A glial K⁺/Cl⁻ cotransporter modifies temperature-evoked dynamics in Caenorhabditis elegans sensory neurons

A. Yoshida†,1, S. Nakano†,1, T. Suzuki†,4,∗∗
K. Ihara§, T. Higashiyama†,4,§ and I. Mori†,†

†Division of Biological Science, Graduate School of Science, Science, JST ERATO Higashiyama Live-Holonics Project, §Center for Gene Research, †Institute of Transformative Bio-Molecules (ITbM), Nagoya University, Nagoya, Aichi, Japan, and ** Present address: College of Biocience and Biotechnology, Chubu University, Kasugai, Aichi, Japan

© 2015 The Authors.

K⁺/Cl⁻ cotransporters (KCCs) are known to be crucial in the control of neuronal electrochemical Cl⁻ gradient. However, the role of these proteins in glial cells remains largely unexplored despite a number of studies showing expression of KCC proteins in glial cells of many species. Here, we show that the Caenorhabditis elegans K⁺/Cl⁻ cotransporter KCC-3 is expressed in glial-like cells and regulates the thermosensory behavior through modifying temperature-evoked activity of a thermosensory neuron. Mutations in the kcc-3 gene were isolated from a genetic screen for mutants defective in thermotaxis. KCC-3 is expressed and functions in the amphid sheath glia that ensheathes the AFD neuron, a major thermosensory neuron known to be required for thermotaxis. A genetic analysis indicated that the regulation of the thermosensory behavior by KCC-3 is mediated through AFD, and we further show that KCC-3 in the amphid sheath glia regulates the dynamics of the AFD activity. Our results show a novel mechanism by which the glial KCC-3 protein non-cell autonomously modifies the stimulus-evoked activity of a sensory neuron and highlights the functional importance of glial KCC proteins in modulating the dynamics of a neural circuitry to control an animal behavior.

Keywords: AFD neuron, amphid sheath, behavior, Caenorhabditis elegans, glia, neural circuit, neuronal activity, potassium-chloride cotransporter, temperature sensing, thermotaxis

Received 15 July 2015, revised 30 September 2015, accepted for publication 8 October 2015
neural activity by the glial KCC-3 protein, and suggest a new mode of actions by KCC proteins that modulate the circuit dynamics and influence animal behaviors.

Materials and methods

Caenorhabditis elegans strains

Caenorhabditis elegans strains were grown as described (Brenner, 1974). N2 (Bristol) was the wild-type strain. Germline transformation experiments were performed as described (Mello et al., 1991). The following extrachromosomal arrays, integrants and mutations were generated in this study or otherwise indicated: LG I: nljIs24[gcya-6p::GCaMP3, gcya-8p::TagRFP]. LG II: kcc-3[nj90], nj94, nj100, tm3649, ok228] (tm3649 was provided by the National BioResource Project-C. elegans, and ok228 was provided by the C. elegans Gene Knockout Consortium). LG IV: gcya-23[nj37] (Inada et al., 2006), gcya-8[nj44] (Inada et al., 2006), gcya-18[nj38] (Inada et al., 2006). LG V: nljIs2[H13p::GFP, tx-3p::GFP] (Sasakura et al., 2005). LGX: nsls113[F16F9.3p::DT-A(653E), unc-122p::GFP] (Bacaj et al., 2008).

Extrachromosomal arrays: njEx921[hlh-17p::kcc-3 cDNA, ges-1p::GFP], njEx922[ptr-10p::kcc-3 cDNA, ges-1p::GFP], njEx972[hlh-17p::kcc-3 cDNA, ges-1p::GFP], njEx973[ptr-10p::kcc-3 cDNA, ges-1p::GFP], njEx975[VRM0637h12, ges-1p::GFP]. The complete list of the strains used in this study is described in Table S1, Supporting Information.

Isolation of nj90, nj94 and nj100

We mutagenized wild-type animals, and F2 progeny cultivated at 17°C were subjected to thermotaxis assays. nj90, nj94 and nj100 were isolated in animals that migrated to the 23°C region. Strains carrying each mutation were backcrossed at least twice.

Mapping of nj94

We crossed nj94 animals with the wild-type polymorphic CB4858 (Hillier et al., 2000) strain, isolated F2 animals carrying nj94 and identified crossover sites essentially as described (Wicks et al., 2001). We mapped nj94 to a 5.3-Mb interval between nucleotides 3,712,597 and 9,005,052 on LG II.

Molecular biology

We injected the fosmid VRM0637h12 that carries the kcc-3 locus into kcc-3[nj100] mutants at 10 ng/μl. To generate a kcc-3::gfp translational fusion (pAY37), we inserted a gfp-coding sequence into the 3′ end of the kcc-3-coding sequence flanked by the 3.9 kb 5′ upstream and the 2.2 kb 3′ downstream genomic sequence. We injected this plasmid at 10 ng/μl into kcc-3[tm3469] animals. To generate fig-1::kcc-3 cDNA (pAY28), hih-17p::kcc-3 cDNA (pAY30) and ptr-10p::kcc-3 cDNA (pAY34), we fused the 2.2, 2.5 and 0.3 kb promoter sequences of fig-1, hih-17 and ptr-10, respectively, to a kcc-3 cDNA attached with the unc-64 3′ UTR sequence. A kcc-3 cDNA clone (yk611h3) was provided by Yuji Kohara. We injected each of these plasmids at 10 ng/μl into kcc-3[tm3469] animals. A description of the plasmid constructs used is available from the authors.

Behavioral assays

Population thermotaxis (TTX) assays were performed as described (Ito et al., 2006). Briefly, animals cultivated at 17, 20 or 23°C were placed on the center of the assay plate with the temperature gradient of 17–23°C and were allowed to freely move for 60 min. The assay plate was divided into eight sections along the temperature gradient, and the number of the animals in each section was counted.

Chemotaxis assays were performed as previously described (Bargmann et al., 1993), except that assay plates contained slightly different medium (2% agar, 1 mM CaCl₂, 1 mM MgSO₄, 25 mM potassium phosphate at pH 6.0; 0.1% diacetyl and 0.5% benzaldehyde, diluted with ethanol, were used in the assays. The chemotaxis index was calculated according to Bargmann et al. (1993).

Imaging analyses

For calcium imaging, a single animal expressing GCAM3 (Tian et al., 2009) and TagRFP in AFD was placed on a 10% agar pad on a cover slip (Kim et al., 2013). Animals were immobilized by 0.1 μm polystyrene beads (Polysciences, Warrington, PA, USA) (Kim et al., 2013) and covered by another cover slip. The samples were placed onto a Peltier-based temperature controller (Tokai Hit, Fujinomiya, Japan) and imaged under Olympus BX61WI microscope equipped with the Dual-View optics system (Molecular Devices, Sunnyvale, CA, USA) to separate the fluorescence signals from GCaMP3 and TagRFP. The samples were initially kept at 14°C for 40 seconds and then subjected to a linear temperature increase to 24°C over 200 seconds followed by 90 seconds of incubation at 24°C. The fluorescence intensities were quantified by the MetaMorph imaging system (Molecular Devices).

For imaging the morphology of the AFD sensory ending, animals carrying nljIs2[H13p::GFP, tx-3p::GFP] were observed under Zeiss LSM880 equipped with the Airyscan unit. The AFD sensory endings were imaged with the super resolution mode.

Statistical analysis

The error bars in all figures indicate standard error of the mean (SEM). One-way analyses of variance (ANOVA) were performed, followed by multiple comparison tests using methods indicated in each figure legend. The double asterisks (**), single asterisks (*) and NS in all figures represent P < 0.01, P < 0.05 and not significant, respectively. The value of P is probability.

Results

Isolation of kcc-3 mutants defective in thermotaxis

To show the mechanisms underlying the C. elegans thermotaxis behavior, we undertook a genetic screen to look for mutants defective in this behavior. We mutagenized wild-type animals and recovered mutant isolates that migrated to the 23°C region even though they had been cultivated at 17°C. Among the isolates, we recovered three mutations, nj90, nj94 and nj100. While wild-type animals cultivated at 17°C migrated to this cultivation temperature 17°C, a significant portion of these mutant animals preferred the 23°C region [Fig. 1a–c; one-way ANOVA, F (8) = 7.05, t(8) = 5.32 and t(8) = 8.61, respectively, all P < 0.01]. They also displayed thermophilic defects when cultivated at 20°C [Fig. 1d; one-way ANOVA, F (8) = 35.0, P < 0.01; Dunnett’s multiple comparison test, WT vs. kcc-3[nj90], kcc-3[nj94] and kcc-3[nj100], t(8) = 10.2, t(8) = 9.43 and t(8) = 11.9, respectively, all P < 0.01], while their thermotaxis behavior appeared relatively normal after the 23°C cultivation [Fig. 1f; one-way ANOVA, F (8) = 9.93, P < 0.01; Dunnett’s multiple comparison test, WT vs. kcc-3[nj90], kcc-3[nj94] and kcc-3[nj100], t(8) = 2.28, NS; WT vs. kcc-3[nj94], t(8) = 4.51, P < 0.01; WT vs. kcc-3[nj100], t(8) = 0.80, NS]. We performed complementation tests among these isolates and found that nj90 failed to complement nj94 and nj100 [Fig. 1h; one-way ANOVA, F (4) = 26.4, P < 0.01; Dunnett’s multiple comparison test, WT vs. kcc-3[nj90]/kcc-3[nj94], t(4) = 78.5, P < 0.01;
Cultivation with food (E. coli) at a certain temperature (17, 20 or 23 °C) 

\[ \text{TTX index} = \sum_{x=1}^{8} x \cdot F_x \]

\[ \text{Dispersion index} = 4 \times (F_1 + F_8) + 3 \times (F_2 + F_7) + 2 \times (F_3 + F_6) + 1 \times (F_4 + F_5) \]

Fx: Fraction of animals in the X region

**Figure 1:** Isolation and characterization of kcc-3(nj90, nj94, nj100) mutants defective in thermotaxis. (a) A schematic diagram of the thermotaxis assay. Animals cultivated with food at a certain temperature were placed at the center of the linear temperature gradient. After 60 min, the number of animals in each region was scored. The TTX and dispersion indices are calculated using the equations as described. (b–g) Thermotaxis of wild-type and kcc-3 mutant animals cultivated at 17° C (b, c), 20° C (d, e) or 23° C (f, g). The distributions of the animals (b, d, f), their TTX indices (c, g) and dispersion indices (e) are shown. (h) Complementation test among nj90, nj94 and nj100. Dispersion indices are shown. Note that dispersion index was used to quantify the defects of kcc-3 mutants cultivated at 20°C. The marks on the bars indicate statistically significant differences compared with WT [Dunnett’s multiple comparison test; \( N = 5 \) (b–g), \( N = 3 \) (h)]. Double asterisks indicate \( P < 0.01 \). Error bars represent SEM.
WT vs. kcc-3(nj90)/kcc-3(nj100), t(4) = 8.55, P < 0.01. These results suggest that the three mutations are allelic and cause thermotaxis defects.

To identify the gene mutated in these mutants, we performed single nucleotide polymorphism (SNP) mapping of nj94 and identified that nj94 maps onto a 5.3-Mbps interval of chromosome II. We conducted a whole-genome sequencing of nj94 and nj100 and looked within this genomic interval for genes that were mutated in both mutants. The gene kcc-3 was the sole candidate gene that was mutated to alter amino acid sequences or splice sites. We also identified that nj90 mutants harbor a mutation in kcc-3 (Fig. 2a). Introduction of a genomic fragment carrying the kcc-3 gene rescued the thermotaxis defect of nj100 (Fig. 2b,c; one-way ANOVA, F_{2,9} = 26.5, P < 0.01; Tukey’s multiple comparison test, WT vs. kcc-3(nj100), t(6) = 9.31, P < 0.01; kcc-3(nj100) vs. kcc-3(nj100); njEx[kcc-3(+)], t(6) = 8.45, P < 0.01). Furthermore, two deletion mutants of kcc-3, ok228 and tm3649 displayed abnormal thermotaxis behaviors [Fig. 1b,c; Dunnett’s multiple comparison test, WT vs. kcc-3(tm3649), t(8) = 5.89, P < 0.01; WT vs. kcc-3(ok228), t(8) = 6.15, P < 0.01]. These results indicate that nj90, nj94 and nj100 are alleles of kcc-3 and that kcc-3 is required for the thermotaxis behavior.

**kcc-3 encodes a potassium-chloride cotransporter**

The kcc-3 gene is predicted to encode a potassium-chloride cotransporter that resembles the previously characterized *C. elegans* KCC-1 protein, which contains 12 putative transmembrane domains and exhibits K+/Cl− cotransport activity when expressed in HEK-293 cells (Holtzman et al. 1998). A phylogenetic analysis indicated that KCC-3 exhibits a higher similarity to the *C. elegans* KCC-1 protein than to the four human KCCs, KCC1–4 (Fig. 2d). The *C. elegans* KCC-3 protein displays 50% identity to the *C. elegans* KCC-1 and 36–39% identities to the four human KCCs. The kcc-3 mutations isolated from our genetic screens, nj90, nj94 and nj100, were found to alter conserved residues (Fig. S1). These observations suggest that like two other *C. elegans* KCC proteins (Holtzman et al. 1998; Tanis et al. 2009), KCC-3 functions as a K+/Cl− cotransporter.
**kcc-3 is expressed in glial-like cells**

To identify where kcc-3 is expressed, we generated a translational kcc-3::gfp transgene that contains 3.9 kb 5' upstream and 2.2 kb 3' downstream sequence of the predicted kcc-3 coding region (Fig. 3a). We found that this kcc-3::gfp transgene rescued the thermotaxis defect of kcc-3(tm3649) (Fig. 3b,c; one-way ANOVA, $F_{2,9}=28.1, P<0.01$; Tukey's multiple comparison test, WT vs. kcc-3(tm3649), $t(6)=10.1, P<0.01$; kcc-3(tm3649) vs. kcc-3(tm3649); njEx[kcc-3(+):gfp], $t(6)=7.85, P<0.01$), indicating that this transgene is functional. Consistent with the previous expression analysis of a kcc-3 promoter::gfp (Tanis et al. 2009), this kcc-3::gfp translational fusion was expressed in glial-like cells, including amphid sheath, cephalic sheath and phasmid sheath cells (Fig. 3d–f). We also observed that the KCC-3::GFP signal was localized at the amphid sheath ending (Fig. 3g). These results indicate that kcc-3 is mainly expressed in glial-like cells.

**kcc-3 functions in the amphid sheath glia**

To identify where kcc-3 acts to regulate thermotaxis, we performed cell-specific rescue experiments. We fused a kcc-3 cDNA with three promoters that have been shown to drive expressions in a subset of glial cells: fig-1 promoter for the amphid sheath cells (Bacaj et al. 2008); hlh-17 promoter for the cephalic sheath cells (McMillan & Johnson 2005) and
ptr-10 promoter for the cephalic sheath cells as well as the inner and outer labial sheath and socket cells (Yoshinura et al. 2008). We observed that introduction of fig-1p::kcc-3 rescued the thermotaxis defects of kcc-3(tm3649) cultured at 17 and 20 °C (Fig. 4a,b; one-way ANOVA, \( F_{1,15} = 16.5, P < 0.01 \); Tukey’s multiple comparison test, WT vs. kcc-3(tm3649), \( t(6) = 6.43, P < 0.01 \); kcc-3(tm3649) vs. kcc-3(tm3649); \( t(6) = 6.62, P < 0.01 \), Fig. 4c,d; one-way ANOVA, \( F_{4,15} = 32.3, P < 0.01 \); Tukey’s multiple comparison test, WT vs. kcc-3(tm3649), \( t(6) = 10.7, P < 0.01 \); kcc-3(tm3649) vs. kcc-3(tm3649); \( t(6) = 10.2, P < 0.01 \). By contrast, neither hlh-17p::kcc-3 nor ptr-10p::kcc-3 transgene rescued the kcc-3(tm3649) mutant phenotypes (Fig. 4a,b; kcc-3(tm3649) vs. kcc-3(tm3649); \( t(6) = 1.85, NS \); kcc-3(tm3649) vs. kcc-3(tm3649); \( t(6) = 0.51, NS \)); Fig. 4c,d; kcc-3(tm3649) vs. kcc-3(tm3649); \( t(6) = 0.37, NS \); kcc-3(tm3649) vs. kcc-3(tm3649); \( t(6) = 0.68, NS \). As previously reported, animals lacking the amphid sheath cells displayed a thermophilic defect when cultured at 20 °C (Fig. S2; one-way ANOVA, \( F_{3,12} = 80.9, P < 0.01 \); Tukey’s multiple comparison test, WT vs. amphid sheath ablated, \( t(6) = 7.53, P < 0.01 \), although the abnormality appears less severe than kcc-3 mutants (Fig. S2; kcc-3(tm3649) vs. amphid sheath ablated, \( t(6) = 5.48, P < 0.05 \); kcc-3(nj100) vs. amphid sheath ablated, \( t(6) = 13.8, P < 0.01 \). These results indicate that kcc-3 acts in the amphid sheath cells to regulate the thermotaxis behavior.

**kcc-3 regulates thermotaxis through the AFD thermosensory neuron**

A previous ultrastructural study showed that the amphid sheath cells display glial-like characteristics and ensheathe the sensory ending of the AFD thermosensory neuron at the nose tip of the animal (Perkins et al. 1986). Given this anatomical interaction of the amphid sheath cell with AFD at the sheath ending and the localization of KCC-3 at this site (Fig. 3g), we set out to examine whether thermotaxis regulation by kcc-3 is mediated through the AFD neuron and asked whether the effect of kcc-3 mutation on thermotaxis requires the presence of the functional AFD neuron. We have previously shown that AFD responds to warming by increasing intracellular calcium concentration and that this response requires three guanylyl cyclases, gcy-8, gcy-18 and gcy-23, that are expressed in AFD (Inada et al. 2006; Kimura et al. 2004; Kuhara et al. 2011). We therefore assessed whether the kcc-3 mutations can cause a thermophilic defect in animals lacking the three guanylyl cyclase genes. While kcc-3(nj100) and kcc-3(tm3649) mutants displayed thermophilic phenotypes, these mutations did not cause the thermophilic phenotype of animals carrying gcy-23(nj37) gcy-8(nj44) gcy-18(nj38) and 32.3, P < 0.01). As previously reported, animals lacking the amphid sheath cells displayed a thermophilic defect when cultured at 20 °C (Fig. S2; one-way ANOVA, \( F_{3,12} = 80.9, P < 0.01 \); Tukey’s multiple comparison test, WT vs. amphid sheath ablated, \( t(6) = 7.53, P < 0.01 \), although the abnormality appears less severe than kcc-3 mutants (Fig. S2; kcc-3(tm3649) vs. amphid sheath ablated, \( t(6) = 5.48, P < 0.05 \); kcc-3(nj100) vs. amphid sheath ablated, \( t(6) = 13.8, P < 0.01 \). These results indicate that kcc-3 acts in the amphid sheath cells to regulate the thermotaxis behavior.

**kcc-3 regulates thermotaxis through the AFD thermosensory neuron**

A previous ultrastructural study showed that the amphid sheath cells display glial-like characteristics and ensheathe the sensory ending of the AFD thermosensory neuron at the nose tip of the animal (Perkins et al. 1986). Given this anatomical interaction of the amphid sheath cell with AFD at the sheath ending and the localization of KCC-3 at this site (Fig. 3g), we set out to examine whether thermotaxis regulation by kcc-3 is mediated through the AFD neuron and asked whether the effect of kcc-3 mutation on thermotaxis requires the presence of the functional AFD neuron. We have previously shown that AFD responds to warming by increasing intracellular calcium concentration and that this response requires three guanylyl cyclases, gcy-8, gcy-18 and gcy-23, that are expressed in AFD (Inada et al. 2006; Kimura et al. 2004; Kuhara et al. 2011). We therefore assessed whether the kcc-3 mutations can cause a thermophilic defect in animals lacking the three guanylyl cyclase genes. While kcc-3(nj100) and kcc-3(tm3649) mutants displayed thermophilic phenotypes, these mutations did not cause the thermophilic phenotype of animals carrying gcy-23(nj37) gcy-8(nj44) gcy-18(nj38) and 32.3, P < 0.01). As previously reported, animals lacking the amphid sheath cells displayed a thermophilic defect when cultured at 20 °C (Fig. S2; one-way ANOVA, \( F_{3,12} = 80.9, P < 0.01 \); Tukey’s multiple comparison test, WT vs. amphid sheath ablated, \( t(6) = 7.53, P < 0.01 \), although the abnormality appears less severe than kcc-3 mutants (Fig. S2; kcc-3(tm3649) vs. amphid sheath ablated, \( t(6) = 5.48, P < 0.05 \); kcc-3(nj100) vs. amphid sheath ablated, \( t(6) = 13.8, P < 0.01 \). These results indicate that kcc-3 acts in the amphid sheath cells to regulate the thermotaxis behavior.

**kcc-3 regulates thermotaxis through the AFD thermosensory neuron**

A previous ultrastructural study showed that the amphid sheath cells display glial-like characteristics and ensheathe the sensory ending of the AFD thermosensory neuron at the nose tip of the animal (Perkins et al. 1986). Given this anatomical interaction of the amphid sheath cell with AFD at the sheath ending and the localization of KCC-3 at this site (Fig. 3g), we set out to examine whether thermotaxis regulation by kcc-3 is mediated through the AFD neuron and asked whether the effect of kcc-3 mutation on thermotaxis requires the presence of the functional AFD neuron. We have previously shown that AFD responds to warming by increasing intracellular calcium concentration and that this response requires three guanylyl cyclases, gcy-8, gcy-18 and gcy-23, that are expressed in AFD (Inada et al. 2006; Kimura et al. 2004; Kuhara et al. 2011). We therefore assessed whether the kcc-3 mutations can cause a thermophilic defect in animals lacking the three guanylyl cyclase genes. While kcc-3(nj100) and kcc-3(tm3649) mutants displayed thermophilic phenotypes, these mutations did not cause the thermophilic phenotype of animals carrying gcy-23(nj37) gcy-8(nj44) gcy-18(nj38) and 32.3, P < 0.01). As previously reported, animals lacking the amphid sheath cells displayed a thermophilic defect when cultured at 20 °C (Fig. S2; one-way ANOVA, \( F_{3,12} = 80.9, P < 0.01 \); Tukey’s multiple comparison test, WT vs. amphid sheath ablated, \( t(6) = 7.53, P < 0.01 \), although the abnormality appears less severe than kcc-3 mutants (Fig. S2; kcc-3(tm3649) vs. amphid sheath ablated, \( t(6) = 5.48, P < 0.05 \); kcc-3(nj100) vs. amphid sheath ablated, \( t(6) = 13.8, P < 0.01 \). These results indicate that kcc-3 acts in the amphid sheath cells to regulate the thermotaxis behavior.
**Figure 5:** KCC-3 regulates thermotaxis through the AFD thermosensory neuron. (a, b) Thermotaxis analysis after 17°C cultivation. The distributions of WT, kcc-3(tm3649) or nj100) mutants, gcy-23(nj37) gcy-8(nj44) gcy-18(nj38) triple mutants (gcy triple) and kcc-3(tm3649 or nj100; gcy-23(nj37) gcy-8(nj44) gcy-18(nj38) quadruple mutants (kcc-3; gcy triple) are shown. (c) Fraction of animals in the eighth section of the data in (a) and (b). (d) and (e) Thermotaxis analysis after 20°C cultivation. The distributions of animals are shown as in (a) and (b). (f) Fraction of animals in the eighth section of the data in (d) and (e). Note that the data of WT and gcy triple in (a) and (b) and in (d) and (e) are identical and displayed separately for the presentation purpose. The marks on the lines indicate statistically significant differences between indicated genotypes (Tukey’s multiple comparison test; \(N = 4\)). Double asterisks indicate \(P < 0.01\). NS, not significant. Error bars represent SEM.

mutations [Fig. 5a–c; one-way ANOVA, \(F_{7,24} = 10.5, P < 0.01\); Tukey’s multiple comparison test, kcc-3(tm3649) vs. gcy triple, \(t(6) = 6.27, P < 0.01\); gcy triple vs. kcc-3(tm3649); gcy triple, \(t(6) = 0.42, \text{NS}\); kcc-3(nj100) vs. gcy triple, \(t(6) = 8.13, P < 0.01\); gcy triple vs. kcc-3(nj100); gcy triple, \(t(6) = 0.86, \text{NS}\), Fig. 5d–f; one-way ANOVA, \(F_{7,24} = 35.7, P < 0.01\); Tukey’s multiple comparison test, kcc-3(tm3649) vs. gcy triple, \(t(6) = 9.20, P < 0.01\); gcy triple vs. kcc-3(tm3649); gcy triple, \(t(6) = 0.89, \text{NS}\); kcc-3(nj100) vs. gcy triple, \(t(6) = 14.8, P < 0.01\); gcy triple vs. kcc-3(nj100); gcy triple, \(t(6) = 0.62, \text{NS}\)]. These results indicate that the effect of kcc-3 mutations requires the presence of a functional AFD neuron and suggest that kcc-3 regulates thermotaxis by acting through AFD.

A previous study showed that the amphid sheath cell is required to maintain the ciliated structures of several sensory neurons and that ablation of the amphid sheath cell resulted in elimination of most microvilli-like extensions of the AFD sensory ending (Bacaj et al. 2008). These observations raised a possibility that kcc-3 might affect development or maintenance of the AFD neuron. We assessed whether kcc-3 mutations disrupt the structure of the AFD sensory ending and observed the morphological structure of AFD using an AFD-specific green fluorescence protein (GFP) reporter under a high-resolution laser scanning confocal microscopy. As previously reported, animals lacking the amphid sheath cell displayed a severe morphological defect in the AFD sensory ending and often lacked the most of microvilli-like extensions (Fig. 6). By contrast, we did not detect an apparent defect in the microvilli-like extensions in kcc-3(tm3649) or kcc-3(nj100) mutants. These observations suggest that kcc-3 is not required for the development or maintenance of AFD neuron but rather regulates the function of AFD neuron.
The morphology of the AFD sensory ending.

Figure 6: The morphology of the AFD sensory ending. (a–d) Three representative images of the AFD sensory endings of WT (a), animals lacking the amphid sheath glia (AMsh ablated) (b), kcc-3(tm3649) mutants (c) and kcc-3(nj100) mutants (d). The AFD sensory ending of animals expressing H13p::GFP, an AFD reporter, was observed, and their microvilli-like extensions were examined.

The effects of kcc-3 mutations on chemotaxis behavior

In addition to AFD, the dendrites of several other sensory neurons are embedded in amphid sheath cells (Perkins et al. 1986). Given the previous observation that animals without amphid sheath cells fail to chemotax toward several odorants (Bacaj et al. 2008), mutations in kcc-3 might also affect these behaviors. We thus tested kcc-3 mutants for AWA-mediated chemotaxis toward diacetyl and AWC-mediated chemotaxis toward benzaldehyde, and found that kcc-3(tm3649) mutants showed abnormal responses to both odorants although in a moderate level to benzaldehyde (Fig. S3). kcc-3(nj100) mutants, on the other hand, appeared to be defective in chemotaxis only toward diacetyl, although not significantly (Fig. S3; a diacetyl: one-way ANOVA, $F_{3,12} = 5.06, P < 0.05$; Tukey’s multiple comparison test, WT vs. kcc-3(tm3649), $t(6) = 4.36, P < 0.05$; WT vs. kcc-3(n100), $t(6) = 2.83$, NS; Fig. S3b; benzaldehyde: one-way ANOVA, $F_{3,12} = 47.4$, $P < 0.01$; Tukey’s multiple comparison test, WT vs. kcc-3(tm3649), $t(6) = 7.37$, $P < 0.01$; WT vs. kcc-3(n100), $t(6) = 0.50$, NS). These results suggest that disruption of KCC-3 might also result in deficits in chemotaxis, in a way that is different from ablation of amphid sheath cells.

kcc-3 regulates temperature-evoked response of AFD

To address whether kcc-3 regulates temperature-evoked response of AFD neurons, we set out to monitor the calcium dynamics of AFD neurons. We and others have previously reported that in response to warming, wild-type AFD neurons depolarized and increased intracellular calcium concentration and that this response occurs only when the temperature rises above the previous cultivation temperature, suggesting that AFD neurons can sense and memorize the temperature information (Kimura et al. 2004; Ramot et al. 2008). We conducted calcium imaging of AFD using a genetically encoded calcium indicator GCaMP3. As previously reported, wild-type AFD neurons responded to warming stimulus with a sharp calcium increase followed by a slow return to the baseline level, and this response occurred when the temperature reached the cultivation temperature (Fig. 7). When AFD neurons of kcc-3 mutants were subjected to the warming stimulus, they increased the calcium level at the temperatures similar to that observed with wild-type AFD neurons (Fig. 7; Fig. S4). However, AFD neurons of kcc-3 mutants did not retain high calcium level after the initial rise upon warming and displayed abnormally fast return to the baseline level [Fig. 7d; Steel–Dwass multiple comparison tests, WT vs. kcc-3(tm3649), $t_{44} = 4.93$, $P < 0.01$; WT vs. kcc-3(nj100), $t_{44} = 4.51$, $P < 0.01$]. This drop of the calcium
Figure 7: KCC-3 modulates the temperature-evoked activity of the AFD neuron. Calcium imaging of AFD in wild-type animals, kcc-3(tm3649) mutants, kcc-3(nj100) mutants, kcc-3(tm3649) mutants carrying an fig-1p::kcc-3 transgene and animals lacking the amphid sheath glia (AMsh ablated). (a) A representative calcium response of each strain. (b) Heat map of GCaMP3/RFP ratio changes is shown (blue = 0%, reddish brown = 100%, N = 23 for each strain). (c) The temperature program used in the analyses. Animals were given temperature stimuli of 14–24°C linear warming. (d) Quantification of the abnormally fast drop of the AFD activity in kcc-3 mutants and AMsh-ablated animals. $R(t_{\text{max}})$ indicates the maximum ratio change of GCaMP3/RFP during the time period of 40–240 seconds. $R(t_{\text{max}} + 50)$ represents the ratio change at 50 seconds after the GCaMP3/RFP ratio change reached $R(t_{\text{max}})$. The Y axis shows the average ratio of $R(t_{\text{max}} + 50)$ to $R(t_{\text{max}})$. Steel–Dwass multiple comparison tests were performed. The marks on the bars represent comparisons with WT. The mark on the line represents comparisons between indicated genotypes. Double asterisks indicate $P < 0.01$. NS, not significant. Error bars represent SEM.
level was occasionally followed by another calcium increase in kcc-3 mutants, resulting in multiple peaks of calcium concentration during a single warming stimulus. We examined whether this abnormal response of AFD is caused by the lack of the kcc-3 activity in amphid sheath cell. We introduced fig-1p::kcc-3 into kcc-3(tm3649) mutants and found that expression of kcc-3 in amphid sheath cells rescued the abnormal response of AFD neurons (Fig. 7; Steel–Dwass multiple comparison tests, kcc-3(tm3649) vs. kcc-3(tm3649); njExfig-1p::kcc-3(+)), t(44) = −4.73, P < 0.01). Similar to kcc-3 mutants, animals in which amphid sheath cells were ablated showed an abnormally fast decrease in the calcium level of AFD after response to the warming stimulus, albeit to a lesser degree than that observed in kcc-3 mutants [Fig. 7; Steel–Dwass multiple comparison tests, WT vs. amphid sheath ablated, t(44) = 3.70, P < 0.01]. These results indicate that KCC-3 functions in glial-like amphid sheath cells to modulate the temperature-evoked neural response of AFD.

Discussion

The roles of KCC proteins in neurons have been characterized, and in particular, establishment of the chloride concentration gradient necessary for the hyperpolarizing inhibitory effect of GABA has been reported in many organisms ranging from C. elegans to mammals (Rivera et al. 1999; Tanis et al. 2009; Zhang et al. 2013). By contrast, little has been known for the function of glial KCC proteins, in spite of its expression in glial cells of several species examined to date (Byun & Delpère 2007; Le Rouzic et al. 2006; Payne et al. 1996; Rusan et al. 2014; Sun et al. 2010). In this study, we showed that the C. elegans KCC-3 protein functions in a glial cell and regulates the thermosensory behavior. KCC-3 is expressed in the amphid sheath glial cells that ensheath the sensory endings of the AFD thermosensory neurons. Our genetic study indicated that the effect of KCC-3 on the thermotaxis behavior is mediated through the AFD neuron, and we further showed that KCC-3 modulates the temperature-evoked activity of AFD. Our results show that the glial KCC-3 protein regulates a sensory behavior by modifying the dynamics of a stimulus-evoked activity of a sensory neuron.

Our genetic study suggests that KCC-3 acts through AFD to regulate thermotaxis. We note, however, that the effect of KCC-3 might also be mediated through other neurons embedded in amphid sheath glia, especially those that are temperature-sensitive such as AWC (Kuhara et al. 2008). Nevertheless, the observation that the thermophilic phenotypes of the kcc-3 mutants were suppressed in mutant animals lacking functional AFD neurons, together with our calcium imaging data, supports the idea that AFD is the major neuron that mediates the effect of KCC-3 on thermotaxis behavior.

Among genes that found to be important for thermotaxis behavior in C. elegans, kcc-3 is the first of its kind that functions in glia to regulate this behavior. Glia themselves have been reported to be essential for thermotaxis, and ablation of these cells causes a thermophilic defect (Bacaj et al. 2008; Fig. S2). The severity of the defect, however, appears rather moderate when compared with kcc-3 mutants. Moreover, our calcium imaging analysis showed that the AFD neuron of animals without the amphid sheath glia exhibits an abnormal response property, similar to that of kcc-3 mutants, but with less severity (Fig. 7). We speculate that the differences in their phenotypes might result from the presence of a molecule that functions in amphid sheath glia and counteracts KCC-3, creating a balance via opposing effects. The absence of one side, KCC-3, in kcc-3 mutants would make the situation ill balanced and result in an adverse impact on the regulation of the AFD function, whereas the loss of both sides by ablation of amphid sheath glia would offset the imbalance and generate a less harmful effect on AFD. As candidates for such a molecule, ion transporters such as Na⁺–K⁺–2Cl⁻ cotransporter or Na⁺/K⁺ ATPase might reciprocally act with KCC-3 in amphid sheath glia to modulate the activity of the AFD neuron. Further investigation should dissect out the molecular basis underlying glial regulation of sensory neurons.

Our results indicated that the initial response of the AFD neuron in kcc-3 mutants did not show strong abnormality, and that they showed a sharp calcium increase at around the cultivation temperature during warming, indicating the ability to sense and memorize temperature. The loss of KCC-3 rather affects the fine-tuning of gradual calcium decrease, exhibiting a radical drop and unstably fluctuating calcium wave. While previous studies have found the threshold temperature and response amplitude as key aspects of the AFD function (Kimura et al. 2004; Kuhara et al. 2011), this study suggests the functional significance of post-activated calcium dynamics for proper neuronal output of AFD. Although the relationship between these calcium responses and behavior has not been fully uncovered, the unprecedented phenotype of kcc-3 mutants can be a unique model to investigate the fundamental principle of how a single neuron utilizes both the acute calcium increase in response to stimuli and the following calcium dynamics to represent and transmit neural information to the downstream circuit, thereby orchestrating the nervous system function.

Previous studies in mammals suggested that KCC proteins expressed in non-neuronal cells regulate extracellular ion concentration necessary for nearby cells to survive and function. For example, the mice Kcc3 and Kcc4 genes were reported to be expressed in the supporting cells of hair cells (Boettger et al. 2002, 2003). The lack of either Kcc3 or Kcc4 causes degeneration of hair cells and results in deafness. These observations suggested the non-cell autonomous effect of KCC proteins whereby the absence of KCC from the supporting cells causes abnormal intracellular and extracellular ion concentrations and leads to degeneration of the hair cells. In addition, a previous study in C. elegans showed that ablation of the amphid sheath glia caused malfunction of the ciliated sensory endings of several neurons, including AFD, that are associated with the glia (Bacaj et al. 2008). These observations raised the possibility that kcc-3 functions in the amphid sheath glia and regulates the development or the maintenance of AFD. However, we did not detect a severe malfunction of AFD sensory endings in kcc-3 mutants when observed under a high-resolution laser scanning confocal microscope. Rather, this study showed that kcc-3 mutations
altered the dynamics of the temperature-evoked activity of AFD. These results indicate that glial KCC proteins can modulate the neural activity without severely degenerating the neurons, and suggest a new mode of action by the glial KCC proteins that regulate the neuronal activity.

How does KCC-3 in glia affect the activity of the AFD neuron? Our results indicated that in the absence of KCC-3 from the amphid sheath glia, the AFD calcium concentration initially increased at a rate comparable to that of wild-type AFD neurons but displayed an abnormally fast return to the baseline level, suggesting that kcc-3 regulates a process during the repolarizing phase. Indeed, a previous electrophysiological study of AFD indicated that voltage-gated outward currents are mediated by K+ (Ramot et al. 2008). These observations suggest that KCC-3-dependent ionic homeostasis around the AFD dendrite plays an important role in setting the rate of outward currents through K+ channels, which inactivate the neuron properly. Depending on the direction of K+Cl− transport by KCC-3, further speculation can be made in two ways: if KCC-3 transports these ions outwardly from the glial cell, the loss of KCC-3 would cause a decrease in the extracellular K+ concentration and result in an increase of the K+ chemical driving force for AFD, leading to an abnormally fast repolarization; if, on the other hand, KCC-3 takes up K+Cl− into the glial cell, its absence would yield a chronically high level of extracellular Cl− concentration, which might serve to activate Cl−-sensitive K+ channels (Yuan et al. 2000) in AFD, expediting repolarization.

Our results suggest a novel mechanism by which the KCC family proteins act in the glial cells to influence the dynamics of sensory neurons and ultimately control the property of the neural circuit required to generate animal behaviors.

References

Bacaj, T., Tevlin, M., Lu, Y. & Shaham, S. (2008) Glia are essential for sensory organ function in C. elegans. Science 322, 744–747.
Bargmann, C.I., Hartwig, E. & Horvitz, H.R. (1993) Odorant-selective genes and neurons mediate olfaction in C. elegans. Cell 74, 515–527.
Beverly, M., Anbil, S. & Sengupta, P. (2011) Degeneracy and neuro-modulation among thermosensory neurons contribute to robust thermosensory behaviors in Caenorhabditis elegans. J Neurosci 31, 11718–11727.
Blaesse, P., Airaksinen, M.S., Rivera, C. & Kaila, K. (2009) Blocking KCC proteins act in the glial cells to influence the dynamics of sensory neurons and ultimately control the property of the neural circuit required to generate animal behaviors.

Regulation of neural activity by a glial KCC

Hedgecock, E.M. & Russell, R.L. (1975) Normal and mutant thermotaxis in the nematode Caenorhabditis elegans. Proc Natl Acad Sci USA 72, 4061–4065.
Hillier, L.W., Marth, G.T., Quinlan, A.R., Dooling, D., Fekry, G., Barnett, D., Fox, P., Glasscock, J.J., Hickenbotham, M., Huang, W., Magrini, V.J., Righi, R.J., Sander, S.N., Stewart, D.A., Stromberg, M., Tsung, E.F., Wylie, T., Schedl, T., Wilson, R.K. & Mardis, E.R. (2008) Whole-genome sequencing and variant discovery in C. elegans. Nat Methods 5, 183–188.
Holzmann, E.J., Kumar, S., Faaland, C.A., Warner, F., Logue, P.J., Erickson, S.J., Ricken, G., Waldman, J. & Dunham, P.B. (1998) Cloning, characterization, and gene organization of K-Cl co-transporter from pig and human kidney and C. elegans. Am J Physiol 275, F550–F564.
Howard, H.C., Mount, D.B., Rochefort, D. et al. (2002) The K-Cl cotransporter KCC3 is mutant in a severe peripheral neuropathy associated with agenesis of the corpus callosum. Nat Genet 32, 394–399.
Hübner, C.A., Stein, V., Hermans-Borgmeyer, I., Meyer, T., Ballanyi, K. & Jentsch, T.J. (2001) Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. Neuron 30, 515–524.
Inada, H., Ito, H., Satterlee, J., Sengupta, P., Matsumoto, K. & Mori, I. (2006) Identification of guanylyl cyclases that function in thermosensory neurons of Caenorhabditis elegans. Genetics 172, 2239–2252.
Ito, H., Inada, H. & Mori, I. (2006) Quantitative analysis of thermotaxis in the nematode Caenorhabditis elegans. J Neurosci Methods 154, 45–52.
Karadsheh, M.F., Byun, N., Mount, D.B. & Delpire, E. (2004) Localization of the KCC4 potassium-chloride cotransporter in the nervous system. Neuroscience 123, 381–391.
Kim, E., Sun, L., Gabel, C.V. & Fang-Yen, C. (2013) Long-term imaging of Caenorhabditis elegans using nanoparticle-mediated immobilization. PLoS One 8, e53419.
Kimura, K.D., Miyawaki, A., Matsumoto, K. & Mori, I. (2004) The C. elegans thermosensory neuron AFD responds to warming. Curr Biol 14, 1291–1295.
Kuhara, A., Okumura, M., Kimata, T., Tanizawa, Y., Takano, R., Kimura, K.D., Inada, H., Matsumoto, K. & Mori, I. (2008) Temperature sensing by an olfactory neuron in a circuit controlling behavior of C. elegans. Science 320, 803–807.
Kuhara, A., Ohnishi, N., Shimowada, T. & Mori, I. (2011) Neural coding in a single sensory neuron controlling opposite seeking behaviours in Caenorhabditis elegans. Nat Commun 2, 395.
Le, M.T., Rozic, P., Ivanov, T.P., Stanley, P.J., Baudoin, F.M., Chan, F., Pinteaux, E., Brown, P.D. & Luckman, S.M. (2006) KCC3 and KCC4 expression in rat adult forehead. Brain Res 1100, 39–45.
McMillner, T.L. & Johnson, C.M. (2005) Molecular characterization of HLH-17, a C. elegans bHLH protein required for normal larval development. Gene 356, 1–10.
Mello, C.C., Kramer, J.M., Stinchcomb, D. & Ambros, V. (1991) Efficient gene transfer in C. elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J 10, 3959–3970.
Mori, I. & Ohshima, Y. (1995) Neural regulation of thermotaxis in Caenorhabditis elegans. Nature 376, 344–348.
Payne, J.A., Stevenson, T.J. & Donaldson, L.F. (1996) Molecular characterization of a putative K-CI cotransporter in rat brain. A neuronal-specific isofrom. J Biol Chem 271, 16245–16252.
Perkins, L.A., Hedgecock, E.M., Thomson, J.N. & Culotti, J.G. (1986) Mutant sensory cilia in the nematode Caenorhabditis elegans. Dev Biol 117, 456–487.
Puskarjov, M., Seja, P., Heron, S.E., Williams, T.C., Ahmad, F., Iona, X., Oliver, K.L., Grinton, B.E., Vutsikis, L., Scheffer, I.E., Petrov, S., Blaesse, P., Dibbens, L.M., Berkovic, S.F. & Kaila, K. (2014) A variant of KCC2 from patients with febrile seizures impairs neuronal Cl− extrusion and dendritic spine formation. EMBO Rep 15, 723–729.
Yoshida et al.

Ramot, D., Macnissi, B.L. & Goodman, M.B. (2008) Bidirectional temperature-sensing by a single thermosensory neuron in C. elegans. Nat Neurosci 11, 908–915.

Rivera, C., Voipo, J., Payne, J.A., Ruusuvuori, E., Lahtinen, H., Lamma, K., Pirvola, U., Saarma, M. & Kaila, K. (1999) The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397, 251–255.

Roayaie, K., Crump, J.G., Sagasti, A. & Bargmann, C.I. (1998) The G alpha protein ODR-3 mediates olfactory and nociceptive function and controls cilium morphogenesis in C. elegans olfactory neurons. Neuron 20, 55–67.

Rusan, Z.M., Kingsford, O.A. & Tanouye, M.A. (2014) Modeling glial contributions to seizures and epileptogenesis: cation-chloride cotransporters in Drosophila melanogaster. PLoS One 9, e101117.

Sasakura, H., Inada, H., Kuhara, A., Fusaoka, E., Takemoto, D., Takeuchi, K. & Mori, I. (2005) Maintenance of neuronal positions in organized ganglia by SAX-7, a Caenorhabditis elegans homologue of L1. EMBO J 24, 1477–1488.

Sun, Y.T., Lin, T.S., Tseng, S.F., Delpire, E. & Shen, M.R. (2010) Deficiency of electroneutral K+−Cl− cotransporter 3 causes a disruption in impulse propagation along peripheral nerves. Glia 58, 1544–1552.

Tanis, J.E., Bellemer, A., Moresco, J.J., Forbush, B. & Koelle, M.R. (2009) The potassium chloride cotransporter KCC-2 coordinates development of inhibitory neurotransmission and synapse structure in Caenorhabditis elegans. J Neurosci 29, 9943–9954.

Tian, L., Hires, S.A., Mao, T., Huber, D., Chiappe, M.E., Chalasani, S.H., Petreanu, L., Akerboom, J., McKinney, S.A., Schreiter, E.R., Bargmann, C.I., Jayaraman, V., Svoboda, K. & Looger, L.L. (2009) Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Proc Natl Acad Sci U S A 106, 20410–20415.

Uvarov, P., Ludwig, A., Markkanen, M., Pruunisild, P., Kaila, K., Delpire, E., Timmus, T., Rivera, C. & Airaksinen, M.S. (2007) A novel N-terminal isoform of the neuron-specific K-Cl cotransporter KCC2. J Biol Chem 282, 30570–30576.

White, J.G., Southgate, E., Thomson, J.N. & Brenner, S. (1986) The structure of the nervous system of the nematode Caenorhabditis elegans. Philos Trans R Soc Lond B Biol Sci 314, 1–340.

Wicks, S.R., Yeh, R.T., Gish, W.R., Waterston, R.H. & Plasterk, R.H. (2001) Rapid gene mapping in Caenorhabditis elegans using a high density polymorphism map. Nat Genet 28, 160–164.

Wood, N.S., Lu, J., England, R., McClellan, R., Dufour, S., Mount, D.B., Deutch, A.Y., Lovinger, D.M. & Delpire, E. (2002) Hypereexcitability and epilepsy associated with disruption of the mouse neuronal-specific K-Cl cotransporter gene. Hippocampus 12, 258–268.

Yoshimura, S., Murray, J.L., Lu, Y., Waterston, R.H. & Shaham, S. (2008) mtl-2 and vab-3 control glia development, hth-17/Olig expression and glia-dependent neurite extension in C. elegans. Development 135, 2263–2275.

Yuan, A., Dourado, M., Butler, A., Walton, N., Wei, A. & Salkoff, L. (2000) SLO-2, a K+ channel with an unusual CI− dependence. Nat Neurosci 3, 771–777.

Zhang, P.W., Zhang, S.Y. & Du, J.L. (2013) KCC2-dependent subcellular E(Cl) difference of ON-OFF retinal ganglion cells in larval zebrafish. Front Neural Circuits 7, 103.

Acknowledgments

We thank Yuji Kohara for kcc-3 cDNA; Shohei Mitani at the National BioResource for kcc-3(tm3649); Shai Shaham for strains; Yumi Murakami, Jun Okada and Kazue Sawayama, Fumika Takeshige for technical assistance. Some strains were provided by the Caenorhabditis Genetic Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). This work was supported by JSPS KAKENHI Grant numbers 24247001 (to I.M.), 24770204 (to S.N.), MEXT KAKENHI Grant number 22123010 (to I.M.) and a grant from the Nakajima Foundation (to S.N.). The authors declare no competing financial interests.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

Figure S1: Multiple alignments of the amino acid sequences of KCC-3 homologues and mutation sites of kcc-3. Black and gray boxes highlight identical and similar residues, respectively. Black bars above the sequences indicate transmembrane domains of C. elegans KCC-1, predicted in Holtzman et al. (1998). The amino acid sequence of KCC-3 is derived from the sequence of a kcc-3 cDNA clone yk611h3.

Figure S2: Animals lacking the amphid sheath cells display abnormal behavior in thermotaxis. Thermotaxis of WT, kcc-3(tm3649), kcc-3(n100) and AMsh-ablated animals cultivated at 20°C. The distributions of the animals (a) and dispersion indices (b) are shown. The marks on the bars represent comparisons with WT (Tukey’s multiple comparison test; N=4). The marks on the lines represent comparisons between indicated genotypes. Single and double asterisks indicate P<0.05 and P<0.01, respectively. Error bars represent SEM.

Figure S3: The effects of kcc-3 mutations on chemotaxis behavior. Chemotaxis toward diacetyl (a) and benzaldehyde (b) of WT, kcc-3(tm3649), kcc-3(n100) and odr-3(n1605). odr-3(n1605) mutants were defective as described previously (Roayaie et al. 1998) and used as a control. Each bar represents the average of chemotaxis index. The marks on the bars represent comparisons with WT (Tukey’s multiple comparison test; N=4). Single and double asterisks indicate P<0.05 and P<0.01, respectively. NS, not significant. Error bars represent SEM.

Figure S4: Calcium imaging of AFD after 17 and 23°C cultivation. Heat map of GCaMP3/RFP ratio changes after 17°C (a) or 23°C (b) cultivation are shown (blue=0%, reddish brown=100%, N=7 for each strain). (c) The temperature program was used in the analyses. Animals were given temperature stimuli of 14–24°C linear warming.

Table S1: The list of the strains used in this study.