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Effects of sex and insulin/IGF-1 signaling on performance in an associative learning paradigm in *Caenorhabditis elegans*

Tibor Vellai[*,1], Diana McCulloch[†], David Gems[†] and Attila L. Kovács[§]

[*]*Department of Genetics, Eötvös Loránd University, Budapest, H-1117, Hungary*

[†]*Centre for Research on Ageing, Department of Biology, University College London, London, UK*

[§]*Department of Anatomy, Cell and Developmental Biology, Eötvös Loránd University, Budapest, H-1117, Hungary*

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[1]*Corresponding author:* Tibor Vellai

Address: Department of Genetics, Eötvös Loránd University, Pázmány Péter sétány 1/C, Budapest, H-1117, Hungary

Tel.: (36-1)-209-0555 Ext: 8684

Fax: (36-1)-209-0555 Ext: 1841

E-mail: vellai@falco.elte.hu

ABSTRACT

Learning is an adaptive change in behavior in response to environmental stimuli. In mammals, there is a distinct female bias to learn skills that is still unprecedented in other animal taxa. Here we have investigated the biological determinants of performance in an associative learning paradigm in the nematode *Caenorhabditis elegans*. Using an assay of chemotactic reactions associated with food deprivation, wild-type male worms show inferior learning ability relative to hermaphrodites. Sex-based learning difference is therefore an ancient evolutionary feature appearing even in relatively simple animals. *C. elegans* mutants with reduced insulin/IGF-1 signaling (IIS) also exhibit a greatly reduced learning ability in this learning assay. In addition, hyperactivation of IIS through loss-of-function mutations in the *PTEN* phosphatase *daf-18*, a negative regulator of IIS, enhances learning ability beyond that of wild type. According to our epistasis analysis, the effect of DAF-2 on learning acts via phosphatidylinositol 3,4,5-trisphosphate (PIP_3) production, but not the DAF-16 FOXO transcription factor. This implies that the signaling pathway from DAF-2 affecting this learning paradigm branches between PIP_3 production and DAF-16. However, learning capacity of nematodes is lowered by loss-of-function mutations in *daf-16*, suggesting involvement of non-IIS-dependent DAF-16 activation in learning. Potentially, sex and IIS affect performance in this learning assay via effects on the neurobiology of learning.

INTRODUCTION

ASSOCIATIVE learning occurs when an animal learns to link a stimulus or behavior with a second temporally associated stimulus (JORGENSEN and RANKIN 1997). Progress in discovering conserved signaling pathways involved in learning is greatly facilitated by studying animals with a relatively simple and tractable nervous system (HIRSCH and BOUDREAU 1958; HOTTA and BENZER 1970; KELLEHER *et al*. 2004). The free-living nematode *C. elegans* with 302 neurons in adult hermaphrodites is an excellent model organism to analyze neuronal function (WHITE *et al.* 1986; BARGMANN and MORI 1997; KENYON 1997).

In *C. elegans*, a male-specific behavior, mate searching, is influenced by a conserved neuroendocrine system, the insulin/IGF signaling (IIS) pathway (LIPTON *et al.* 2004). Aging and reproductive growth are also regulated hormonally by IIS in this organism (KENYON 1997; RIDDLE and ALBERT 1997; GUARENTE and KENYON 2000), and it is possible that this regulation is sexually dimorphic. For example, wild-type males are more predisposed than hermaphrodites to form diapausal dauer larvae in response to starvation and high population density (AILION and THOMAS 2000). (The self-fertilizing *C. elegans* hermaphrodite is essentially a female that generates sperm for a brief period before oogenesis.) Given that reduction in IIS or signaling via the *daf-7* TGF-β pathway increase dauer formation (RIDDLE and ALBERT 1997), this suggests that flux through either pathway might be constitutively reduced in males. In addition, wild-type males live about 20% longer than hermaphrodites (GEMS and RIDDLE 2000).

This male longevity advantage requires the IIS-regulated transcription factor DAF-16; however, in *daf-2(m577)* mutants a male longevity advantage was seen similar to that in wild-type, suggesting that while DAF-16 levels are higher in males, IIS level is not lower (GEMS and RIDDLE 2000). In spite of these observations, the nature of sexual dimorphism in endocrine function in *C. elegans* remains almost unexplored. In addition, the tissues at which the IIS pathway controls lifespan appear to include the nervous system (WOLKOW *et al.* 2000; LIBINA *et al.* 2003).

In this study, we investigate the role of IIS in the learning process in *C. elegans*, and whether this regulation operates in a different way in males and hermaphrodites. We show that male nematodes perform less than hermaphrodites in a learning paradigm in which paired presentation of salt and starvation induces a change in behavior. We also demonstrate a strong influence of the IIS pathway on performance in this simple learning assay. Mutant worms with reduced IIS are defective in learning, while mutational hyperactivation of the IIS cascade results in enhancement of learning ability beyond that of wild type.

MATERIALS AND METHODS

***C. elegans* strains:** Strains were maintained on nematode growth medium (NGM) as described (BRENNER 1974). The strain var. Bristol N2 was used as wild-type. Single and double mutant strains used in this study were: DR466 *him-5(e1490)V*, CB1489 *him-8(e1489)IV*, CB1370 *daf-2(e1370)III*, DR1564 *daf-2(m41)III*, DR1572 *daf-2(e1368)III*, TJ1052 *age-1(hx546)II*, JT709 *pdk-1(sa709)X*, GR1307 *daf-16(mgDf50)I*, CF1038 *daf-16(mu86)I*, NS3227 *daf-18(nr2037)IV*, CB1375 *daf-18(e1375)IV*, GR1308 *daf-2(e1370); daf-16(mgDf54)*, GR1309 *daf-2(e1370); daf-16(mgDf47)*, BU050 *daf-2(e1370); him-5(e1490)*, BU051 *daf-2(e1368); him-8(e1489)*, BU052 *daf-18(nr2037) him-8(e1489)*, BU053 *daf-18(e1375) him-8(e1489)*, CB2590 *tra-1(e1099)/+ dpy-18(e1096)/+III*, CB2810 *tra-1(e1575)/+III; unc-42(e270)him-5(e1490)dpy-21(e428)V*.

RNA interference:

To generate a *daf-18/PTEN* RNA interference (RNAi) construct, reverse-transcriptase (RT) -PCR experiment was performed to amplify a *daf-18* cDNA fragment that was cloned into the vector pPD129.36 (kindly provided by A. Fire). Forward and reverse primers were used as follows: 5-CAT GCC ATG GCA TGT TCC ATC ACA ACG ACG CTA C-3 and 5'-CAT GCC ATG GCA TGC CGA ACA CTT CGC TCT TTT C-3'. RNAi experiments were performed as described (KAMATH *et al.* 2000).

Behavioral assays: Chemotaxis towards NaCl was assayed as described (SAEKI *et al.* 2001), with some modifications. For preparing the assay plate, an agar plug from a 100

mM NaCl-containing NGM plate was placed on a NaCl-free NGM plate for 12 hours. The plug was then removed, and 1 µl of 0.5M Sodium-azide (NaN_3) was added onto the same position to anaesthetize the worms. As a control, 1 µl NaN_3 was also spotted at 4 cm away from the centre of the NaCl gradient. For assaying changes in chemotaxis, well-fed animals were washed off from NGM plates, and transferred either directly onto the assay plates (naive) or onto food-free NGM plates (conditioned). Bacterium-free NGM plates without NaCl were used as controls. After 4 hours of conditioning, approximately 100 young adults were transferred individually by platinum wire, equidistant from the two NaN_3 spots, onto an assay plate. Worms were left there to move freely for 30 minutes, and the number of animals within 1.5 cm of each spot was counted. The chemotaxis index was calculated as $CI=N_N-N_C/N_T$ (N_N: the number of animals around the NaCl gradient, N_C: the number of animals around the control spot, N_T: total number of animals). Assays were performed in triplicates, thus in each single experiment N represents around 300 animals.

Dauer formation analysis: The progeny of mated hermaphrodites (50-50% male/hermaphrodite ratio) were grown at a temperature (for wild-type, 25˚C; for *daf-2* mutants, 22.5˚C) that gives a mix of dauers and non-dauers in the population (AILION and THOMAS 2000). Dauer formation of wild-type worms was induced by applying partially purified exogenous dauer pheromone. Dauers were then recovered under permissive conditions, and the sex of non-dauers and recovered dauers was scored.

RESULTS

Effect of sex on the capacity of *C. elegans* to associate NaCl with starvation: Grown under well-fed conditions, *C. elegans* shows chemoattraction to 0.1 - 200 mM NaCl (WARD 1973). However, migration of worms toward salt falls dramatically following starvation on agar plates with NaCl (SAEKI *et al*. 2001). Since this altered response to salt is induced by paired presentation of NaCl and food deprivation, it involves experience and thus represents a form of associative learning. Chemotaxis towards Na^+ and Cl^- ions in *C. elegans* requires a sensory amphid neuron pair called ASE (BARGMAN and HORVITZ 1991), but little is known about the mechanisms underlying such behavioral plasticity (SAEKI *et al*. 2001; ISHIHARA *et al*. 2002). This study is an exploration of several aspects of these mechanisms.

First, we compared learning ability in wild-type *C. elegans* males and hermaphrodites. To quantify changes in chemotactic behavior, we deprived young adult worms of food for 4 hours, and then transferred them individually from conditioning plates to assay plates (Figure 1A). Interestingly, wild-type males conditioned with paired presence of starvation and 100 mM NaCl (salt) were less repelled by salt than hermaphrodites. Whereas 44% of wild-type males continued to move toward salt after conditioning, only 23.5% of hermaphrodites behaved in the same way under identical conditions (Figure 1B). This weaker behavioral change of males was also apparent in mutant strains that segregate males with high frequency (HODGKIN *et al*. 1979). Both *him-8(e1489)* and *him-5(e1490)* mutant males were able to respond to conditioning, and they were less likely to

avoid the salt than the corresponding hermaphrodites (Figure 1B). The fact that both sexes showed an equal degree of attraction to NaCl rules out the explanation that different response to paired NaCl and starvation is due to the slight hyperactivity of males.

The primary determinant of sex in *C. elegans* is karyotype: animals with one sex chromosome (XO) are normally males, and animals with two sex chromosomes (XX) are hermaphrodites. To test whether the sex difference in learning ability is a direct consequence of karyotype, or of the resultant state of differentiation, we monitored behavioral plasticity in mutant nematode populations where the relationship between genotypic and phenotypic sex was reversed. *tra-1(e1099)* loss-of-function mutant XX animals form fertile males (pseudomales), while *tra-1(e1575)* gain-of-function mutant XO animals are fertile females (HODGKIN 1987). In this learning assay, *tra-1(e1099)* XX males behaved like wild-type XO males (i.e. they learned less well), and *tra-1(e1575)* XO females behaved like wild-type XX hermaphrodites (i.e. they showed superior learning ability) (Figure 1B). Thus, the sex difference in learning is the result of phenotypic rather than genotypic sex.

One possibility is that the sex difference in learning is the result of a sex difference in the capacity to sense salt. To test this possibility we determined the male and hermaphrodite threshold responses in this associative learning paradigm. We first compared chemoattraction to salt of hermaphrodites and males over a range of salt concentrations. Over a 1 – 100 mM concentration range, in both sexes, a similar degree of attraction was

seen (Figure 1C). However, both sexes showed a significant reduced attraction to 0.1 mM NaCl ($p = 0.001$, unpaired *t*-test). Importantly, there was no significant sex difference in chemoattraction, even at 0.1 mM NaCl, implying sexual equality in the ability to sense salt ($p = 0.65$, unpaired *t*-test).

We also compared associative learning in hermaphrodites and males over this range of salt concentrations (Figure 1C). The threshold salt concentration at which animals showed full associative learning proved to be lower in hermaphrodites (0.1-1 mM) than in males (1-10 mM). Furthermore, at all NaCl concentrations tested, males learned less well than hermaphrodites (Figure 1C). Because males are no less able to sense salt than hermaphrodites (as judged by their chemo-attractive responses), this difference in threshold salt concentration for full associative learning is likely to reflect a sex difference in learning rather than capacity to sense salt.

Our results imply the existence of a sex difference in learning ability in *C. elegans*. However, it remains possible that this difference is specific to the assay used here. For example, it could reflect a sex difference in the biology of nutrition. Potentially, males may be less sensitive to the nutritional environment, either in terms of chemosensation of food, or internal sensing of starvation or satiety. This could explain why they show less avoidance of salt after conditioning.

We probed this possibility by examining the effect of food on locomotion in males. Nutritionally replete males moved more slowly in the presence of food (22 ± 0.7 bends in

the anterior body region of moving animals during a 30 second interval) than in the absence of food (28 ± 1.5 bends). This is similar to the basal locomotion response of wild-type hermaphrodites to food (SAWIN *et al.* 2000). In addition, males moved much more slowly on food after starvation (enhanced slowing response; 14 ± 1.9 bends on plates with bacteria versus 29 ± 0.9 bends on plates without bacteria). Thus, the gross behavioral responses to food are similar in the two sexes. This would seem to suggest that the sex difference in learning reported here reflect sex differences in associative learning rather than in response to the nutritional environment.

Effects of IIS on the capacity of nematodes to associate NaCl with starvation: We next investigated the possible genetic basis of this sex-dependent behavioral response. In *C. elegans*, reproductive growth, lifespan and several aspects of behavior are regulated hormonally by a conserved neuroendocrine system, the IIS pathway (Figure 2A). Several findings are consistent with sex differences in neuroendocrine function in *C. elegans* (see Introduction). We therefore asked whether IIS influences associative learning in *C. elegans.*

As shown in Figure 2B, reduction-of-function mutations in the gene *daf-2*, which encodes the worm insulin/IGF-1 receptor (KIMURA *et al.* 1997), blocked associative learning in hermaphrodites at 25°C where *daf-2* mutant traits are highly temperature sensitive. In the assay used these *daf-2* mutant animals continued to migrate toward NaCl instead of avoiding it. We also tested two downstream components of this signal transduction pathway, *age-1* and *pdk-1* (Figure 2A), which encode a phosphatidylinositol-3-OH kinase

(PI 3-kinase) catalytic subunit and a phosphoinositide-dependent kinase, respectively (MORRIS *et al.* 1996; PARADIS *et al.* 1999). *age-1(hx546)* and *pdk-1(sa709)* mutants were defective in associative learning, although only moderately in the latter (Figure 2B).

The poor performance in this learning assay of mutants with reduced IIS could reflect a role of IIS in learning; alternatively, it could simply reflect general defects in neuronal or other function in IIS mutants. Only if the former were true would an engineered increase in IIS be predicted to result in an enhancement of learning ability. We therefore tested whether increased IIS results in nematodes with enhanced capacity to associate salt with starvation, employing *nr2037*, a null mutation in *daf-18*. This gene encodes an ortholog of the human tumor suppressor protein PTEN, a negative regulator of the IIS pathway (OGG and RUVKUN 1998; GIL *et al.* 1999; MIHAYLOVA *et al.* 1999; ROUAULT *et al.* 1999). *daf-18(0)* elevates IIS activity by increasing the level of phosphatidylinositol 3,4,5 trisphosphate (PIP_3), the lipid secondary signal produced by the AGE-1/AAP-1 PI 3-kinase. Significantly, *daf-18(0)* resulted in associative learning that was enhanced relative to wild-type (Figure 2B).

How does IIS influence behavior in this learning paradigm? There are many possibilities, given that IIS affects various aspects of the biology of *C. elegans*, including development, morphology, behavior, metabolism and aging (RIDDLE and ALBERT 1997; GEMS *et al.* 1998; LIPTON *et al.* 2004). One possibility is that it directly controls the neurobiological processes involved in associative learning. In mammals, insulin and IGF-1 signaling mediate effects of food on metabolism and growth. Thus, mutational

reduction of IIS may result in a lack of response to the nutritional environment, due to an internal perception of starvation. To probe this latter possibility, we monitored basal and enhanced slowing responses in IIS deficient worms (SAWIN *et al.* 2000). Both well-fed and starved *daf-2(e1370)* mutant nematodes showed a significant response, in terms of locomotion, to food and to starvation (data not shown). This is consistent with at least some perception of food in *daf-2* mutants. Moreover, mutations in *daf-2* and *age-1* were shown recently to influence behavioral plasticity of temperature-induced responses (MURAKAMI et al. 2005), supporting the role of IIS in learning *per se*.

Associative learning is partially dependent on IIS-independent action of the DAF-16 FOXO transcription factor: A range of mutant phenotypes of IIS-deficient worms requires the activity of the forkhead transcription factor DAF-16, for example constitutive dauer formation (RIDDLE and ALBERT 1997), increased lifespan (Kenyon *et al.* 1993; OGG *et al.* 1997) and reduced fecundity and movement (GEMS *et al.* 1998). Interestingly, mutational inactivation of *daf-16* only partially suppressed the learning defective phenotype of *daf-2(e1370)* hermaphrodites at all (Figure 2B). Thus, DAF-2 acts independently of DAF-16 in its effects on this learning paradigm. In fact, in contrast to *daf-18*, mutation of *daf-16* in an otherwise wild-type background markedly reduced hermaphrodite learning capacity (Figure 2B).

Possible link between sex bias in associative learning and IIS: Maleness and lowered IIS in *C. elegans* lead to inferior ability to associate salt with starvation. Is it possible that a constitutive reduction in IIS in wild-type male nematodes accounts for their inferior

performance in this assay? We probed further the relationship between sex and DAF-2 activity. Wild-type males exposed to dauer pheromone form more dauer larvae than hermaphrodites (AILION and THOMAS 2000). This male bias to dauer larva formation was not seen when dauers were produced constitutively as the result of reduction-of-function mutations in *daf-2* (Table 1). We also compared associative learning in *daf-2* mutant males and hermaphrodites, and observed the same failure to learn in each (Figure 3A).

If the inferior learning capacity of wild-type male nematodes described here results from a constitutive reduction in IIS, then increasing IIS levels should increase associative learning in males to that of hermaphrodites. To this we used the *daf-18(0)* mutation, as described above. Strikingly, *daf-18(0)* resulted in an elevated learning capacity which was not different to that in mutant hermaphrodites (Figure 4). *nr2037* also weakly suppressed the learning defective phenotype of *daf-2(e1370)* mutants in both sexes (Figure 4). The results for *daf-2* or *daf-18* could imply that the sex difference reflects constitutive reduction in IIS in males. Alternatively, *daf-2* and *daf-18* mutations could be epistatic to the sex bias, or the sex bias could arise from a parallel pathway.

DISCUSSION

Sex differences in learning ability has been observed in higher animal taxa, including primates, where males have an inferior learning ability (LONDSDORF *et al.* 2004; BLOTE and VAN GOOL 1989; VEDERHUS and KREKLING 1996). In this study we showed the existence of a distinct sex bias in associative learning in *C. elegans* (Figure 1B, C). Male nematodes learn less well than hermaphrodites in a learning paradigm in which paired presentation of salt and starvation induces changes in chemotactic behavior (SAEKI *et al*. 2001). Because males respond to the presence and absence of food, this learning bias is not a simple consequence of the male's incapability to sense food. However, it cannot be excluded that there might be differences in the efficiency of food sensation between males and hermaphrodites that could influence the learning process. So far there is no indication of this possibility.

We also provide evidence of an apparent regulatory role of IIS in the nematode rudimentary learning capacity (Figure 2B, C). *daf-2* and *age-1* mutant nematodes exhibit inferior learning ability relative to wild-type (Figure 2B). Furthermore, mutations in the *PTEN* phosphatase *daf-18*, which increase IIS, result in an enhancement of learning ability. The role of IIS in nematode learning is further supported by a recent study of IIS pathway mutants, which demonstrated their influence on behavioral plasticity in response to changing temperature (MURAKAMI et al. 2005).

There is accumulating evidence implicating IIS in cognitive functions in mammals. In mice, for example, the intestinal glucagon-like peptide GLP-1, which influences glucose metabolism by stimulating insulin synthesis and secretion, improves learning and memory (DURING *et al.* 2003). The presence of insulin receptor and IGF-1 receptor in discrete regions of the rat brain (ADAMO *et al.* 1989; UNGER *et al.* 1991; ZHAO *et al.* 1999) suggests their functional involvement in brain cognition function. Lesions to the hippocampus produce severe loss of learning ability and memory that can be significantly reversed by insulin treatment (DE CASTRO and BALAGURA 1976; HOREL 1978; HUPPERT and PIERCY 1979; SPIERS *et al.* 2001). Both insulin receptor substrate-1 and phosphatidyinositol-3 phosphate kinase are abundantly expressed in the rat hippocampus (FOLLI *et al.* 1994). The PI3K inhibitor LY294002 blocks learning and memory in mice (BARROS *et al.* 2001; LIN *et al.* 2001). Together, these data point to a role of IIS in cognitive functions in divergent animal phyla. The mechanisms by which IIS controls mammalian cognition is poorly understood (DURING *et al.* 20003; ZHAO *et al.* 2004). This study shows that *C. elegans* represents a tractable genetic model organism for unraveling the details of how the IIS hormonal system affects learning and memory.

It is intriguing that the effect of mutation of *daf-2* on *C. elegans* performance in the salt-starvation association assay involves PI3-kinase and PIP_3 generation, but not the forkhead transcription factor DAF-16 FOXO (Figure 2B). This implies that the IIS pathway branches at PIP3 to control learning, via a distinct PIP_3-sensitive pathway, whose identity it would be interesting to learn. Interestingly, our findings clearly show that *daf-16* is partially required for learning in *daf-2(+)* animals. Together, these results suggest that

daf-16 plays a role in learning in the assay used that is *daf-2* independent. One possibility is that starvation results in DAF-16 activation, as part of the stress response, and that this response constitutes part of the experience of starvation that is associated with salt. In this context, DAF-16 could be responding to the stress-sensitive c-Jun N-terminal kinase (JNK) pathway, which activates DAF-16 (OH et al. 2005). However, a positive role of DAF-16 in learning argues against that a constitutive decrease of IIS and increase in DAF-16 activity accounts for the poor learning ability of males. This working hypothesis was probed by testing the effect of mutations of *daf-2* and *daf-18* on sex differences in learning. In both cases, the sex difference was suppressed; however, this could imply a) that the sex difference reflects constitutive reduction in IIS in males; b) that *daf-2* and *daf-18* mutations are epistatic to the sex bias, or c) that the sex bias arises from a parallel pathway.

Recently, it was shown that IIS interacts with the nutrient-sensing TOR (for "Target Of Rapamycin") kinases to control lifespan, metabolism and development (SALTIEL and KAHN 2001; VELLAI *et al.* 2003; JIA *et al*. 2004). The results presented here suggest a possible interrelationship between nutrition and learning through metabolism regulated by IIS. Potentially, the coordinated hormonal control of food intake, energy metabolism and learning by the IIS cascade may improve the ability of an organism to utilize its limited food resources in such a way as to maximize fitness.

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TABLES

Table 1. Suppression of the male bias to form dauers by mutations in *daf-2*.

Genotype	**Temp (°C)**	**% Males forming dauers**	**% Herms. forming dauers**	**M:H dauer ratio**	**N**	**P**
Wild-type, pheromone	25	35.8 ± 8.9	29.9 ± 5.0	1.20	369	> 0.1
daf-2(e1370)	22.5	31.9 ± 5.2	40.0 ± 5.4	0.80	766	> 0.1

Progeny of mated hermaphrodites were raised at a temperature that gave a mix of dauers and non-dauers. The sex of recovered dauers was scored and the overall ratio of male:hermaphrodite dauer formation was determined. ± represents mean standard error. Herms, hermaphrodites.

FIGURE LEGENDS

Figure 1. - Sex differences in the ability of young adult worms to modulate behavior. (A) Schematic diagram of an assay plate. The dark area indicates a local NaCl-containing region. Young adults were picked up individually from naive or conditioning plates, placed on the starting position of the assay plate and left to move freely. The number of worms around each NaN_3 spot was counted. (B) Reduced capacity in starvation-induced suppression of chemotaxis of wild-type (N2) males as well as of *him-8(e1489)*, *him-5(e1490)* and *tra-1(e1099)* mutant males. $p < 0.001$, except for *him-5(e1490)* males where $p < 0.01$ (unpaired t-test). For the chemotaxis index ($CI=N_N-N_C/N_T$, where N_N: the number of animals around the NaCl gradient, N_C: the number of animals around the control spot, N_T: total number of animals), positive values demonstrate attraction to NaCl and negative values repulsion from NaCl. Worms were assayed for chemotaxis directly (naive), or conditioned by starving on NGM plate either without (-) or with (+) NaCl for 4 hours. Neither mutants exhibited significant changes in behavioral response from the corresponding naives when they were conditioned on NaCl-free plates (data not shown). *tra-1(e1575)* gain-of-function mutants with XO karyotype are hermaphrodites whereas *tra-1(e1099)* loss-of-function mutant animals with XX karyotype are fertile males. (C) Chemotaxis toward various concentration of NaCl. At 0.1 mM NaCl, where only a fraction of nematodes responds to salt, males are still less repelled by salt than hermaphrodites ($p < 0.01$, unpaired *t*-test). Herm: Hermaphrodite. Error bars indicate mean ± standard error.

Figure 2. - Mutant worms with decreased insulin/IGF-1 signaling are defective in associative learning. (A) Regulatory hierarchy among the components of the insulin/IGF-1 genetic cascade in *C. elegans*. Arrows indicate activations, bars indicate negative regulatory interactions. DAF-2, the worm insulin/IGF-1 receptor; AGE-1, phosphatidylinositol-3-OH kinase (PI3K); DAF-18, PTEN phosphatase - the only known negative regulator of insulin/IGF-1 signaling; PDK-1, AKT-1, AKT-2 and SGK-1, phosphoinositide-dependent serine/threonine kinases; DAF-16, FOXO forkhead transcription factor. (B) Wild-type and insulin/IGF signaling-defective mutant hermaphrodites maintained at 20°C were shifted to 25°C, and assayed for chemotaxis directly (naive) or conditioned on food-free NGM plates with NaCl. None of these strains displayed significant changes in behavior from the corresponding naives when they were conditioned on NaCl-free plates (data not shown). A genetic null mutation in *daf-18*, *nr2037*, improves the ability of hermaphrodites to change behavior. Error bars indicate mean ± standard error.

Figure 3. – The sex bias in associative learning in *C. elegans* is suppressed by mutations in *daf-2*. $P < 0.001$ (unpaired *t* test). Error bars indicate mean ± standard error.

Figure 4. - Mutations in the *PTEN* phosphatase *daf-18* increase the ability of nematodes to modulate behavior. The *daf-18* null mutation *nr2037* restores the ability of males to change behavior to levels comparable with those of mutant hermaphrodites. $p < 0.22$ (unpaired *t*-test). *nr2037* partially suppresses the learning defective phenotype of *daf-*

2(e1370) males. $p < 0.01$ (unpaired *t*-test). Herm, Hermaphrodite. Error bars indicate mean ± standard error.

Figure 1

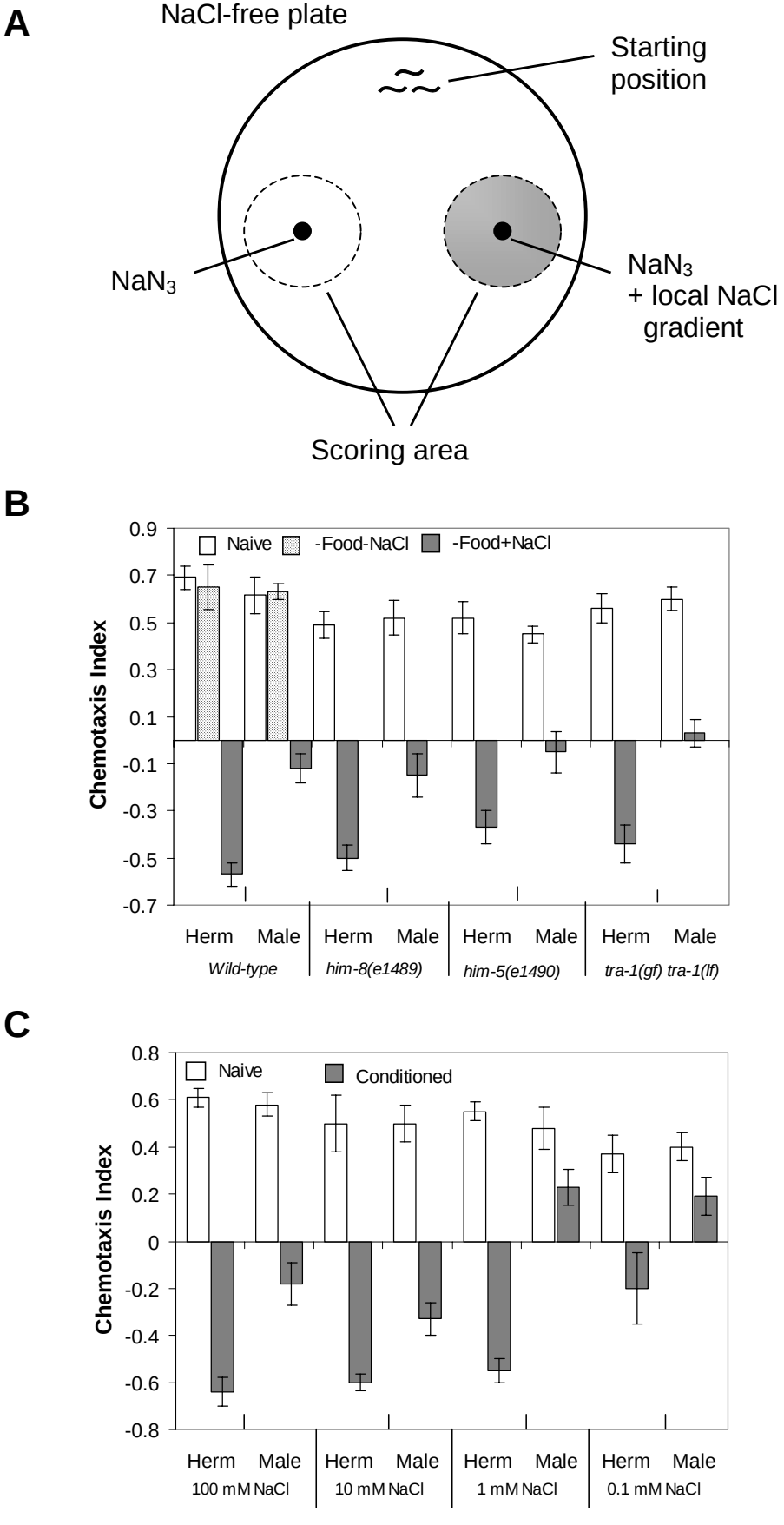

A
NaCl-free plate
Starting position
NaN3
NaN3 + local NaCl gradient
Scoring area
B
Naive
-Food-NaCl
-Food+NaCl
Chemotaxis Index
0.9
0.7
0.5
0.3
0.1
-0.1
-0.3
-0.5
-0.7
Herm Male Herm Male Herm Male Herm Male
Wild-type
him-8(e1489)
him-5(e1490)
tra-1(gf) tra-1(lf)
C
Naive
Conditioned
Chemotaxis Index
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
Herm Male Herm Male Herm Male Herm Male
100 mM NaCl
10 mM NaCl
1 mM NaCl
0.1 mM NaCl

Figure 2

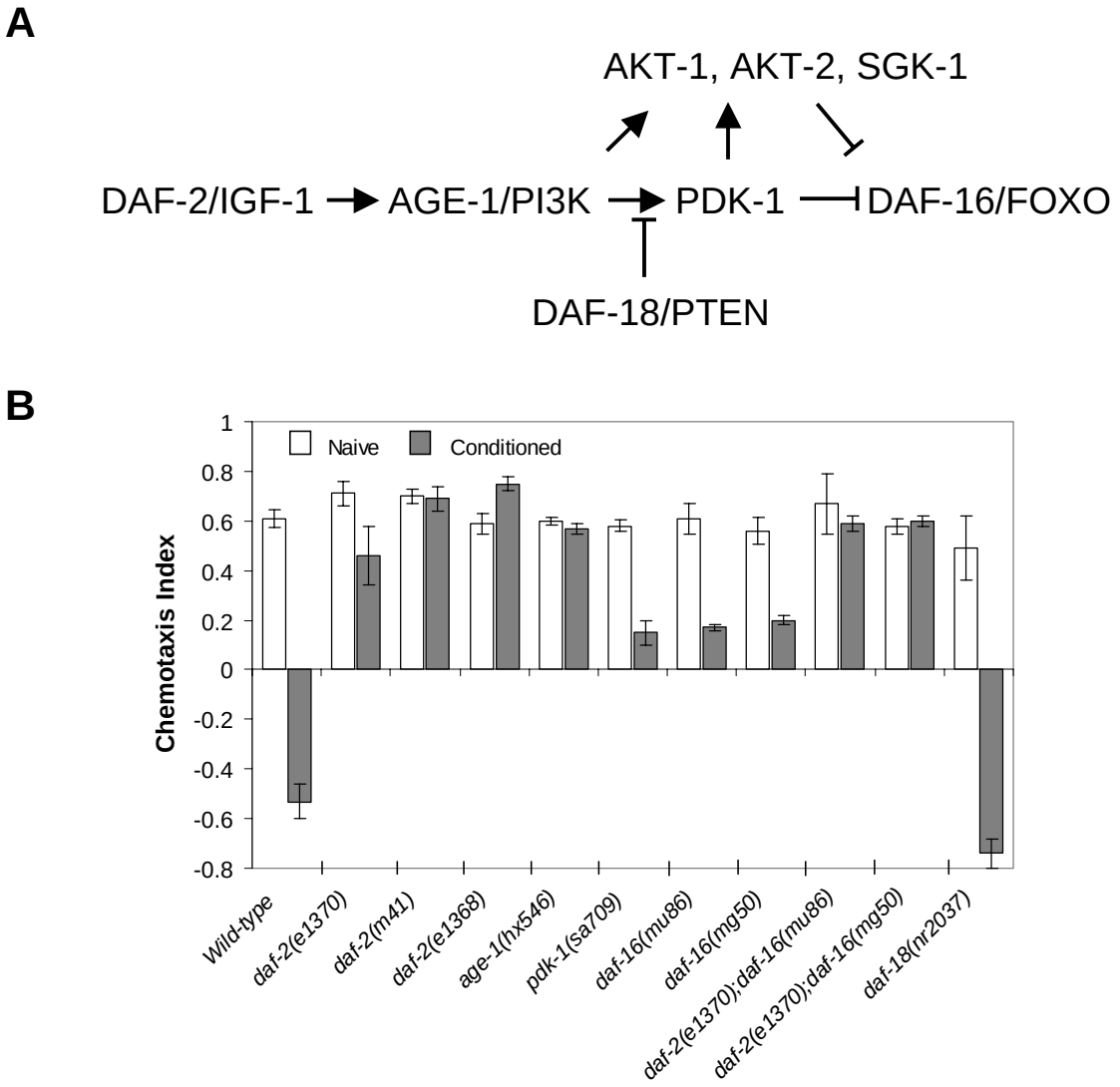

A
AKT-1, AKT-2, SGK-1
DAF-2/IGF-1
AGE-1/PI3K
PDK-1
DAF-16/FOXO
DAF-18/PTEN
B
Naive
Conditioned
Chemotaxis Index
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
Wild-type
daf-2(e1370)
daf-2(m41)
daf-2(e1368)
age-1(hx546)
pdk-1(sa709)
daf-16(mu86)
daf-16(mg50)
daf-2(e1370);daf-16(mu86)
daf-2(e1370);daf-16(mg50)
daf-18(nr2037)

Figure 3

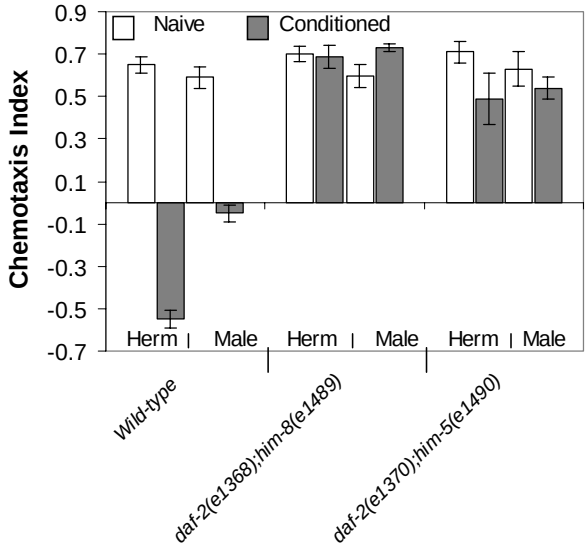
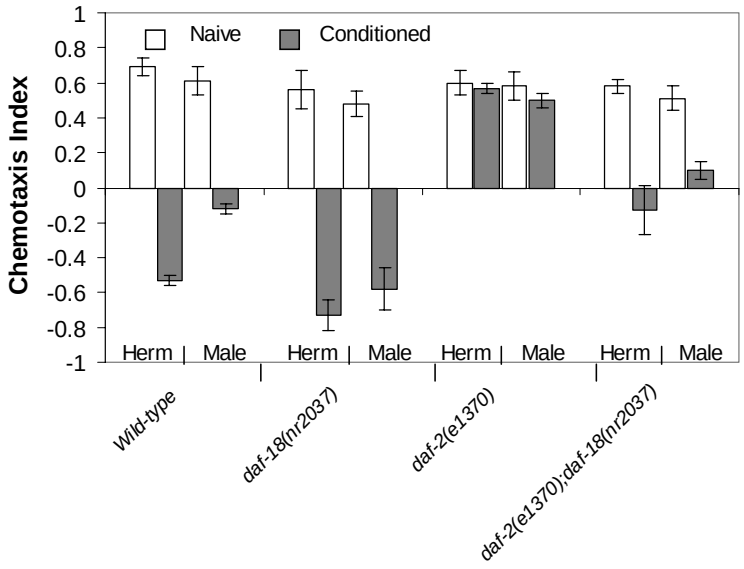

Naive
Conditioned
Chemotaxis Index
0.9
0.7
0.5
0.3
0.1
-0.1
-0.3
-0.5
-0.7
Herm
Male
Herm
Male
Herm
Male
Wild-type
daf-2(e1368);him-8(e1489)
daf-2(e1370);him-5(e1490)

Figure 4


Chemotaxis Index
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
Naive
Conditioned
Herm
Male
Herm
Male
Herm
Male
Herm
Male
Wild-type
daf-18(nr2037)
daf-2(e1370)
daf-2(e1370);daf-18(nr2037)