaling in AFD. n = 3 or more assays. Error bar indicates SEM. Single asterisk, double asterisk and n.s. indicate $p < 0.05$, $p < 0.01$, and $p > 0.05$, respectively, in post hoc Tukey-Kramer tests for a comparison of each genotype.



**genetic epistasis between
glc-3 and **AWC** defective mutations**

Figure 34. Analysis of genetic epistasis between *glc-3* and AWC-defective mutants. The population thermotaxis assays of AWC defective mutants, and *glc-3* mutants in the background of abnormal neural signaling in AWC. n = 3 or more assays. Error bar indicates SEM. Single asterisk, double asterisk and n.s. indicate p < 0.05, p < 0.01, and p > 0.05, respectively, in post hoc Tukey-Kramer tests for a comparison of each genotype.

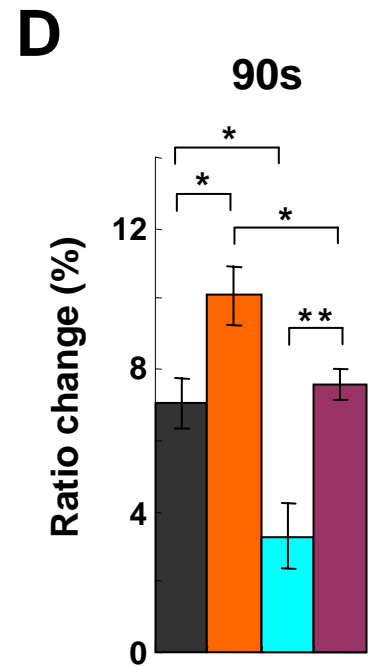
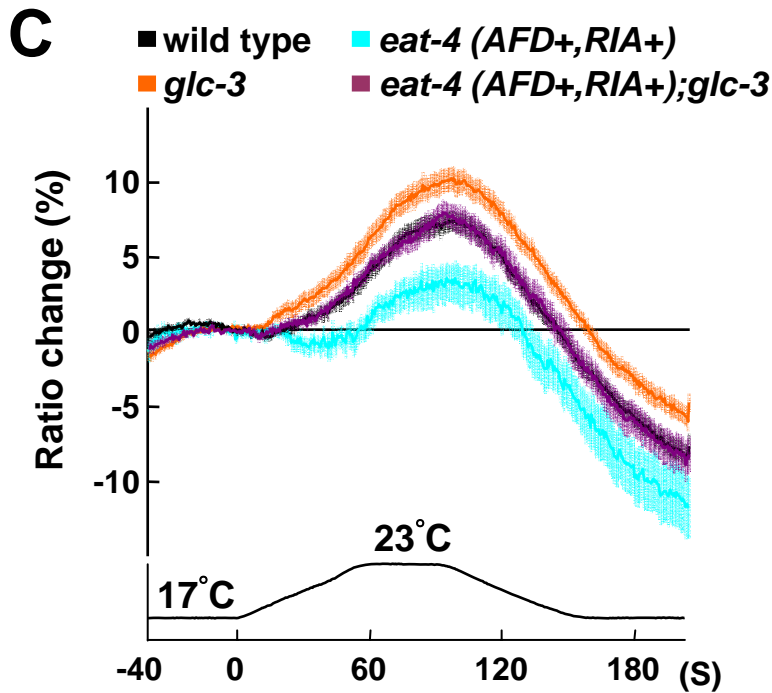
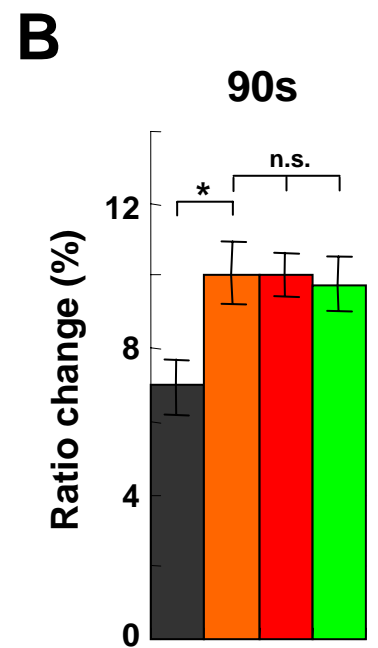
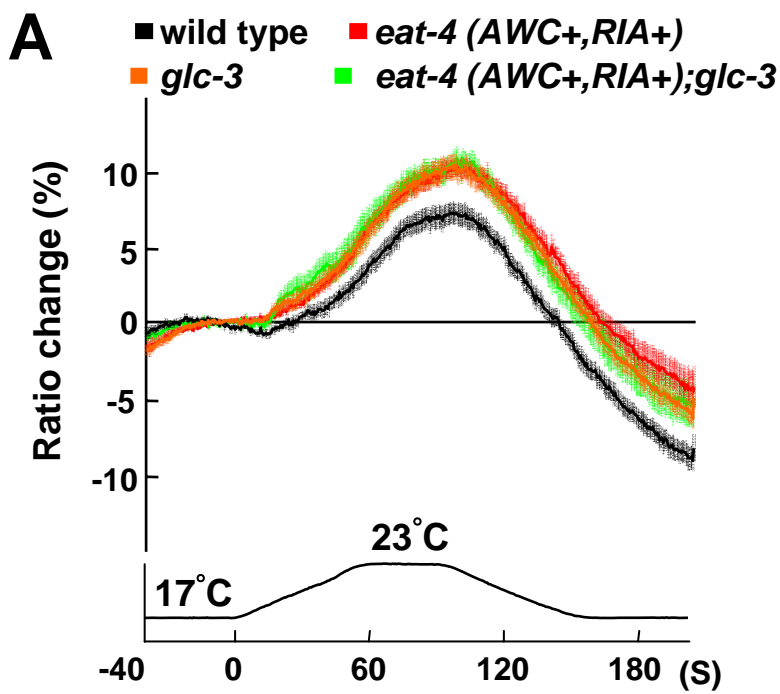


Figure 35. Effect of AFD or AWC defect on activity of AIY in *glc-3(ok321)* mutants.

(A, B) *in vivo* calcium imaging of AIY in *eat-4 (AWC+,RIA+); glc-3* and each single mutants. (A) Ratio change of *yc3.6* in AIY according to temperature change. (B) The average of Ratio changes to temperature stimuli at 90s regarding the results shown in Figure 35A.

(C, D) *in vivo* calcium imaging of AIY in *eat-4 (AFD+,RIA+); glc-3* and each single mutants. (C) Ratio change of *yc3.6* in AIY according to temperature change. (D) The average of Ratio changes to temperature stimuli at 90s regarding the results shown in Figure 35C.

(A, B, C, D) $n = 22$ or more animals. Error bar indicates SEM. Single asterisk, double asterisk and n.s. indicate $p < 0.05$, $p < 0.01$, and $p > 0.05$, respectively, in post hoc Tukey-Kramer tests for a comparison of each genotype.

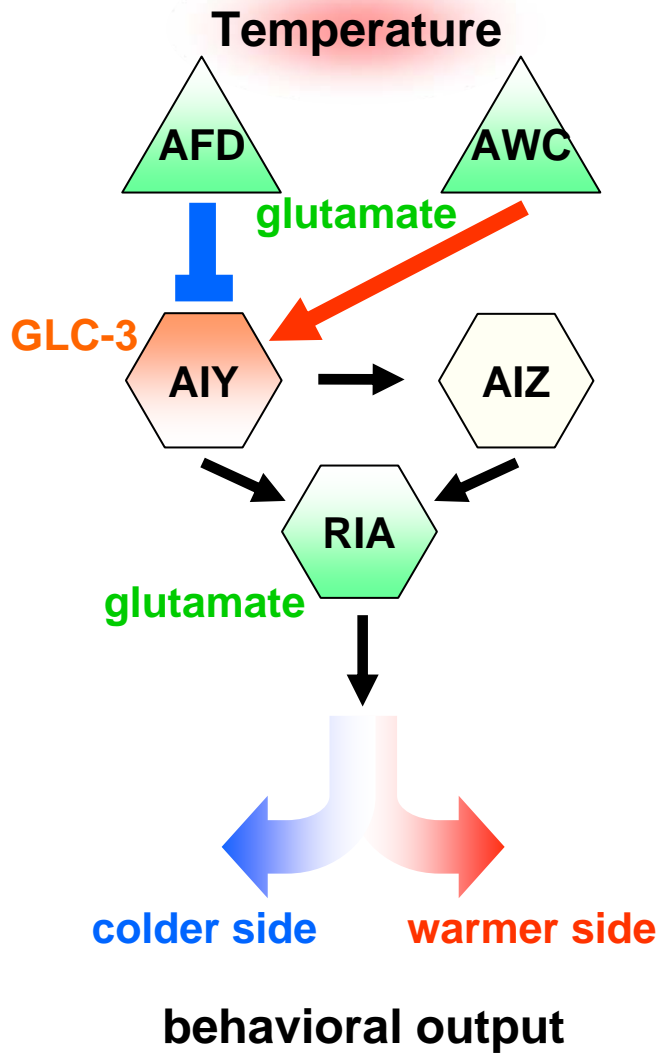
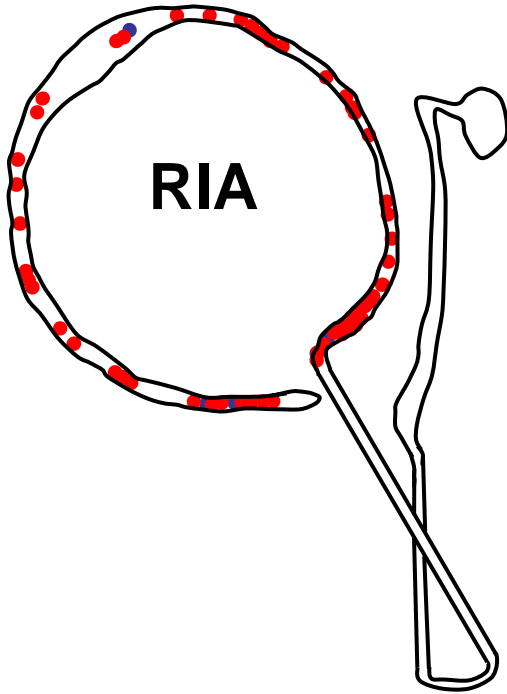


Figure 36. A model for glutamatergic neurotransmission in the thermotaxis neural circuit.

AFD, AWC, and RIA release glutamate in EAT-4 dependent manner (green). GLC-3 acts in AIY (orange). EAT-4 dependent glutamate signals from AFD inhibit the activity of AIY through activation of GLC-3 inhibitory glutamate receptors, and induce eventual migration to colder side (blue). By contrast, EAT-4 dependent glutamate signals from AWC stimulate the activity of AIY, and induce eventual migration to warmer side (red).

A**B**

downstream neurons of RIA	percentage of total presynapses in RIA
SMD	40%
RMD	50%
RIV	8%
SIA	1%
RIA	1%

(White *et al.*, 1986 modified)

Figure 37. Postsynaptic neurons of RIA.

(A) Schematic structure of RIA interneurons. RIA has a single neurite. All dots represent presynapses in the neurite of RIA. Of those, red dots represent presynapses connecting to SMD or RIA head motor neurons.

(B) Percentages of precynapses connecting to each neurons in RIA.

Ionotropic (channel)	
AMPA/KA type	<i>glr-1 glr-2 glr-3 glr-4 glr-5 glr-6 glr-7 glr-8</i>
NMDA type	<i>nmr-1 nmr-2</i>
Cl ⁻ channel type	<i>glc-1 glc-2 glc-3 glc-4 avr-14 avr-15</i>

metabotropic	<i>mgl-1 mgl-2 mgl-3</i>

Table 1. Glutamate receptors in *C. elegans*.

Four classes of glutamate receptors are predicted in *C. elegans*.

Appendix

pNR8 (*eat-4* cDNA)

```
10      20      30      40      50      60      70      80
ATGACCATGATTACGCCAAGCTTGCATGCCCTGCAGGTCGACTAGAGAGATCCCGGGATTGGCAAAGGACCCAAAGGT
TACTGGTACTAATGCGGTTTCGAACGTACGGAGCTCCAGCTGAGATCTCCTAGGGGCCCTAACCGGTTCCCTGGGTTTCCA

90      100     110     120     130     140     150     160
ATGTTTGAATGATACTAACAATAGAACATTTTCAGGAGGACCCTTGGCTAGCGTGCAGGTTACCAGAAACATGT
TACAAAGCTTACTATGATTGTTATGTTATCTTGAATAAGCTCCTCGGGAACCCGATCGCAGCTGCCATGGCTTTGGTACA

170     180     190     200     210     220     230     240
CGTCATGGAACGAGGCTTGGGATCGTGGCAACAATGGTTGGAGAGCCACTCGCCAAAATGACTGCAGCAGCTGCATCT
GCAGTACTTGCCTCGAACCTAGCACCGTTTGTTCACCAACTCTCGGTGAGCGGTTTTACTGACGCTGCTGCAGCTAGA
GENE=EAT-4CDNA

250     260     270     280     290     300     310     320
GCAACTGGTGCAGCACCCACAGCAAATGCAAGAAGAAGGAAACGAAAACCCGATGCAAAATGCATTCAAACAAAGTGT
CGTTGACCAGCTGCTGGGGGTGCTGTTTACGCTTTTACGGCTTTTGCACCCGAAAGATCGATAAAGACGTTTATACCC
GENE=EAT-4CDNA

330     340     350     360     370     380     390     400
TCAAGTTTGGAGCAAATGGATCGGAAAATGCCGAAAACCTGGCTCTAGCTATCTTGGCAAAATGGGATTCATGA
AGTTCAATACCTCGTTGAACCTAGCCTTTTACGGCTTTTGCACCCGAAAGATCGATAAAGACGTTTATACCC
GENE=EAT-4CDNA

410     420     430     440     450     460     470     480
TTTCATTTGGAATTCGATCGAAATTCGGTGCAGCAAACTCATATGTATAAAAAATACAGACTCCATACGGAAGTT
AAAGTAAACCTTAAAGTACGTTAAAGCCAGCTCGGTTTTGAGTATACATATTTTAAATATGTCTAGGTATGCTTTTCAA
GENE=EAT-4CDNA

490     500     510     520     530     540     550     560
CATATGCATGAATCAATGGCAATAGATGAGCTTAGTGTCTATGGAGAGTTCGTAACCTTTACGGGTACTTAGTCACTCA
GTATACGTAATTAACCTGTTATCTACTCGAATCAGAGTACTCTCAAGCATGAAAATGCCCATGAATCAGTGTGAGT
GENE=EAT-4CDNA

570     580     590     600     610     620     630     640
GATTCGCGCTGGTTTCTTGCCTCAAGATTTCCACCAACAACATATTTCGGATTGGAAATCGGAGTCCGGAGCATTTTGA
CTAAGGCCGACCAAAAAGACGAGCTTTCAAAGGTGGTTTTGTTGATAAGCCATAAACCTTAGCTCAGCCTCGTAAAAC
GENE=EAT-4CDNA

650     660     670     680     690     700     710     720
ATATTCCTTACCATATGGATTTAAAGTGAAGAGTACTCTTGGCTTTTATTCAAAATTAACCAAGGCTTGTTCAG
TATAAGAGGATGGTATACCTAAATTTCACTTTTCACTGATGGAACCCGAAAAGTAAAGTTAATGAGTTCCTCCGAAAC
GENE=EAT-4CDNA

730     740     750     760     770     780     790     800
GGAGTTTGTATCCCTGCATGCATGGTGTCTGGAGATATGGGCCCCCAATGGAAGATCTAAATAGCAACACACAGC
CCTCAAAACATGGGACGATACGTACACAGACCTCTATAACCCGGGGTGGTTACCTTTAGATTTGATGTTGGTGTGCG
GENE=EAT-4CDNA

810     820     830     840     850     860     870     880
ATTCACAGGATCATATGCTGGAGCTGTTCTGGGTACTCTTATCTGCTTCTTGGTTTCATACGCTCTCATGGCTGCTC
TAAGTGTCTAGTATACGACCTCGACAAGAGCCAGATGGAATAAGACGAAAGAACAAAGATGACAGAGTACCCGACGAG
GENE=EAT-4CDNA

890     900     910     920     930     940     950     960
CATTTTATCTTTATGGAGTATGTGGTGCATTTGGGCGATCTTTGGTCTGTGTCACTTTGAGAAACCCCGCTTTTCA
GTAATAAGAAATACCTCATACACACAGTAAACCCGCTAAGAAACCAAGACACAGTGAATACTTTTGGGCGCAAAGTA
GENE=EAT-4CDNA

970     980     990     1000    1010    1020    1030    1040
CCGACCATATCGCAAGAAGAGAAAATATTTATCGAAGATGCAATTTGGTCAAGTCTCAAAACCCCAACGATCCGATC
GGCTGGTATAGCGTCTTCTCTTTTATAAATAGCTTCTACGTTAACCAAGTGCAGAGTTTGGTGGGTTGCTAGGCTAG
GENE=EAT-4CDNA

1050    1060    1070    1080    1090    1100    1110    1120
GATTCATGGAAGCAATGTCACTTCAAGCCTGTGGGCAATTTATGTTGCGAATCTTGCAGAGCTGGACTTCTCT
CTAAGGTACCTTTCTTAACAGTGAAGTTTCGGACACCCGTTAATAACAACGCTTGAACGAGCTTGCACGTGAAGA
GENE=EAT-4CDNA
```

```
1130    1140    1150    1160    1170    1180    1190    1200
ATCTCCTTTTGCAAAATCAATTGACTTATATGAAGGAAGCATTTGGAAATGAAGATCGCAGACTCCGGACTTCTTGGTGA
TAGAGAAAACGTTTTAGTAACTGAATATACCTTCTTCAACCTTACTTCTAGCGTCTGAGGCTGAAGAAGACGACGT
GENE=EAT-4CDNA

1210    1220    1230    1240    1250    1260    1270    1280
ATCCCCATTTGGTAATGGGATGCGTGGTCTTATGGTGGACAACTCGCTGATTATCTCCGATCCAAACAAGATCCTTTT
TAGGGGTAAACCATTACCCTACGCACCAAGAAATACCACCTGTTGAGCGACTAATAGAGGCTAGGTTGTTCTAGGAAAG
GENE=EAT-4CDNA

1290    1300    1310    1320    1330    1340    1350    1360
CACCACGCGTTCGGAAGATTTTCAATTTGGTGGTGTGGGAGGAAAGTGCATTCGTAATTTGTTGCATATACAA
GTGGTGACGGCAAGCCTTCTAAAAGTTAAACACCACCAAAACCTCTCTTCGACGTAAAGTACGATTAACACGCTATATGTT
GENE=EAT-4CDNA

1370    1380    1390    1400    1410    1420    1430    1440
CAAGTGATCAACTGCTATAATGGCATTGATTCGCTGTTGGAAATGCTGGAATTTCTTGGTTTCAACGTAAT
GTTCTACTATGTTGACGATATTACCCTAACACGACGACACCTTACAGACCTAAACGATAAAGACCAAAGTGTCAATTTA
GENE=EAT-4CDNA

1450    1460    1470    1480    1490    1500    1510    1520
CATTTGGACATTTGCCCGGTTATGACGACCTCAATGGGATTCGGAATGGAATCGGAACACTTGGCGGATTAACCTTG
GTAAACCTGTAAAGGGGCAATACGTCGGTAGGATTACCTAAGAGCTTACCTTAGCCTTTGGAACGGCCATTTGAAC
GENE=EAT-4CDNA

1530    1540    1550    1560    1570    1580    1590    1600
CCCCATTTGACTGAAGCATTACGCTGCTACTGAAACCGGATGGACGAGTGTGTTTCTTATTAGCCAGTCTTATCTCACT
GGGTAAACACTGACTTCTGAAGTGCAGGAGTGAAGCTTTGCTACTCTGCTCAAAAAGAAATAACTCGTCAGAATAAGTGA
GENE=EAT-4CDNA

1610    1620    1630    1640    1650    1660    1670    1680
TTACTGGAGTCACTTTTTATGCTGCTATGCTTCCGGAGAATTCGAAGAATGGGCAAGCAAAAAGAGGAGGAAGATGG
AATGACCTCAGTGAATAACAGCAGATACGAGGCTCTTGAAGTTCTTACCCGCTTGGTTTTCTCTCTCTTCTTACC
GENE=EAT-4CDNA

1690    1700    1710    1720    1730    1740    1750    1760
TCGAATAAGGAATTTGGTGAATAAACTGGAATCAATGGAACTGGATACGGAGTCTGAGACTACATTCACACAGTACC
AGCTTATTTCTTAACCACTTATTTGACTTAGTACTCTTACCTTACCTGCTGCTCAAAAAGAAATAACTCGTCAGAATA
GENE=EAT-4CDNA

1770    1780    1790    1800    1810    1820    1830    1840
AGCCGGTGTGATTTCTTATCAAGCTCAGGCGGCTCCAGCTCCTGGAACCAACCCGTTCCGCTCAGCTTGGGATGAAC
TCGGCCCAACTAAGAAGAATAGTTCGAGTCCGCCAGGTCGAGGACTTGGTTGGGCAAGCGAAGTCAACCTTACTTG
GENE=EAT-4CDNA

1850    1860    1870    1880    1890    1900    1910    1920
ATGGATCCTCGGAGTTGTGGAAAATCCGCTATACAGCAGTGGTAGGATATCTGAGCTCCGATCGGCGGCTGTCATCA
TACCTAGGAGACTCAACACTTTTAGGCGTAATAGTCTGACCATCTTATAGCTCAGGCGTACGCGGCGACAGTAGT
GENE=EAT-4CDNA

1930    1940    1950    1960    1970    1980    1990    2000
GATCGCCATCTCGCGCCGCTCTGACTTCTAAGTCCAATACTTCAACATCCCTACATGCTCTTCTCCCTGTGC
CTAGCGGTAGAGCGCGGGCACGGAGACTGAAGATTCAGTTAATGAGAAAGTTGAGGATGTACGAGAAAGAGGGACAGC
NOTE=3' UTR OF UNC-54 VER. PPD49.26

2010    2020    2030    2040    2050    2060    2070    2080
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AGGGTGGGGATAAAAACAATAAGTTTTTTGGAAGAAGAAATTAAGAAACAAAATCGAAGAAAATTCAGTGGAGAT
NOTE=3' UTR OF UNC-54 VER. PPD49.26

2090    2100    2110    2120    2130    2140    2150    2160
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NOTE=3' UTR OF UNC-54 VER. PPD49.26

2170    2180    2190    2200    2210    2220    2230    2240
ATAATAATCTATCCAAAATCTACAAATGTTCTGTGTACACTTCTATGTTTTTTTACTTCTGATAAATTTTTTTG
TATTATTAAGTAGGGTTTTAGATGTTTACAAGACACATGTGGAAGAAATCAAAAATAAAGAGACTATTTAAAAA
NOTE=3' UTR OF UNC-54 VER. PPD49.26

2250    2260    2270    2280    2290    2300    2310    2320
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AAACATCATAGAAAAACCGCACAAAAACCTTATCATATGTTACGTTTCAGTTTTATGACCGCAATTTTTATTTCTT
TTTGAGTATCTTTTTGGCGTGTGTTTTATGGAAATGATACAAAGTCAAACTGCGGCTAAAAATAAAGAAG
NOTE=3' UTR OF UNC-54 VER. PPD49.26 >
2330 2340 2350 2360 2370 2380 2390 2400
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CGTGCAGACCCGGAGAGTACTGACAGTTAGTACAGTAGTACTTTTTCAAACCTCATAAAAAACCTTAAAAAGTTAGTTC
NOTE=3' UTR OF UNC-54 VER. PPD49.26 >
2410 2420 2430 2440 2450 2460 2470 2480
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ACTTTCAAATCTTAATTAAGGAGCAAAACGAAAAACCCCAAGGGGATAACAACAGTTCTCAAAGCTCTCCGCC
NOTE=3' UTR OF UNC-54 VER. PPD49.26 >
2490 2500 2510 2520 2530 2540 2550 2560
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CAAAAAGAACGATTTTAGTGTTTCAACTACTGCTGCTACTGTTCTTCTAGCCTTCTTCCAAACCAAACTCCGAGTCA
NOTE=3' UTR OF UNC-54 VER. PPD49.26 >
2570 2580 2590 2600 2610 2620 2630 2640
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CTTCCACTCATCTTCAACTTAACTTCCACTCATCAGATACCCAAAAACGGAATTAATGCTTATGTAAGGTT
NOTE=3' UTR OF UNC-54 VER. PPD49.26 >
2650 2660 2670 2680 2690 2700 2710 2720
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NOTE=3' UTR OF _____ >
2730 2740 2750 2760 2770 2780 2790 2800
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2810 2820 2830 2840 2850 2860 2870 2880
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2890 2900 2910 2920 2930 2940 2950 2960
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2970 2980 2990 3000 3010 3020 3030 3040
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3050 3060 3070 3080 3090 3100 3110 3120
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3130 3140 3150 3160 3170 3180 3190 3200
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3210 3220 3230 3240 3250 3260 3270 3280
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3290 3300 3310 3320 3330 3340 3350 3360
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3370 3380 3390 3400 3410 3420 3430 3440
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3450 3460 3470 3480 3490 3500 3510 3520
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3530 3540 3550 3560 3570 3580 3590 3600
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3610 3620 3630 3640 3650 3660 3670 3680
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3690 3700 3710 3720 3730 3740 3750 3760
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3770 3780 3790 3800 3810 3820 3830 3840
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3850 3860 3870 3880 3890 3900 3910 3920
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4010 4020 4030 4040 4050 4060 4070 4080
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4090 4100 4110 4120 4130 4140 4150 4160
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4170 4180 4190 4200 4210 4220 4230 4240
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4250 4260 4270 4280 4290 4300 4310 4320
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4330 4340 4350 4360 4370 4380 4390 4400
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4490 4500 4510 4520 4530 4540 4550 4560
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4570 4580 4590 4600 4610 4620 4630 4640
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4650 4660 4670 4680 4690 4700 4710 4720
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4730 4740 4750 4760 4770 4780 4790 4800
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4810 4820 4830 4840 4850 4860 4870 4880
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4890 4900 4910 4920 4930 4940 4950 4960
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4970 4980 4990 5000 5010 5020 5030 5040

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5050 5060 5070 5080 5090 5100 5110 5120
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5130 5140
GATAACAATTTACACAGGAAACAGCT
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pNR57 (*avr-14a* cDNA)

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90 100 110 120 130 140 150 160
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/NOTE=3' UTR OF UNC-54 >
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1690 1700 1710 1720 1730 1740 1750 1760
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1770 1780 1790 1800 1810 1820 1830 1840
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/NOTE=3' UTR OF UNC-54 >
1850 1860 1870 1880 1890 1900 1910 1920
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/NOTE=3' UTR OF UNC-54 >
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2010 2020 2030 2040 2050 2060 2070 2080
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2170 2180 2190 2200 2210 2220 2230 2240
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2330 2340 2350 2360 2370 2380 2390 2400
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2410 2420 2430 2440 2450 2460 2470 2480
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2490 2500 2510 2520 2530 2540 2550 2560
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2570 2580 2590 2600 2610 2620 2630 2640
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2650 2660 2670 2680 2690 2700 2710 2720
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2730 2740 2750 2760 2770 2780 2790 2800
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2970 2980 2990 3000 3010 3020 3030 3040
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3210 3220 3230 3240 3250 3260 3270 3280
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3290 3300 3310 3320 3330 3340 3350 3360
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3370 3380 3390 3400 3410 3420 3430 3440
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pNR59 (*avr-14b* cDNA)

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pNR94 (*mgl-3* cDNA)

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/GENE=MGL-3 CDNA

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CGTGCAGCTCGACTTTTACCTTAAACATAACTACTCTACATGCTCTTAAAGTCTTCTACCTGACTCTTTTAAAGTC
/GENE=MGL-3 CDNA

970     980     990     1000    1010    1020    1030    1040
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/GENE=MGL-3 CDNA

1050    1060    1070    1080    1090    1100    1110    1120
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/GENE=MGL-3 CDNA

1130    1140    1150    1160    1170    1180    1190    1200
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ACCCATAGTTTGTAGTCAACAGGCACTAACCTGTAGCTTGTATGCTCGTGTAGTGTAAAGAGGTTACCATGCTCT
/GENE=MGL-3 CDNA

1210    1220    1230    1240    1250    1260    1270    1280
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TCTTTGACCTATAAACCTTATAAAGCTAGTGAAGGGGTTTTCTCAAACAAAAGAACCTTCTCAAAGCTCATAGAGC
/GENE=MGL-3 CDNA

1290    1300    1310    1320    1330    1340    1350    1360
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CTACAAGCCGGTACATCTACAGTCTGAAAACCTTAAACAAAATACCAAAATTAATGAAGCTTTGTCCAAATGTAG
/GENE=MGL-3 CDNA

1370    1380    1390    1400    1410    1420    1430    1440
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/GENE=MGL-3 CDNA

1450    1460    1470    1480    1490    1500    1510    1520
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/GENE=MGL-3 CDNA

1530    1540    1550    1560    1570    1580    1590    1600
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/GENE=MGL-3 CDNA

1610    1620    1630    1640    1650    1660    1670    1680
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/GENE=MGL-3 CDNA

1690    1700    1710    1720    1730    1740    1750    1760
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/GENE=MGL-3 CDNA

1770    1780    1790    1800    1810    1820    1830    1840
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/GENE=MGL-3 CDNA

1850    1860    1870    1880    1890    1900    1910    1920
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/GENE=MGL-3 CDNA

1930    1940    1950    1960    1970    1980    1990    2000
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/GENE=MGL-3 CDNA

2010    2020    2030    2040    2050    2060    2070    2080
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CTTAGCTAGCTGTGATAAACAACAGAGCCAAAGAAATTTTAAAGGTTATGAGGACACTATTACGATAGACCTTCTCT
/GENE=MGL-3 CDNA

2090    2100    2110    2120    2130    2140    2150    2160
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/GENE=MGL-3 CDNA

2170    2180    2190    2200    2210    2220    2230    2240
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TACGAGTTACTGAGCCTAAGATTACAGATAGTTACAGCTCGGTAATACGCTGTTAGTGTGTTTTGATTAGCTGAAC
/GENE=MGL-3 CDNA

2250    2260    2270    2280    2290    2300    2310    2320
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 /GENE=MGL-3 CDNA >

2330 2340 2350 2360 2370 2380 2390 2400
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 /GENE=MGL-3 CDNA >

2410 2420 2430 2440 2450 2460 2470 2480
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 /GENE=MGL-3 CDNA >

2490 2500 2510 2520 2530 2540 2550 2560
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 /GENE=MGL-3 CDNA >

2570 2580 2590 2600 2610 2620 2630 2640
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 /GENE=MGL-3 CDNA >

2650 2660 2670 2680 2690 2700 2710 2720
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 /GENE=MGL-3 CDNA >

2730 2740 2750 2760 2770 2780 2790 2800
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2810 2820 2830 2840 2850 2860 2870 2880
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 /GENE=MGL-3 CDNA >

2890 2900 2910 2920 2930 2940 2950 2960
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 /GENE=MGL-3 CDNA >

2970 2980 2990 3000 3010 3020 3030 3040
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 /GENE=MGL-3 CDNA >

3050 3060 3070 3080 3090 3100 3110 3120
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3130 3140 3150 3160 3170 3180 3190 3200
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3210 3220 3230 3240 3250 3260 3270 3280
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3290 3300 3310 3320 3330 3340 3350 3360
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3370 3380 3390 3400 3410 3420 3430 3440
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3' UTR OF UNC-54 >

3450 3460 3470 3480 3490 3500 3510 3520
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3530 3540 3550 3560 3570 3580 3590 3600
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3610 3620 3630 3640 3650 3660 3670 3680
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3690 3700 3710 3720 3730 3740 3750 3760
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3850 3860 3870 3880 3890 3900 3910 3920
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3930 3940 3950 3960 3970 3980 3990 4000
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4010 4020 4030 4040 4050 4060 4070 4080
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4090 4100 4110 4120 4130 4140 4150 4160
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4250 4260 4270 4280 4290 4300 4310 4320
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4330 4340 4350 4360 4370 4380 4390 4400
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4410 4420 4430 4440 4450 4460 4470 4480
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4490 4500 4510 4520 4530 4540 4550 4560
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4570 4580 4590 4600 4610 4620 4630 4640
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4650 4660 4670 4680 4690 4700 4710 4720
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4730 4740 4750 4760 4770 4780 4790 4800
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4810 4820 4830 4840 4850 4860 4870 4880
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4890 4900 4910 4920 4930 4940 4950 4960
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4970 4980 4990 5000 5010 5020 5030 5040
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5050 5060 5070 5080 5090 5100 5110 5120
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5130 5140 5150 5160 5170 5180 5190 5200
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5210 5220 5230 5240 5250 5260 5270 5280
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5290 5300 5310 5320 5330 5340 5350 5360
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5370 5380 5390 5400 5410 5420 5430 5440
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5450 5460 5470 5480 5490 5500 5510 5520
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5530 5540 5550 5560 5570 5580 5590 5600
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5610 5620 5630 5640 5650 5660 5670 5680
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5690 5700 5710 5720 5730 5740 5750 5760
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5770 5780 5790 5800 5810 5820 5830 5840
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5850 5860 5870 5880 5890 5900 5910 5920
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6010 6020 6030 6040 6050 6060 6070 6080
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6090 6100 6110 6120 6130 6140 6150 6160
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6250 6260 6270 6280 6290 6300 6310 6320
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6330 6340 6350 6360 6370 6380 6390 6400
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6490 6500 6510 6520 6530 6540 6550 6560
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6570 6580 6590 6600 6610 6620 6630 6640
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6650 6660 6670 6680 6690 6700 6710 6720
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6730 6740
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pNR56 (*glc-3* cDNA)

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10      20      30      40      50      60      70      80
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90      100     110     120     130     140     150     160
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      /GENE=GLC-3 CDNA >

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      /GENE=GLC-3 CDNA >

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      /GENE=GLC-3 CDNA >

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      /GENE=GLC-3 CDNA >

410     420     430     440     450     460     470     480
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      /GENE=GLC-3 CDNA >

490     500     510     520     530     540     550     560
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      /GENE=GLC-3 CDNA >

570     580     590     600     610     620     630     640
GCTAATTCGAATTCATAGAGAGGGTGAATCTTTTACTCGGTTTCAATTTTCTGATGGTTTGTCTGCTCGGATGCAATTC
CGATTAAGCTTAAATGATCTCTGCCAGCTTAAAGAAATAGGCAAGCTTAAAGCTACAAAACAGTACAGGCTACGTAATG
      /GENE=GLC-3 CDNA >

650     660     670     680     690     700     710     720
AATACTATCCAATGGATGTTCAAACGTGCTGATTGATCTTGCCTTACGCATATACTGAAAATGATATCGAGTATCGA
TTATGATAGGTTACCTACAGTTTGCACAGACTAAGTACGAGGAGGAGTGGTATGACTTTTACTATAGCTCATAGCT
      /GENE=GLC-3 CDNA >

730     740     750     760     770     780     790     800
TGGAGAAGACTGATCCAGTTCAATTGAAGAAAGGACTGCATTCTTCACTTCCAAGTTTGAACCTAATAATGTTGATAC
ACCTTCTTCTGACTAGGCAAGTTAACTCTTTCTGACGTAAGAAGTGAAGGTTCAAAACTTGAATTTATACAACATG
      /GENE=GLC-3 CDNA >

      >/note=A_to_G_silent
810     820     830     840     850     860     870     880
AACTTTATGACGAGTAAAACAAATACAGGGCTTACTTCTGCTACGAACAGTCTTGGAACTGAGACGACAGTTCAGTT
TTGAAATACATGCTCATTTTGTATGTCCTGAATGAGGACAGATGCTTGTGCAAGCTTACTGCTGTCAGTCAAGTCAA
      /GENE=GLC-3 CDNA >

890     900     910     920     930     940     950     960
ATTATCTTCTCAATTATATATTCCATCAACATGTTAGTATGCTCTATGGGATATGTTTGGCTAGACAGAGTGTCT
TAATAGAAGAAGTAAATATAAAGTATGTTGTTACAACTACAGAGTACCCATAGCAAACCGATCTGCTCCACGA
      /GENE=GLC-3 CDNA >

970     980     990     1000    1010    1020    1030    1040
GTCCCGCAGCAGTCACTTTGGGAGTAACTACTCTACTACAATGACACACAGGCTCCGGAAATCAACGCCAAACTCC
CAGGGGCTGCTCAGTGAACCTCATTTGATGAGATGAGTGTACTGGTGTCCGAGGCTTACTGCGGTTTGAAGG
      /GENE=GLC-3 CDNA >

1050    1060    1070    1080    1090    1100    1110    1120
ACCGGTCATACACAAAAGCGATCGATGTTGGATTGGCGCTGCTTACTTTTATTTTGGTCTTTATTAGAATTTG
TGGCCAGAGTATGTTTTCGCTAGCTACAAACCTAACCGGGACGGAATGAAAATAAAAACACGAAATAATCTTAAAC

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      /GENE=GLC-3 CDNA >
1130    1140    1150    1160    1170    1180    1190    1200
CATGGGTAACCTACATATCTGCGAGGAGTTTTTATAAACGAAATAAAAACGCTCAAGTCAAATTCACCTATTGATCGAA
GTACCCATTGAATGTATAGCAGCTCTCAAAAATTTGCTTTATTTTGGACGAGTTCAGCTTTAAGTGATAACTAGCTT
      /GENE=GLC-3 CDNA >

1210    1220    1230    1240    1250    1260    1270    1280
ACAAAACAGGCACTGATTATTCGAAACACAGTGGTAGCTCAGTTTCCAGAGCATCCCGAGGAAGTGGAAATGGGGTTGGC
TGTTTTGCTGCTGACTAATAAGGCTTGTGTCACCATCGATCAAAGTCTCGTAGGGCTCTTACCCTTACCCCAACCG
      /GENE=GLC-3 CDNA >

1290    1300    1310    1320    1330    1340    1350    1360
TCAGGCTCCAGATGTTTGGATTCGACAGTCTGAAATGAAAACCTGTAAGTAGAGTAAATGGGCATATCAATCAACA
AGTCCGAGGCTCAAAAACCTAAGCTGTGACAGCTTACCTTTTGGACATTCATCTATTACCCGTATAGTTAGTGTGT
      /GENE=GLC-3 CDNA >

1370    1380    1390    1400    1410    1420    1430    1440
ATGATGAGGCGCGGAGCTCATCTTTTTCAGCCAAACGACAAAATCGCCGATTTGTTGGTGGACTAACTTTCGAAAT
TACTACTCCGGCGCTCGAGTAGTAAAAACTGCGGTTGCTGTTTTAGCCGCTAAACAAACACCTGATTGAAAGCTTTA
      /GENE=GLC-3 CDNA >

1450    1460    1470    1480    1490    1500    1510    1520
ATTGCACTAATAGATGGATTGAAATCGATTAAGTTGATGATGATGAAAGGCGAAAGGGCGGATTTAATATCAAGAGCTCT
TAAGCTGATTAATCTACTAAGCTTTAGCTAATTTTCAACTACTATTGCGCTTTTCCGCCCTAAATATAGTTCTCAGGA
      /GENE=GLC-3 CDNA >

1530    1540    1550    1560    1570    1580    1590    1600
ATTTCTACTCTCTCGTAGTGTTCACACTTTGCTACTCGGACCAAGTATTACAATATCACGCCACCGAAAGCCAAATGAG
TAAAGGATGAGAGAAGCATACAAAGTTGAAACAGATGACCTGGTTCATAGGTTATAGTCCGCTGGCCTTCGGTTTACT
      /GENE=GLC-3 CDNA >

1610    1620    1630    1640    1650    1660    1670    1680
GTACCATGGTATTGATATCTGAGCTCCGATCGGCGCTGTCAATCAGTCCGATCCGCTCCGCGCCGCTGCTGACTTCTA
CATGGTACCATACTATAGACTCGAGGCGTAGCGGCGACAGTAGTCTAGCCGTAGAGCGCGGCGCAGGAGACTGAAGAT
      /NOTE=3' UTR OF UNC-5 >

1690    1700    1710    1720    1730    1740    1750    1760
AGTCCAATTACTTCAACATCCCTACATGCTCTTCTCCCTGCTGCTCCACCCCTATTTTGTATTATCAAAAAAC
TCAGGTTAATGAGAAGTTGTAGGGATGACGAGAAGAGGCAACGAGGTTGGGGATAAAAAACAATAATAGTTTTTTTG
      /NOTE=3' UTR OF UNC-5 >

1770    1780    1790    1800    1810    1820    1830    1840
TTCTCTTAAATTTCTTTGTTTTTGTAGTTCTTTTAACTGACCTTCAACAATGAAATGTTGATGATTCAAAAATAGAATTA
AAGAAGAATTAAGAAACAAAAATCGAAGAAATTCAGTGGAGATGTTACTTTAACACATCAAGTTTTTATCTTAAT
      /NOTE=3' UTR OF UNC-5 >

1850    1860    1870    1880    1890    1900    1910    1920
ATTGTAATAAAAAAGTGAAAAAAATGTCCTCCCTCCCCCTTAAATAAATTCATCCCAAAATCTACACATGTTCT
TAAGCATATTTTTTTCAGCTTTTTTAAACAGAGGGAGGGGGTAAATTTATTAAGATAGGGTTTTAGATGTTTACAAG
      /NOTE=3' UTR OF UNC-5 >

1930    1940    1950    1960    1970    1980    1990    2000
TGTGTACACTTCTATGTTTTTTTTTCTTCTGATAAATTTTTTGAACATCATAGAAAAACCGCACACAAATACCT
ACACATGTGAAGAATCAAAAAAATGAAGACTTTTAAAAAACTTTGATGATCTTTTTTGGCGTGTGTTTTATGGA
      /NOTE=3' UTR OF UNC-5 >

2010    2020    2030    2040    2050    2060    2070    2080
TATCATATGTTACGTTTTCAGTTTATGACCGCAATTTTATTTCTGCACTGCGGCTCTCATGACGTCAAATCATGC
ATAGTATACAATGCAAAAGTCAAATCTGGGTTTAAAAAATAAGAAGCTGAGACCCGGAGAGTACTGCAAGTTAGTACG
      /NOTE=3' UTR OF UNC-5 >

2090    2100    2110    2120    2130    2140    2150    2160
TCATCGTGA AAAAGTTTGGAGTATTTTTGGAATTTTTCAATCAAGTGAAGTTTATGAAATTTTCTGCTTTTTC
AGTAGCACTTTTCAAAACCTCATAAAAACCTTAAAAAGTTAGTTCACTTTCAAAATCTTTAATTAAGGACGAAAACG
      /NOTE=3' UTR OF UNC-5 >

2170    2180    2190    2200    2210    2220    2230    2240
TTTTTGGGGTTTTCCCTATGTTTGTCAAGGATTCGAGGACGGGTTTTTCTTGTCAAAATCAAGATTTGATGAGC
AAAAACCCCAAGGGGATAACAACAGTCTCAAAGCTCTGCCGCAAAAAGAACGATTTTTAGTGTTCATAACTACTCG
      /NOTE=3' UTR OF UNC-5 >

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2250 2260 2270 2280 2290 2300 2310 2320
ACGATGCAAGAAAGATCGGAAGAAGGTTTGGGTTTGGGCTCAGTGGAAAGGTGAGTAGAAGTTGATAATTTGAAAGTGGA
TGCTACGTTCTTTCTAGCTCTTCCAAACCCAACTCCGAGTCCCTCCACTCATCTTCAACTATTAACCTTTACCT
/NOTE=3' UTR OF UNC-54 >

2330 2340 2350 2360 2370 2380 2390 2400
GTAGTGTCTATGGGGTTTTGCTTAAATGACAGAATACATTCCTCAATACCAAACTAAGTGTTCCTACTAGTCGGC
CATCACAGATACCCCAAAACCGAATTACTGTCTTATGTAAGGTTATATGTTGTTGATTGACAAAAGATGATCAGCGG
/NOTE=3' UTR OF UNC-54 >

2410 2420 2430 2440 2450 2460 2470 2480
CGTACGGGCCCTTTCGTCTCGCGCTTTCGGTGTAGCGGTGAAACCTCTGACACATGCAGCTCCCGGAGACGGTCA
GCATGCCGGGAAAGCAGAGCGCCAAAGCCACTACTGCCACTTTTGGAGACTGTGTACGCTCGAGGGCTCTGCGCAGTG

2490 2500 2510 2520 2530 2540 2550 2560
GCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCTCAGGCGCGCTCAGCGGGTGTGGCGGGTCTGGGGGTGGCT
CGAACAGACATTCCGCTACGGCCCTCGTCTGTTGGCGAGTCCCGCGCAGTCGCCCAACCCGCCACAGCCCCGACCGA

2570 2580 2590 2600 2610 2620 2630 2640
TAACATGCGGCATCAGAGCAGATTGACTGAGAGTGCACCATATCGGGTGTGAAATACCGCAGAGATGCGTAAGGAGAA
ATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACGTGGTATACGCCACACTTTATGGCGTGTCTACGACTCTCTTT

2650 2660 2670 2680 2690 2700 2710 2720
AATACCGCATCAGGCGGCCCTTAAGGGCCTCGTGTACGCCCTATTTTATAGGTTAATGTCATGATAATAAGTTTCTTA
TTATGGCGTAGTCCGGCAATTCCTGGAGCATTGCGGATAAAAATATCCAATACAGTACTATTATACCAAAGAAAT

2730 2740 2750 2760 2770 2780 2790 2800
GACGTGAGTGGCCATTTTCGGGAAATGTGCGGGAACCCCTATTTGTTTATTTTCTAAATACATTTCAAATATGATC
CTGCAGTCCACCGTGAAAGCCCTTTACAGCGCCCTTGGGGATAAAAATAAAAAGATTTATGTAAGTTTATACATAG

2810 2820 2830 2840 2850 2860 2870 2880
CGCTCATGAGACAATAACCTTGATAAATGCTTCAATAATATGAAAAGGAAGATAGTATGAGTATCAACATTTCCGTGTC
GCGAGTACTCTGTTATTGGGACTATTAGCAAGTATTATAACTTTTTCTCTCATCTCATAGTTGTAAAGGCACAG

2890 2900 2910 2920 2930 2940 2950 2960
GCCCTTATCCCTTTTTTGGGGCATTTCGCTTCTGTTTGTCTCACCAAGAAAGCGTGTGAAAGTAAAGATGCTGA
CGGAAATAAGGAAAAAAGCCGTAACCGAAAGCAGAAACAGTGGGTTCTTTCGACCACTTTCATTTTCTACGACT

2970 2980 2990 3000 3010 3020 3030 3040
AGATCAGTTGGGTGCAGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCTTGGAGATTTTTGCCCCGAAG
TCTAGTCAACCCAGTGTCTACCAATGTAGCTGACTAGAGTTGTCCGCTTAGGAACTCTCAAAAGCGGGCTTC

3050 3060 3070 3080 3090 3100 3110 3120
AACGTTTTCAATGATGAGCAGTTTTAAAGTTCTGTGATGCGCGGATATTACCCGATTGACCGCGGGAAGAGCA
TTGCAAAAGTTACTACTCGTGAATAATTAAGACGATACACCGGCCATAATAGGGCATAACTGCGGCCGCTTCTCGTT

3130 3140 3150 3160 3170 3180 3190 3200
CTCGTCCGCGCATACACTATTCTCAGAATGACTGGTTGAGTACTCACAGTACACAGAAAAGCATCTTACGGATGGCAT
GAGCCAGCGCGTATGTGATAAGAGTCTTACTGAAACCACTCATGAGTGGTCAAGTGTCTTTCTGAGAATGCCTACCGTA

3210 3220 3230 3240 3250 3260 3270 3280
GACAGTAAGAGAATTATGCACTGTGCCATAACCATGAGTGATAAACAATGCGGCCAATTTACTTCTGACAACGATCGGAG
CTGTACTCTCTTAATAGCTCAGCAGCGTATTGGTACTCACTATTGTGACGCGGTTGAATGAAGACTGTTGCTAGCCTC

3290 3300 3310 3320 3330 3340 3350 3360
GACCAAGGAGCTAACCGCTTTTTGACAACTATGGGGATCATGTAACCTCCCTGATCTGTTGGGAACCGGAGCTGAAT
CTGGCTTCTCGATTGGCGAAAAACGTGTTGACCCCTAGTACTATTAGCGGAACCTAGCAACCTTTGGCTCGACTTA

3370 3380 3390 3400 3410 3420 3430 3440
GAAGCCATAACCAACGACGAGCTGACACCAAGTCCGTAGCAATGGCAACAAGTTGCGCAACTATTAACGGCGA
CTTGGTATGGTTTGTCTCGCACTGTGGTGTACCGACATCTGTTACCGTTGTTGCAACGCGTTGATAATTGACCGCT

3450 3460 3470 3480 3490 3500 3510 3520
ACTACTACTTAGCTTCCCGCAACAATAAGACTGGATGGAGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGG
TGATGAATGAGATCGAAGGGCCCTGTTAATTAATCTGACTACCTCCGCTATTTCAACGCTCTGGTGAAGACGGAGCC

3530 3540 3550 3560 3570 3580 3590 3600
CCCTTCCGGCTGGCTGTTTATTGCTGATAAATCTGGAGCCGGTGAAGCGGCTCGCGGTATCATTGACGACTGGGG
GGGAAAGCCGACCAAAATACGACTATTAGACTCTGGCCACTCGACCCAGAGCGCCATAGTAACGCTGTAACCT

3610 3620 3630 3640 3650 3660 3670 3680
CCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACAGCAGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGAT
GGTCACTACCTCGGGAGGGCATAGCATCAATAGATGTGCTGCCCTCAGTCCGTTGATACCTACTGCTTTTACTGTCTA

3690 3700 3710 3720 3730 3740 3750 3760
CGCTGAGATAGGTCCCTCAGTATTAAGCATTGGTAACCTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAA
GCGACTCTACCCAGGAGTACTAATTCGTAACCATTTGACAGTCTGGTTCAAATGAGTATATGAAACTAACAATAATT

3770 3780 3790 3800 3810 3820 3830 3840
AATCTCATTTTAAATTTAAAAGGATCTAGTGAAGATCCTTTTGATAATCTCATGACCAAAATCCCTTAACTGAGTTT
TTGAAGTAAAAATTAATTTTCTAGATCCACTCTAGGAAAAACTATTAGAGTACTGGTTTTAGGGAAATGCACTCAA

3850 3860 3870 3880 3890 3900 3910 3920
TCGTTCCACTGAGCGTACAGCCCGTAGAAAAGTCAAAAGTCTTCTTGAGATCCTTTTTCGCGGTAATCTGCTG
AGCAAGGTGACTCGAGTCTGGGGCATCTTTCTAGTTTCTAGAAAAGCTTAGGAAAAAAGACCGCCATTAGACGAC

3930 3940 3950 3960 3970 3980 3990 4000
CTTGAACAACAAAAACACCCTACAGCGGTGGTTGTTGCCGATCAAGAGCTACCAACTCTTTTCCGAAAGTAA
GAACGTTGTTTTTTTGGTGGCGATGGTCCCAACCAACCAACCGCCATAGTTCTGATGGTTGAGAAAAAGGCTCCATT

4010 4020 4030 4040 4050 4060 4070 4080
CTGGCTTACAGCAGAGCGGATACCAAACTAGTCTCTAGTGTAGCCGATGTTAGGCCACCACTTCAAGAACTCTGTA
GACCGAAGTCTGCTCGGCTACTAGTTTATGACAGGAAGTACACATCGGCATCAATCCGGTGGTGAAGTTCTTGAGCAT

4090 4100 4110 4120 4130 4140 4150 4160
GCACCGCTACATACCTCCGCTGCTAATCCTGTACAGTGGCTGCTGCCAGTGGCGATAGTCTGCTTACCGGGTT
CTGTTGGGATGATGTAGGAGCGAGCATAGGACAATGGTCCACGACGAGCGTCCAGCTTTTACGACGAAATGGCCCAA

4170 4180 4190 4200 4210 4220 4230 4240
GGACTCAAGCAGATAGTTACCGGATAAGGCGCAGCGGCTGGGCTGAACGGGGGGTTGTGCAACAGCCAGCTGTGCGGGTCAACCTCG
CTGAGTTCTGCTATCAATGGCTATTCCGCGTCCGACGCCAGCTGCCCCCAAGCAGCTGTGTCGGGTCAACCTCG

4250 4260 4270 4280 4290 4300 4310 4320
GAACGACCTACACCGAAGTACAGTACCTACAGCGTGAAGTGTGAGAAAAGCCACGCTTCCGAAAGGAGAAAGCGGAC
CTTGTGGATGTGGCTGACTCTAGGATGTGCACTGTAACCTTTCCGCGTGGGAAGGCTTCCCTTTCCCGCTG

4330 4340 4350 4360 4370 4380 4390 4400
AGGTATCCGGTAAGCGGCGAGGTCGGAACAGGAGAGCGCAGGAGGCTTCCAGGGGAAACGCTGGTATCTTTATAG
TCCATAGGCCATTCCGCTCCAGCTTGTCTCTCGCGTCTCCCTCGAAGGTCCTTTTCCGACCAAGAAATATC

4410 4420 4430 4440 4450 4460 4470 4480
TCCTGTCGGGTTTCCGCACTTGTACTGAGCGTCAATTTTGTGATGCTCGTACGGGGGGGAGGCTATGGAAAAAG
AGGACAGCCCAAGCGGTTGAGACTGAACTCGCAGCTAAAACACTACGAGCAGTCCCCCGCTCGGATACCTTTTTCG

4490 4500 4510 4520 4530 4540 4550 4560
CCAGCAACCGGCTTTTACGGTCTTGGCTTTTGTGGCTTTTGTGCTACATGTTCTTTCTGCTTATCCCTGTAT
GGTCTTGTCCGCAAAATGCAAGGACCGAAAACGACCGAAAACGAGTGTACAAAAGAGGACGCAATAGGGACTA

4570 4580 4590 4600 4610 4620 4630 4640
TCTGTGATAACCGTATTACCGCTTTGAGTGAAGTATACCGCTCGCCGACCGAAGCAGCGAGCGAGCTAGT
AGACACTATTGGATAATGGCGAAACTCACTGACTATGGCAGCGGCTCGGCTGCTGGCTCGCTCAGTCA

4650 4660 4670 4680 4690 4700 4710 4720
GAGCGAGGAAGCGGAAGAGCGCAATACGCAACCGCTCTCCCGCGCTTGGCGATTCTTAATGACAGCTGGCAGC
CTCGCTCTTCCGCTTCTCGCGGTTATGCGTTTGGCGGAGGGGGCGCAACCGCTAAGTAATACGCTGACCGCTG

4730 4740 4750 4760 4770 4780 4790 4800
ACAGGTTTCCGACTGGAAGCGGGCAGTGAAGCAACGCAATTAATGTAGTGTAGTCACTATTAGGCCACCCAGGCT
TGCCAAGGGCTGACTTTCCGCTTCACTCGCTTGTGTTAATACACTCAATCGAGTGAATCCGTTGGGTTCCGA

4810 4820 4830 4840 4850 4860 4870
TTACACTTTATGCTTCCGGCTCGTATGTTGTGGAATTTGAGCGGATAACAATTTACACAGGAACAACGCT
AATGTGAAATACGAGGGCGGACATAACAACACTTTAACACTCGCTCTGTTAAAGTGTGCTTTTGTCTGA

