Insulin-like signaling and the neural circuit for integrative behavior in C. elegans

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Caenorhabditis elegans exhibits a food-associated behavior that is modulated by the past cultivation temperature. Mutations in INS-1, the homolog of human insulin, caused the defect in this integrative behavior. Mutations in DAF-2/insulin receptor and AGE-1/phosphatidylinositol 3 (PI-3)-kinase partially suppressed the defect of ins-1 mutants, and a mutation in DAF-16, a forkhead-type transcriptional factor, caused a weak defect. In addition, mutations in the secretory protein HEN-1 showed synergistic effects with INS-1. Expression of AGE-1 in any of the three interneurons, AY, AIZ, or RIA, rescued the defect characteristic of age-1 mutants. Calcium imaging revealed that starvation induced INS-1-mediated down-regulation of AIZ activity. Our results suggest that INS-1, in cooperation with HEN-1, antagonizes the DAF-2 insulin-like signaling pathway to modulate interneuron activity required for food-associated integrative behavior.

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The secreted peptide hormone insulin modulates neural plasticity. Insulin and insulin receptors are expressed in several regions of the rat brain [Havrankova et al. 1978a,b], insulin receptors localize to post-synapses [Abbott et al. 1999], and insulin can produce long-term depression [LTD] of synaptic transmission through endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazoloproponionic acid (AMPA) receptors in rat hippocampal CA1 neurons [Man et al. 2000]. In addition, Phosphatidylinositol 3 [PI-3]-kinase that functions in the insulin signaling pathway is thought to induce long-term potentiation [LTP] of synaptic transmission in the dentate gyrus of rat

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in association between cultivation temperature and starvation or in recognition of starvation per se. To address these possibilities, we tested the responses of *aho-2(nj32)* animals to changes in feeding state using a locomotory activity assay [Sawin et al. 2000]. Well-fed wild-type animals move more slowly in plates with food than without food, and starved wild-type animals move even more slowly in plates with food than do well-fed animals [Fig. 1M]. *aho-2(nj32)* and wild-type animals exhibited nearly the same responses to changes in feeding state [Fig. 1M], suggesting that *aho-2(nj32)* animals can respond to starvation and exhibit a defect in association between cultivation temperature and starvation (Mohri et al. 2005).

We investigated whether *aho-2(nj32)* mutants had a defect in integration of different chemosensory inputs using an interaction assay [Fig. 1B] that is a behavioral test for the integration of two opposing signals, a signal from an attractive odorant, diacetyl, and one from a repulsive metal, Cu$^{2+}$ ion [Ishihara et al. 2002]. HEN-1 is a secretory protein with an LDL receptor motif and is required for the behavioral task tested in this interaction assay [Ishihara et al. 2002]. In contrast to *hen-1(tm501)-null* mutants, *aho-2(nj32)* mutants responded normally [Fig. 1N]. *aho-2(nj32)* mutants exhibited normal responses to diacetyl and Cu$^{2+}$ separately (data not shown), implying that the integrative process of the two chemical compounds is normal in *aho-2(nj32)* mutants.

The gene *aho-2* was mapped to the 0.08 map unit region in the center of chromosome IV [Mohri et al. 2005; data not shown], which is covered by the three cosmids. We found that only F13B12 rescued the defect of *aho-2(nj32)* [Fig. 2A]. Among six predicted genes in the region covered by F13B12 [data not shown], a PCR product containing *ins-1*, the *C. elegans* gene most closely related to human insulin among 38 insulin-related genes (Pierce et al. 2001; Li et al. 2003), rescued the defect of the *aho-2(nj32)* mutant [Fig. 2A]. The *ins-1(nr2091)* mutant, a previously isolated putative null mutant [Pierce et al. 2001], also showed an Aho phenotype [Fig. 2C]. We found a 130-base-pair [bp] deletion from the first exon to the second exon in *ins-1* of *aho-2(nj32)* mutants [data not shown]. These results led us to conclude that *aho-2* is identical to *ins-1*.

**Neuronal expression of INS-1 is important for integrative thermotaxis behavior**

We observed *ins-1*-expressing cells using an *ins-1* promoter::GFP fusion gene. As previously reported, fluorescence was observed in many head neurons, including ADF, AIA, AIM, ASE, ASG, ASH, ASI, ASJ, AWA, BAG, and NSM, and was also observed in the intestine, hypodermis, and vulval muscle [Fig. 2E; Pierce et al. 2001; data not shown].

Expressing *ins-1* cDNA from its own promoter or in all neurons using the *unc-14* promoter almost fully rescued the Aho phenotype of *ins-1(nr2091)* mutants, whereas no rescue occurred when expressing the *ins-1* cDNA in intestine using the *ges-1* promoter [Fig. 2C]. These results indicate that neuronal expression of INS-1 is sufficient to rescue the food-associated thermotaxis behavior defect of *ins-1(nr2091)* mutants. We conducted cell-specific rescue experiments to determine if the expression of INS-1 in any particular neuron is required for association between temperature and feeding state. The expression of *ins-1* cDNA using the *ins-1*, *nrs-1*, *lin-11*, *unc-86*, and *ceh-14* promoters effectively rescued the defect of *ins-1(nr2091)* mutants, and weak rescue occurred upon *ins-1* cDNA expression from *osm-6* and *gpa-2* promoters [Fig. 2D]. In contrast, no rescue occurred upon expression of *ins-1* cDNA using the *unc-42*, *tph-1*, *gcy-8*, *gcr-3*, or
Figure 2. INS-1 is required in neurons for integrative thermotactic behavior. [A] Rescue experiments for the food-associated thermotactic behavior defect in aho-2(n32) mutants. The asterisks indicate \( p \leq 0.05 \) for a comparison with starved aho-2(n32) mutants. Fed animals, \( n \geq 30 \); starved animals, \( n \geq 60 \). Error bars indicate SEM. [B] The thermotaxis behavior of animals cultivated at 20°C with food. The phenotypic categories are described in the Supplemental Material. The data, presented as horizontal stacked bar graphs, represent the average of three independent thermotaxis assays using 20 animals per assay. Three independent transgenic lines were tested for the INS-1 overexpression (O/E) line (containing 100 ng/µL genomic DNA); \( n = 60 \) for wild type, \( n = 60 \) for the mutant. Single and double asterisks indicate \( p < 0.05 \) and \( p < 0.01 \), respectively, for a comparison with wild type. [C] Rescue experiments of the food-associated thermotaxic behavior of aho-2(n32) mutants. Fed animals, \( n \geq 30 \); starved animals, \( n \geq 60 \). Error bars indicate SEM. [D] Summary of results of the rescue experiments for \( ins-1(nr2091) \) and neuronal expression pattern of INS-1 with different promoters.

AIY promoter (Fig. 2D). Essentially, we did not identify any single neuron where the expression of INS-1 was required for the rescue, suggesting that INS-1 acts cell nonautonomously. Close examination of the rescue results and \( ins-1 \)-::GFP expression in neurons revealed that the defect of the \( ins-1 \) mutant was rescued when INS-1 was expressed in at least one of the neurons that appeared to express INS-1 normally (Fig. 2E).

To address the possibility that the lack of rescue by several promoters was caused by either too low or too high expression of the \( ins-1 \) cDNA, we constructed \( ins-1 \) mutant strains transgenic with different concentrations of \( ges-1 \)-::\( ins-1 \) cDNA or \( gcy-8 \)-::\( ins-1 \) cDNA, and tested those strains for food-associated thermotactic responses. \( ges-1 \)- or \( gcy-8 \)-driven INS-1 expression in the \( ins-1(nr2091) \) strain did not significantly rescue the defect regardless of the concentration used (Supplementary Fig. 1), suggesting that the inability of some of the promoters to rescue the defect was not caused by insufficient or excessive expression of the \( ins-1 \) cDNA.

\( ins-1 \) may be regulated transcriptionally, at the hormone processing level, and/or at the level of secretion. We observed whether there was any difference in INS-1 expression between the fed state and the starved state using an \( ins-1 \) promoter::GFP fusion gene and a resuable \( ins-1 \)-::GFP fusion gene. Light microscopic observation failed to find the difference in expression level or localization of the fusion protein (data not shown). These results argue against transcriptional regulation of \( ins-1 \) expression. If INS-1 is regulated at the level of secretion in response to starvation, the concentration of extracellular INS-1 may be too low to detect with GFP. Overexpression of INS-1 in a wild-type background induced a partially abnormal thermotaxis phenotype (Fig. 2B), which is consistent with the model that the secretion of INS-1 could modulate thermotactic behavior.

**INS-1 antagonizes DAF-2 insulin-like signaling in food-associated thermotactic plasticity**

A previous report suggested that INS-1 antagonizes DAF-2 insulin-like signaling for dauer formation in \( C. elegans \) [Pierce et al. 2001]. We explored whether DAF-2 insulin-like signaling also functions in food-associated thermotactic behavioral plasticity by examining mutants of \( daf-2 \), a homolog of the insulin/IGF-1 receptor [Kimura et al. 1997], \( age-1 \), a homolog of PI-3-kinase [Morris et al. 1996], and \( daf-16 \), a forkhead-type transcription factor [Lin et al. 1997]. The \( daf-16(m26) \) mutant, which is a suppressor of both the \( daf-2 \) and the \( age-1 \) mutant in dauer formation (Gottlieb and Ruvkun 1994; Larsen et al. 1995), showed a weak Aho phenotype (Fig. 3A). The \( daf-2(e1368) \) and \( age-1(hx546) \) mutants, however, normally avoided their cultivation temperature after a 3-h starvation (Fig. 3A). If INS-1 antagonizes DAF-2 insulin-like signaling for this integrative behavior, the behavioral responses of \( daf-2(e1368) \) and \( age-1(hx546) \) mutants to starvation might be opposite to the response of \( ins-1(nr2091) \) mutants. To test this hypothesis, we conducted a time course assay for starvation-induced temperature avoidance. As the cultivation time under starvation conditions increased, the fraction of wild-type animals that migrated to the cultivation temperature gradually decreased (Fig. 3C). Consistent with our hypothesis, \( age-1(hx546) \) mutants started to avoid the cultivation temperature much earlier than wild-type animals (Fig. 3C). These results indicate that \( age-1(hx546) \) could associate cultivation temperature with starvation quicker than wild-type animals. \( daf-2(e1368) \) mutants showed a response similar to wild-type animals (Fig. 3C), which might be due to the fact that \( daf-2(e1368) \) is one of the weakest alleles in dauer formation [Gems et al. 1998]. Because of developmental or behavioral defects, we were unable to examine starvation-induced temperature avoidance of \( daf-2(mg43) \) and \( daf-2(e1370) \) mutants, both of which are stronger alleles than \( daf-2(e1368) \) in dauer formation [Kimura et al. 1997; Gems et al. 1998; data not shown].

Double mutants were constructed and their food-associated thermotactic responses were tested to clarify the genetic interaction between the insulin-like signaling genes. With a 3-h starvation, both \( daf-2 \) and \( age-1 \) mutations partially suppressed the defective starvation-induced temperature avoidance of \( ins-1 \) mutants, although \( daf-2 \) did not suppress \( daf-16 \) (Fig. 3A). These results are consistent with a model that INS-1 acts antagonistically
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**Figure 3.** Insulin-like signaling, acting in cooperation with HEN-1, functions in thermotaxis interneurons. 

(A,B) Results of the thermotaxis assay for well-fed and starved wild-type, single-mutant, and double-mutant animals cultivated at 17°C. Single and double asterisks indicate p < 0.05 and p < 0.002, respectively. Fed animals, n = 40; starved animals, n = 80. Error bars indicate SEM. (C) Results of the thermotaxis assay from 0 [fed] to 3-h-starved animals cultivated at 17°C. Error bars indicate SEM, n = 50 animals for each time point. (D) Rescue experiments of the quicker avoidance defect of the age-1(hx546) mutant. Animals were cultivated under starvation conditions for 30 min at 17°C prior to the thermotaxis assay. Single and double asterisks indicate p < 0.05 and p < 0.01, respectively, for a comparison with starved age-1(hx546) mutants (n = 40 animals). Error bars indicate SEM.

on DAF-2 insulin-like signaling for food-associated thermotaxis behavior. daf-16; ins-1 double mutants showed a stronger defect than daf-16 single mutants [Fig. 3B], suggesting that ins-1 is genetically downstream from daf-16, which is consistent with a feedback loop from daf-16 to ins-1.

**INS-1 and HEN-1 act coordinately in food-associated thermotactic plasticity**

Insulin-like signaling, in cooperation with the TGF-β and cyclic GMP pathways, is one of the important pathways for dauer formation. We tested whether dauer formation pathways other than the insulin-like signaling pathway are also involved in integrative behavior for temperature and feeding state. Dauer-defective [Daf-d] mutants for daf-5, daf-6, and daf-12, which encodes the TGF-β pathway member Sno/Ski (da Graca et al. 2004), a Patched-related protein that functions upstream of both the TGF-β pathway and the cyclic GMP pathway (Schackwitz et al. 1996; Perens and Shaham 2005), and a nuclear receptor [Antebi et al. 2000], respectively, did not show the defects [Fig. 3B]. Recently, Murakami et al. (2005) showed that the secreted protein TGF-β/DAF-7 is involved in memory acquisition of the cultivation temperature. However, daf-7(e1372) and daf-1(m40) mutants that had a deficit in one subunit of the TGF-β receptor did not show the defects, and neither the daf-1 nor daf-7 mutant suppressed the defect of ins-1 mutants [Fig. 3B].

HEN-1, a secretory protein with an LDL receptor motif, is reported to be involved in food-associated thermotactic plasticity (Ishihara et al. 2002). To test for a genetic interaction between insulin-like signaling and HEN-1, we conducted double mutants. daf-2 mutation did not suppress the defect of hen-1 mutants. The ins-1; hen-1 double mutant, however, showed a stronger mutant phenotype than ins-1 or hen-1 single mutants [Fig. 3B]. These results suggest that INS-1 and HEN-1 act in parallel and that insulin-like signaling and HEN-1 signaling are major components in the regulation of food-associated thermotactic plasticity.

**Insulin-like signaling functions in thermotaxis interneurons**

Where is the target cell that receives and processes INS-1 to antagonize the DAF-2 insulin-like signaling pathway for temperature-starvation integrative behavior? To address this question, we conducted a cell-specific rescue experiment on age-1(hx546) mutants with a 30-min starvation. Expressing age-1 cDNA in all neurons using the unc-119 promoter rescued the quicker starvation-induced temperature avoidance defect of age-1(hx546) mutants [Fig. 3D]. Expressing age-1 cDNA in several neurons, including the AIZ, AIY, or RIA interneurons, all of which are essential interneurons for thermotaxis (Mori and Ohshima 1995), almost fully rescued the quicker avoidance defect of age-1(hx546) mutants [cf. Figs. 3D and 2E; Brockie et al. 2001]. By contrast, expressing age-1 cDNA in AFD thermosensory neurons by the gcy-8 promoter, in sensory neurons by the osm-6 promoter or in many neurons by the unc-42 promoter did not rescue the defect [cf. Figs. 3D and 2E]. These results suggest that AGE-1 [and probably the insulin-like signaling pathway] functions in thermotaxis interneurons for food-associated neural plasticity.

**Calcium imaging of the thermotaxis interneurons**

To analyze a physiological aspect of the insulin-like signaling in integrative behavior for food and temperature, we observed the changes in neuronal activity of the AIZ thermotaxis interneuron of live animals by measuring stimulus-evoked Ca2+ concentration changes using cameleon, a genetically encodable calcium indicator (Miyawaki et al. 1997; Kimura et al. 2004). The activity of the AIZ interneuron in wild-type animals cultivated at 17°C with food increased with warming and decreased with cooling, whereas the activity of the AIZ interneuron in the starved wild-type animals was much less responsive to temperature changes [Fig. 4A; Kuhara and Mori 2006]. The AIZ interneuron of starved ins-1(nr2091) animals was as active as that of fed ins-1(nr2091) animals [Fig. 4B]. These results suggest that INS-1 is required for the starvation-induced negative regulation of AIZ neuron activity. Calcium imaging on AFD thermosensory neurons revealed that feeding state did not influence the activity of AFD [Fig. 4C].
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We recently found that the interneuron-deficient calcineurin mutant tax-6(sensory+, inter−) also showed a defect in association between temperature and feeding state at 17°C (Fig. 3A; Kuhara and Mori 2006). TAX-6 calcineurin is required for temperature-starvation integrative behavior in both of two directly connected interneurons, AIZ and RIA, and like INS-1, TAX-6 is required for starvation-induced regulation of AIZ activity (Kuhara and Mori 2006). To investigate the genetic interaction between calcineurin-mediated signaling and insulin-like signaling in thermotaxis interneurons, we constructed daf-2; tax-6(sensory+, inter−) mutants. The food-associated thermotactic behavior defect of the tax-6(sensory+, inter−) mutation was not suppressed by the daf-2 mutation (Fig. 3A), which is consistent with the possibility that TAX-6 calcineurin acts downstream from DAF-2 in thermotaxis interneurons. We also investigated whether DAF-16 is involved in transcription of tax-6 by comparing the expression of a tax-6::GFP translational fusion gene in a wild-type background with expression of the fusion gene in a daf-16(mu86) deletion mutant background. We could not find any differences in expression level [data not shown]. These results are consistent with the possibility that daf-16 is not required for the transcription of tax-6.

Figure 4. In vivo calcium imaging and model for food-associated thermotactic behavioral plasticity. [A,B] Calcium imaging of the AIZ interneuron in animals cultivated at 17°C under fed or starved conditions expressing the cameleon protein. (A) Wild-type animals. (B) ins-1(nr2091) mutants. n = 10–13. Relative increases or decreases in the intracellular calcium concentration were measured as increases or decreases, respectively, in the YFP/CFP fluorescence ratio of the cameleon protein (Ratio Change). Temperature (Temp.) is shown as a black line at the bottom. (C) Calcium imaging of the AFD thermosensory neuron in wild-type animals grown at 20°C expressing the cameleon protein cultivated under fed or starved conditions (n = 18–20). Temperature (Temp.) is shown as a black line at the bottom. (D) Model of the suggestive genetic pathway for modulation of integrative behavior between cultivation temperature and feeding state by insulin-like signaling based on the results presented in this study. DAF-7 is thought to be involved in memory acquisition of cultivation temperature (Murakami et al. 2005).

A neuroendocrine system modulates the neural circuit important for integrative behavior

Our results propose a model for food-associated thermotactic plasticity (Fig. 4D). During association between cultivation temperature and starvation, INS-1 is secreted from several neurons and antagonizes insulin-like signaling by inhibiting the activity of DAF-2 receptor. DAF-16 may be activated, probably through AGE-1, and a feedback loop from DAF-16 to INS-1 might exist. HEN-1 might also be secreted from AIIY or ASE neurons (Ishihara et al. 2002). We thus suggest that a neuroendocrine system is important for modulating the neural circuit that underlies the integrative behavior.

Murakami et al. (2005) argued that AGE-1 acts in AIIY neurons to enhance isothermal tracking, which is one aspect of thermotaxis. Insulin-like signaling is required for salt chemotaxis learning, where animals pre-exposed to the chemotactant NaCl under starvation condition exhibit reduced chemotactic response to NaCl (Tomioaka et al. 2006). These reports also implicate the importance of insulin-like signaling in behavioral plasticity.

What are the targets of DAF-16 in integrative behavior for temperature and food? Insulin-like signaling is a part of the dauer formation pathway, which has many feedback loops (Schackwitz et al. 1996). The results of the present study are consistent with a feedback loop in insulin-like signaling for this integrative behavior. Assuming the existence of a feedback loop, one clue for the targets of DAF-16 in the thermotactic plasticity might be found in a report by Murphy et al. (2003), which suggested that the insulin homologs INS-2, INS-7, INS-18, and INS-21 are likely to be direct or indirect targets of DAF-16. Likewise, it is plausible that any of these insulin-like molecules act agonistically on DAF-2 to activate the insulin-like pathway in food-associated thermotactic plasticity. We believe that these issues are critical for further study.

Materials and methods

Strains and genetics
The standard techniques were used for culturing and handling C. elegans. For details and strains, see Supplemental Material.

Behavioral assays
A radial temperature-gradient assay was performed as described previously (Mori and Ohshima 1995; Mohri et al. 2005). The locomotory rate assay was performed according to a previous report (Sawin et al. 2000). The interaction assay was also performed as previously described (Ishihara et al. 2002). For details, see Supplemental Material.

Molecular biology and germline transformation
Standard methods for molecular biology and germline transformation were used. For details and vectors, see Supplemental Material.

In vivo calcium imaging and data analysis
In vivo calcium imaging was performed essentially according to Kimura et al. (2004) and Kuhara and Mori (2006). For details, see Supplemental Material.

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