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A cyclic nucleotide-gated channel inhibits sensory axon outgrowth in larval and adult *Caenorhabditis elegans:* a distinct pathway for maintenance of sensory axon structure

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SUMMARY

The *tax-2* and *tax-4* genes of *C. elegans* encode two subunits of a cyclic nucleotide-gated channel that is required for chemosensation, thermosensation and normal axon outgrowth of some sensory neurons. Here we show that, in *tax-2* and *tax-4* mutants, young larvae have superficially normal axons, but axon outgrowth resumes in inappropriate regions in late larval stages. Using a temperature-sensitive mutation in *tax-2*, we find that *tax-2* activity is required during the adult stage to preserve normal axon morphology. These results indicate that *tax-2* and *tax-4* are required for the maintenance of correct axon structure, and reveal an unexpected plasticity that allows *C. elegans* axons to be remodeled long after their initial connections have been established. TAX-2 and TAX-4 have been proposed to form a transduction channel for chemosensation and thermosensation, and *tax-2* activity is required in the adult stage for normal chemotaxis to NaCl and odorants. Animals mutant for the *daf-11* gene have axon phenotypes that are similar to those of *tax-2* and *tax 4* mutants; this axon phenotype also has a late time of action. *daf-11* regulates a developmental process called dauer larva formation that is controlled by sensory stimuli, and *tax-2* and *tax-4* can either stimulate or inhibit dauer larva formation in different contexts.

Key words: *Caenorhabditis elegans*, *tax-2*, Sensory axon, Outgrowth, Cyclic nucleotide-gated channel, Axon

INTRODUCTION

Cyclic nucleotide-gated channels have prominent functions in vertebrate and invertebrate sensory transduction, and additional functions during neuronal development that are less well understood (Zagotta and Siegelbaum, 1996). Vertebrate phototransduction is mediated by regulation of cGMP-sensitive channels and vertebrate olfaction utilizes a similar channel that responds to the second messenger cAMP. In the nematode *C. elegans*, olfaction, taste and thermosensation are mediated by cyclic nucleotide-gated channels encoded by the *tax-4* and *tax-2* genes (Dusenbery et al., 1975; Hedgecock and Russell, 1975; Coburn and Bargmann, 1996; Komatsu et al., 1996). *tax-4* and *tax-2* are also required for normal axon guidance of a subset of sensory neurons, and a TAX-2::GFP fusion protein is localized to both developing axons and sensory cilia (Coburn and Bargmann, 1996). Vertebrate cyclic nucleotide-gated channels are also found on axons of developing and mature neurons, where their function is unknown (Bradley et al., 1997).

tax-2::GFP and *tax-4::GFP* fusion genes are expressed in nine pairs of sensory neurons associated with the amphid sensory organs (Coburn and Bargmann, 1996; Komatsu et al., 1996). *tax-4* encodes an α subunit of a cyclic nucleotide-gated channel, while *tax-2* encodes a predicted β subunit. Genetic data suggest that the products of *tax-2* and *tax-4* form a heteromeric channel of α and β subunits, as is observed in vertebrate sensory systems (Coburn and Bargmann, 1996; Komatsu et al., 1996). Many of the neurons that express *tax-2* and *tax-4* are defective in *tax-2* and *tax-4* mutants, including the AFD neurons that sense temperature, the ASE neurons that sense water-soluble attractants, the AWC neurons that sense some attractive odorants and the AWB neurons that sense repulsive odorants (Mori and Ohshima, 1995; Bargmann and Horvitz, 1991a; Bargmann et al., 1993; Troemel et al., 1997). The AWA neurons that sense some attractive odorants and the ASH neurons that sense aversive chemical and mechanical stimuli do not express or require *tax-2* and *tax-4*. Instead, these neurons require an alternative predicted channel, OSM-9, which is similar to the capsaicin receptor channel implicated in mammalian pain sensation (Colbert et al., 1997; Caterina et al., 1997).

The ASE and ASJ neurons have high-penetrance axon outgrowth defects in *tax-2* and *tax-4* mutants, but the other sensory neurons are superficially normal in morphology (Coburn and Bargmann, 1996). In *tax-2* and *tax-4* mutants,

ASE and ASJ axons are found in aberrant posterior ventral and lateral positions. This phenotype implicates the cyclic nucleotide-gated channel in axon outgrowth or guidance. In other systems, cyclic nucleotides have effects on synaptic plasticity (Zhong et al., 1992) and axon guidance (Song et al., 1997); the cyclic nucleotide-gated channel represents one potential target for cAMP and cGMP during development.

tax-2::GFP and *tax-4::GFP* are also expressed in three neurons that regulate a developmental decision between two alternative larval stages. After its second molt, *C. elegans* can become either a third-stage larva that will develop to a fertile adult or an arrested dauer larva. The ASI and ASG neurons express *tax-2* and *tax-4* and regulate entry into the dauer stage in concert with the ADF neurons, which do not express *tax-2* and *tax-4* (Bargmann and Horvitz, 1991b). The ASJ neurons express *tax-2* and *tax-4* and regulate both entry into and exit from the dauer stage (Bargmann and Horvitz, 1991b; Schackwitz et al., 1996). *C. elegans* decides whether to undergo normal or dauer development mainly in response to a pheromone that reflects nematode density, with modulatory cues from food and temperature (Golden and Riddle, 1984). Mutations in ten genes lead to dauer-constitutive mutants that always form dauer larvae, while a separate set of dauer-defective mutants never form dauer larvae (Riddle et al., 1981). The dauer-constitutive genes include the partly redundant group 1 and group 2 dauer-constitutive genes. Animals with a mutation in either a group 1 gene or a group 2 gene can pursue non-dauer development at low temperatures, though they always form dauer larvae at high temperatures (Riddle et al., 1981). However, animals mutant for both group 1 and group 2 genes always form dauer larvae at all temperatures (Thomas et al., 1993). Both group 1 and group 2 genes seem to affect sensory neuron function. The group 2 genes control a TGF-β/BMP-type signalling pathway, including the DAF-7 ligand molecule that appears to be produced by the ASI sensory neurons (Ren et al., 1996; Schackwitz et al., 1996). The group 1 mutant *daf-11* has a more complex defect in the ASJ sensory neuron that causes ASJ to drive dauer formation under inappropriate conditions (Schackwitz et al., 1996); the abnormal dauer formation in these mutants can be rescued by laser killing of the ASJ neurons.

To better understand the axon morphology phenotypes and the sensory phenotypes of *tax-2*, we have analyzed the time of gene action using a temperature-sensitive allele of the gene. *tax-2* is required in the adult stage for normal olfaction and salt taste sensation. Unexpectedly, normal ASJ axon development also requires *tax-2* action until the adult stage, revealing that chemosensory axon morphology remains plastic throughout much of the animal's life. The dauer-constitutive gene *daf-11* has a similar late axon phenotype. Genetic interactions with dauer formation genes indicate that *tax-2* and *tax-4* have activities that can either prevent or promote dauer larva formation, perhaps because of gene activity in different sensory neurons.

MATERIALS AND METHODS

Isolation of new *tax-2* and *tax-4* mutations

General methods of *C. elegans* strain maintenance were as described (Brenner, 1974). The *tax-2* mutants *ks10, ks15* and *ks31* were identified on the basis of thermotaxis-defective phenotypes. *tax-2(ks10)* was isolated by selecting thermophilic F2 animals after mutagenesis of CB1377 *daf-6(e1377)* hermaphrodites with EMS. *tax-2(ks10)* was separated from the *daf-6* mutation for further analysis. *tax-2(ks15)* was isolated in a similar screen for cryophilic animals. *tax-2(ks31)* was isolated by selecting cryophilic F2 animals after mutagenesis of wild-type animals with EMS. *ks10, ks15* and *ks31* mapped to linkage group I. *ks10* and *ks15* failed to complement *p671, p691* or one another for behavioral defects, and *ks10, ks15* and *ks31* failed to complement *p691* for axon guidance defects.

One allele of *tax-2 (ky139)* and three alleles of *tax-4* including *ky89* were identified as suppressors of the dauer-constitutive phenotype of *daf-11(m47)* or *daf-11(m87)* mutants that showed an enhanced amphid axon guidance defect compared to *daf-11*. The suppressors were separated from *daf-11*, mapped, and tested for complementation of *tax-2* and *tax-4*.

Chemotaxis assays

Population chemotaxis assays for olfaction were performed as described (Bargmann et al., 1993). Briefly, washed, well-fed animals were placed equidistant from a point source of odorant and a control area. After 1 hour, the chemotaxis index was calculated as: [(adults at attractant)–(adults at control)/(total number of adults)]. The dilution of odorants in ethanol were the following: 1:200 benzaldehyde, 1:100 isoamyl alcohol, 10 mg/ml pyrazine, 1:1000 diacetyl and 1:1000 2,4,5-trimethylthiazole. For population chemotaxis assays to NaCl, a gradient was allowed to form from an 0.4 M source of NaCl for 12-16 hours. A *tax-2(ks31)* strain with an integrated *tax-2::GFP* transgene was tested for NaCl chemotaxis, so that the ASE chemotaxis phenotype and the ASE axon phenotype could be compared in the same strain (Coburn and Bargmann, 1996).

DiO staining and GFP expression in chemosensory neurons

DiO-staining was performed as described previously (Coburn and Bargmann, 1996). An integrated *tax-2::GFP* fusion gene that includes the first intron of *tax-2* but not sequences upstream of the initiation codon was used to score aberrant ASJ axons in early developmental stages. This fusion gene is expressed in AWB, AWC, ASG, ASI, ASJ and ASK neurons (Coburn and Bargmann, 1996). An integrated *gpa-9::GFP* fusion gene (generously provided by Gert Jansen and Ronald Plasterk) was used to examine ASJ axon morphology in detail. This fusion gene is expressed at high levels only in the ASJ sensory neurons.

Temperature-shift experiments

tax-2(ks31) adult animals were allowed to lay eggs for 1-4 hours at 20°C or 25°C, then adults were removed from the plates. Plates were placed at 20°C or 25°C for varying times, then shifted to the other temperature. Animals were allowed to develop to the adult stage, then chemotaxis was tested and amphid axon guidance defects were observed by DiO staining. In a separate set of experiments, synchronized *tax-2(ks31), daf-11(m84)* or *daf-11(m84); daf-12(m20)* animals were allowed to develop to the adult stage at either 20°C or 25°C and then shifted to the other temperature. Chemotaxis and axon structure were assayed at varying times after the temperature shift. DiO staining was conducted in temperature-controlled incubators so that animals remained at the correct temperature throughout the experiment.

Statistical analysis of temperature-shift experiments was conducted using Primer of Biostatistics software (Stanton A. Glantz, McGraw-Hill publishers). For temperature shifts during development, all shifts within a given larval stage were averaged to generate a mean value for that stage. Mean stage values were compared using the *t*-test. Chemotaxis results for adult temperature shifts were also compared using the *t*-test. Axon outgrowth data for adult temperature shifts were in the form of proportions and were compared using the *z*-test.

Comparisons were conducted by beginning with the earliest stage (or the zero hour shift for adults) and comparing it with each succeeding data point to identify the first significantly different value. Data points at later times and stages were compared to one another to identify additional significant alterations. To correct for multiple comparisons, the significance level for all analyses was set at $P<0.01$.

Construction of *tax-2; daf-c* and *tax-4; daf-c* double mutant strains

Dauer formation at 25°C and dauer recovery at 15°C were tested essentially as described (Vowels and Thomas, 1992), except that animals were grown on *E. coli* strain HB101. Double mutant strains without other marker mutations were made using standard genetic methods. In general, the *daf* mutation was tracked by the Daf-c phenotype and the *tax* mutation was tracked by balancing it in *trans* with a tightly linked marker mutation. In all cases, genotype was confirmed by complementation testing.

The linked *daf-2 tax-4* double mutant strain was isolated from a Daf nonUnc F2 recombinant after mating *tax-4* males to *daf-2 unc-32* hermaphrodites. The *tax-2(p694); daf-11(m87)* strain was constructed using PCR analysis to follow the *tax-2* mutation, which is a deletion in the 5′ end of *tax-2* (Coburn and Bargmann, 1996).

The construction of homozygous double mutant strains between *tax-2* or *tax-4* and *daf-1, daf-4, daf-8* or *daf-14* proved impossible because the homozygous double mutants were highly dauer-constitutive. In these cases, the strain was maintained as a balanced strain in which the *daf* mutation was homozygous and the *tax* mutation was balanced in *trans* to linked marker mutations. *tax-2* was balanced by *unc-13(e251) lin-11(n566) I* and *tax-4* was balanced by *dpy-17(e164) unc-32(e189) III*. 60-80 wild-type F3 animals from *tax-2/marker; daf* F2 parents were cloned to individual plates at 15°C; all yielded 1/4 marker progeny, indicating that all adults were of the genotype *tax/marker; group2 daf-c*, and that the *tax; group2 daf-c* strain could not reach adulthood. The linked double mutant strain *daf-4 tax-4* was maintained as a *daf-4 tax-4/daf-4 unc-32* balanced strain. In all cases, the presence of *tax-2* or *tax-4* in the balanced strain was confirmed by complementation testing.

Laser ablation of ASJ in *tax-2* dauer larvae

The ASJ neurons were killed with a laser microbeam as described (Bargmann and Horvitz, 1991b) and animals were allowed to recover in M9 buffer for 20 hours before concentrated *E. coli* was added to stimulate dauer recovery. This recovery assay in liquid prevents animals from being lost by desiccation on the sides of the plate (Bargmann and Horvitz, 1991b). Cell deaths were confirmed by the absence of DiO filling of the ASJ neurons after testing was complete (Herman and Hedgecock, 1990).

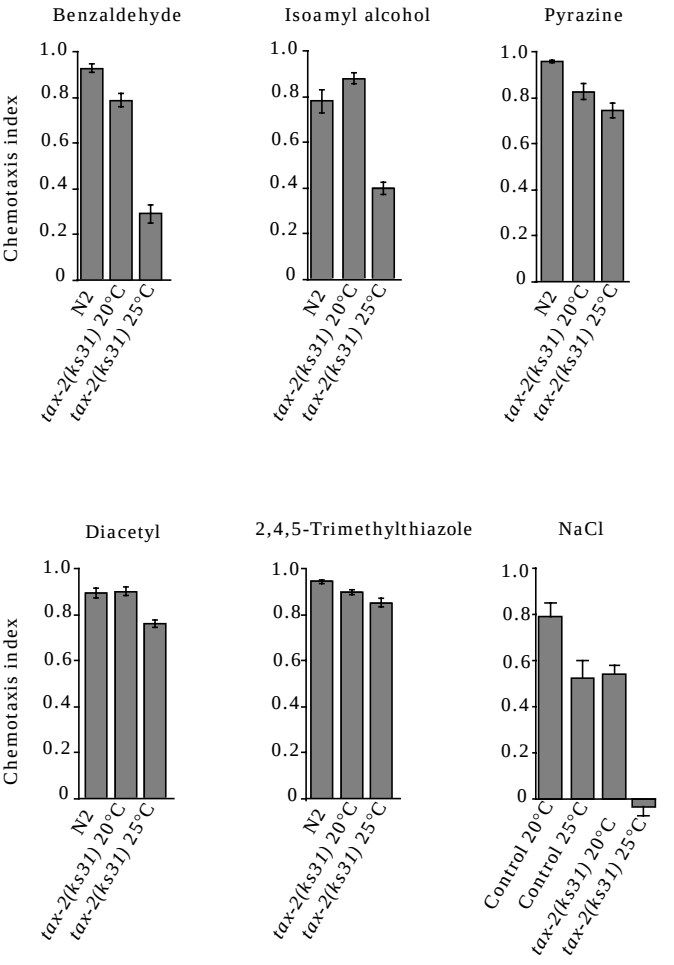

Fig. 1. *tax-2(ks31ts)* is temperature-sensitive for chemotaxis defects. Chemotaxis responses of *tax-2(ks31ts)* adults raised at the permissive temperature (20°C) and the restrictive temperature (25°C). Wild-type animals (N2) behave similarly at both temperatures. For the NaCl assays, both *tax-2(ks31)* and the control strain contained a *tax-2::GFP* transgene (see Methods). Each data point represents the average of 4-8 independent assays using approximately 100-200 animals per assay (error bars equal the s.e.m.). The defects of *tax-2(ks31)* at 25°C are comparable in their severity and odorant-selectivity to those of the strong *tax-2* allele *p691* (Coburn and Bargmann, 1996).

RESULTS

tax-2 function is required in the adult for normal chemotaxis

tax-2(ks31) is a threonine-to-isoleucine missense mutation in the predicted TAX-2 pore domain (Coburn and Bargmann, 1996), and these mutants were strongly temperature-sensitive for both their AWC and ASE chemotaxis defects (Fig. 1). *tax-2* animals raised at 20°C displayed normal or nearly normal chemotaxis, while *tax-2(ks31)* animals raised at 25°C had defects comparable to those of the strongest *tax-2* mutants. Chemotaxis to odorants sensed by AWA (diacetyl, pyrazine and trimethylthiazole) was normal at both temperatures.

To determine the time of *tax-2* gene action, animals were shifted between 20°C and 25°C at different times of development, then scored for their chemotaxis phenotypes as adults (Fig. 2A). The assays were performed about 12 hours after the L4-to-adult molt. When *tax-2* mutants were shifted to permissive temperature as late as the fourth larval stage (L4), most displayed successful AWC chemotaxis behaviors. These results indicate that the *tax-2* gene product can rescue AWC olfactory function at late stages of development. However, *tax-2* activity was not essential in late larval stages. Animals shifted to the restrictive temperature at the L2 stage exhibited a significant ability to detect AWC odorants, although better chemotaxis was observed when animals retained *tax-2* activity until L3 or L4 stages. Active TAX-2 protein from larval stages may persist into the adult, or its function may be less important at later times.

Because of the relatively late time of *tax-2* action, additional temperature-shift experiments were conducted to ask whether

tax-2 could affect chemotaxis in the adult stage. Animals were raised at 20°C or 25°C until the young adult stage and shifted to the other temperature, and chemotaxis was scored in the adults at various times after the temperature shift. These experiments revealed that *tax-2* was required in the adult for normal chemotaxis. AWC chemotaxis was completely defective 60 hours after adults were shifted from the permissive to the restrictive temperature. Conversely, AWC chemotaxis exhibited clear improvement 21 hours after a shift from the restrictive to permissive temperature and recovered almost fully after 72 hours (Fig. 2B). The adult temperature shifts indicate that *tax-2* gene activity in the adult stage is necessary and sufficient for chemotaxis in older adults. Similarly, ASE-mediated chemotaxis to NaCl was defective within 6 hours after adults were shifted to the restrictive temperature (Fig. 2C). These results are consistent with a direct role for the channel in transduction, but also consistent with other models for channel function.

The *tax-2* axon phenotype has an unexpectedly late time of action

The amphid neuron ASJ has a striking axon defect in *tax-2* mutants and *tax-2(ks31)* mutants were highly temperature-sensitive for the ASJ axon phenotypes. They had mostly normal sensory axons (2-5% defective) at 20°C, but 80-90% of the animals were defective at 25°C, a defect comparable to the strongest *tax-2* and *tax-4* alleles (Fig. 3). Surprisingly, temperature shifts with *tax-2(ks31)* revealed that the *tax-2* gene product was not required during the period of initial neural development and axon guidance in the embryo. When *tax-2(ks31)* mutants were raised through embryogenesis at the restrictive temperature and then shifted to lower temperature after hatching, the resulting adults were normal in their ASJ axon morphology (Fig. 3A). Indeed, if they were shifted to permissive temperature as late as the third larval stage (L3), 43% of the adults had normal axons. The converse experiment revealed that the presence of the *tax-2* gene product in the embryo and young larva was not sufficient for normal axon development; instead, the *tax-2* gene product appeared to be required in the third or fourth larval stages for normal adult ASJ axon morphology. The temperature-sensitive period for the ASJ axon phenotype was more discrete than that observed for AWC chemotaxis (compare Figs 2A and 3A), suggesting that *tax-2* activity during a defined time period in the third or fourth larval stage has a major influence on adult axon morphology.

To ask whether the adult axon morphology was established

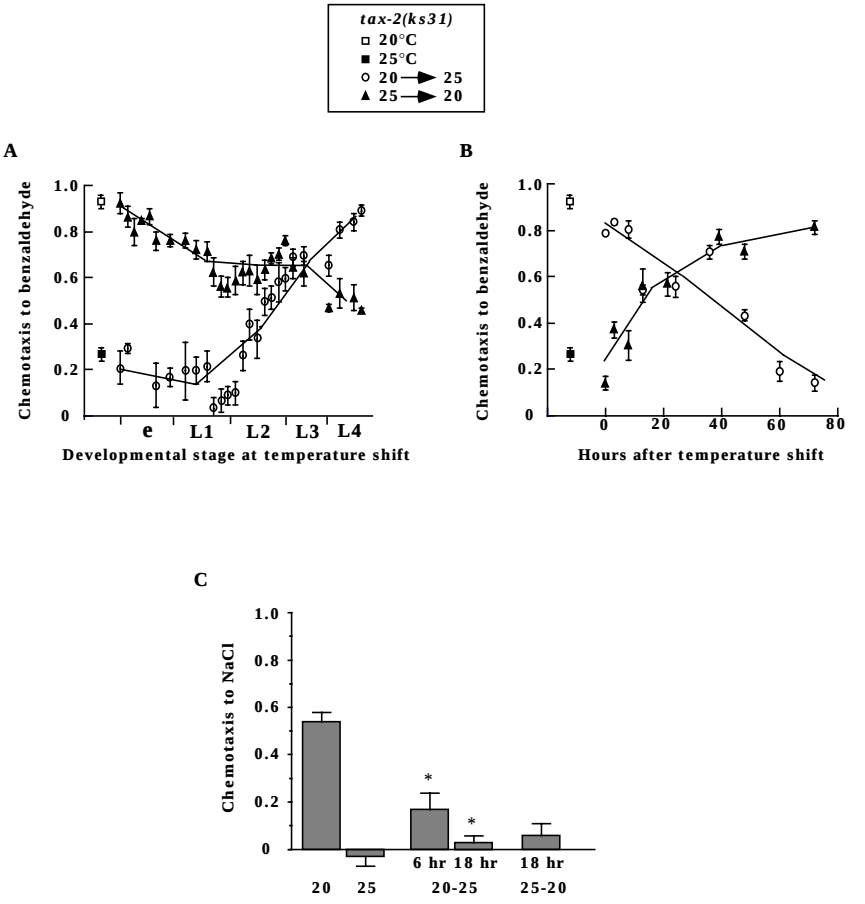

Fig. 2. *tax-2* is required in the adult stage for normal benzaldehyde and NaCl chemotaxis. (A) *tax-2(ks31ts)* animals were shifted between restrictive temperature (25°C) and permissive temperature (20°C) at various points during development. Benzaldehyde chemotaxis was assayed in adults. Curves were generated by averaging all values from a given larval stage. For the downshift, embryo average chemotaxis index=0.83 (s.e.m.=0.02), L1=0.66 (0.04), L2=0.63 (0.02), L3=0.63 (0.06), L4=0.50 (0.02). These numbers displayed a downward trend (Pearson rank order correlation for all 27 data points r_s=-.784, P<0.001). Significant decreases were observed between the embryo and L1 shifts (P=0.002), and the L2 and L4 shifts (P=0.005). For the upshift, embryo average chemotaxis index=0.20 (s.e.m.=0.03), L1=0.13 (0.03), L2=0.38 (0.06), L3=0.66 (0.02), L4=0.85 (0.02). Significant improvements were observed between L1 and L2 shifts (P=0.005), L2 and L3 shifts (P=0.008), and L3 and L4 shifts (P=0.001). (B) *tax-2(ks31)* animals that were raised at the restrictive temperature (25°C) until adulthood were shifted to the permissive (20°C) temperature, or vice versa. Benzaldehyde chemotaxis was assayed after the temperature shift. Curves were generated by averaging each set of three data points. In the upshift, significant defects were observed after 24 hours (P<0.001), with further defects by 60 hours (P=0.008). In the downshifts, significant improvement was observed within three hours (P<0.001), with further improvement after 21 hours (P=0.004) and after 72 hours (P=0.002). (C) *tax-2(ks31)* animals that were raised at the restrictive temperature (25°C) until adulthood were shifted to the permissive (20°C) temperature, or vice versa, and NaCl chemotaxis was assayed after the temperature shift. Significant defects were observed within 6 hours of the upshift (asterisks, P<0.01). Each data point represents the average of 4-8 independent assays using approximately 100-200 animals per assay (error bars denote the s.e.m.). Control values for chemotaxis of animals raised continuously at 20°C or 25°C are indicated at the left of each graph. Abbreviations used are: e, embryo; L1, first larval stage; L2, second larval stage; L3, third larval stage; L4, fourth larval stage.

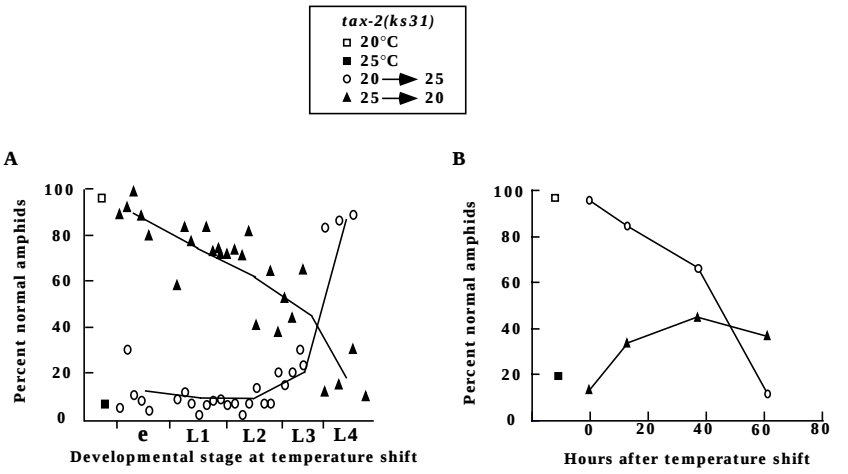

Fig. 3. *tax-2* is required in late larval and adult stages for normal axon structure. (A) *tax-2(ks31ts)* animals were shifted between restrictive temperature (25°C) and permissive temperature (20°C) at various points during development. Amphid axon morphology was examined in adults. Curves were generated by averaging values from each larval stage. For the upshift, embryo average= 10% normal axons (s.e.m.=5%), L1=6% (1%), L2=5% (1%), L3=20% (3%), L4=87% (2%). Significant improvement was found between the L2 and L3 shifts (P<0.001) and between the L3 and L4 shifts (P<0.001). For the downshift, embryo average= 90% normal axons (s.e.m.=3%), L1=74% (3%), L2=61% (8%), L3=43% (12%), L4=17% (6%). Significant decreases were observed between the embryo and L1 shifts (P=0.004), L1 and L3 shifts (P=0.01), and L2 and L4 shifts (P=0.009). (B) *tax-2(ks31)* animals were raised at the restrictive temperature (25°C) until adulthood, then shifted to the permissive (20°C) temperature, or vice versa. Amphid axon morphology was assayed after the temperature shift. For upshifts, all time points were more defective than the preceding time point (P<0.001). For downshifts, improvement was highly significant after 13 hours (P<0.001) and slightly greater after 37 hours (P=0.004). Amphid axon structure was visualized by DiO staining, which reveals predominantly defects in the ASJ sensory neurons (Coburn and Bargmann, 1996). For each point, 100-700 animals were viewed. Control values for animals raised continuously at 20°C or 25°C are indicated on the left of each graph. An amphid was scored as defective if any amphid neuron had a defect. Abbreviations used are: e, embryo; L1, first larval stage; L2, second larval stage; L3, third larval stage; L4, fourth larval stage.

at a specific developmental stage, adult animals were shifted between temperatures. Surprisingly, *tax-2* was required in the adult to maintain the normal ASJ axons; axon structure was slightly defective 13 hours after a shift from permissive to restrictive temperature and highly defective after 61 hours (Fig. 3B). However, the normal *tax-2* gene product was unable to rescue many defective ASJ axons after 61 hours in the adult stage. Thus, the requirement for *tax-2* in the ASJ axons is different from the requirement for *tax-2* in chemotaxis: normal axon morphology requires *tax-2* activity both before and during the adult stage.

Unfortunately, the ASE axon phenotypes of *tax-2(ks31)* mutants were weak at the high temperature (23% defective ASE axons, compared to 54% defective axons for the strong allele *tax-2(p691))*, and some defective axons were observed at the low temperature. These effects precluded temperature-shift experiments for the ASE axon phenotype.

daf-11 and *daf-21* mutations cause sensory axon defects similar to those of *tax-2* and *tax-4*

Animals bearing mutations in the *daf-11* and *daf-21* genes have ASE and AWC chemotaxis defects similar to those of *tax-2* and *tax-4* mutants (Vowels and Thomas, 1994). We found that *daf-11* and *daf-21* also had effects on axon outgrowth that were similar to those of *tax-2* and *tax-4* mutants (Figs 4, 5A). The most common defect was the presence of one or more inappropriate axons running from the nerve ring into the ventral nerve cord (Fig. 4). As was true for *tax-2* mutants, defects in *daf-11* and *daf-21* mutants could be traced to the ASJ sensory neurons (Fig. 6). The ASJ axon defects in *tax-2; daf-11* and *tax-4; daf-11* double mutants were similar to those of the more severe single mutant (Fig. 5A), suggesting that these genes affect a common developmental process. The group 2 dauer-constitutive mutants *daf-1, daf-2, daf-4, daf-7, daf-8* and *daf-14* had normal amphid chemosensory axons (data not shown).

daf-11(m84) is an unusual allele of *daf-11* with a strong dauer-constitutive phenotype, but a minimal defect in dauer larva recovery and chemotaxis to volatile attractants (Vowels and Thomas, 1994). *daf-11(m84)* displayed a temperature-sensitive defect in ASJ axon morphology: when animals were raised at 20°C, the axons appeared to be normal, but animals raised at 25°C showed defects in amphid structure similar to those observed in *tax-2* animals (Fig. 5B,C). To ask when the *daf-11* gene product was required for normal axon structure, temperature-shift experiments were conducted with the *daf-11(m84)* mutation. Synchronized animals were raised to the young adult stage at 20°C or 25°C, shifted to the other temperature and axon structure observed by DiO-staining. As was observed with *tax-2*, *daf-11* activity was required continuously to maintain the normal axon structure in adult animals; axon structure was completely defective 61 hours after adults were shifted from permissive to restrictive temperature (Fig. 5B,C). However, as was observed in the *tax-2(ts)* mutant, defective axon morphology could not be corrected fully in the adult stage in *daf-11(m84)* mutants.

The defective *tax-2, tax-4* and *daf-11* posterior axons extend during larval stages

To further examine the nature of the axon defects in *tax-2*, *tax-4* and *daf-11* mutants, we made use of GFP reporter genes that were expressed in a subset of the chemosensory neurons. A *gpa-9::GFP* fusion gene (generously provided by Gert Jansen and Ronald Plasterk) was used to examine the morphology of the ASJ neurons in detail (Fig. 6A-C). This reporter gene is expressed at high levels in adult ASJ neurons, but not in other sensory neurons. In wild-type animals, the ASJ neurons have a single axon that extends anteriorly to the nerve ring, where it extends dorsally to the dorsal midline. In *daf-11* and *tax-2* mutants, this axon is present and takes a superficially normal

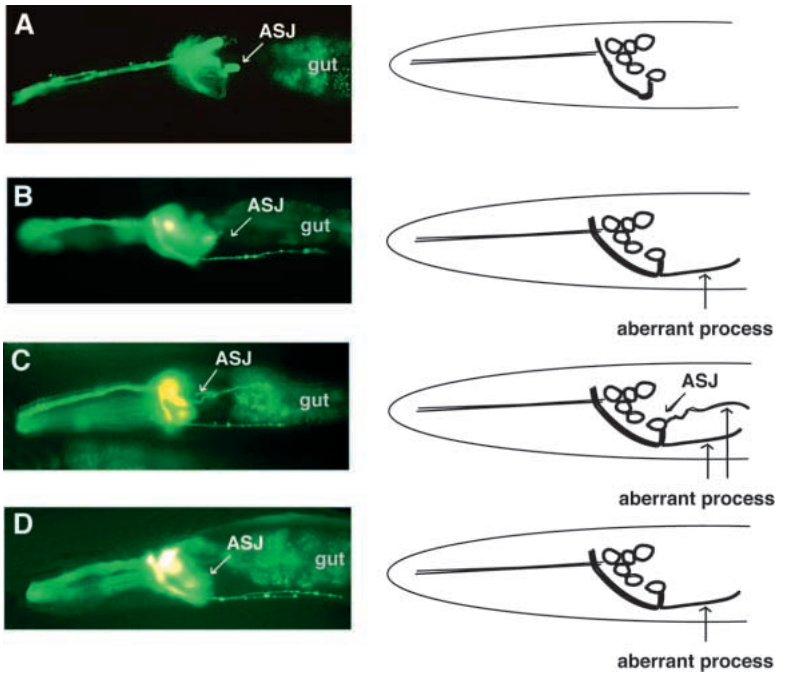

Fig. 4. *daf-11* and *daf-21* mutants have abnormal sensory axon morphology. (A) DiO staining of wild-type animal. Lateral view showing DiO staining of dendrites, cell bodies and axons of six amphid neurons. Positions of the staining neurons are diagrammed. Amphid dendrites grow straight to the anterior tip of the nose, and amphid axons grow in a U-shaped trajectory to the nerve ring just anterior of their cell bodies (see Fig. 6D). The normal position of the ASJ neuron is marked. (B) DiO staining of a typical *daf-11* mutant animal. Lateral view showing an aberrant amphid axon projecting posteriorly from the nerve ring in a ventral position. (C) DiO staining of a *daf-11* mutant animal. Lateral view showing an aberrant process projecting laterally from the ASJ neuron into the posterior body region. *daf-21* animals can have an identical phenotype. In addition, this animal displays an aberrant ventral axon projecting posteriorly from the nerve ring. (D) DiO staining of a *daf-21* mutant animal. Lateral view showing two aberrant axons projecting posteriorly from the nerve ring. Some DiO staining is observed in the gut of the animal. In all cases, anterior is to the left and dorsal is up.

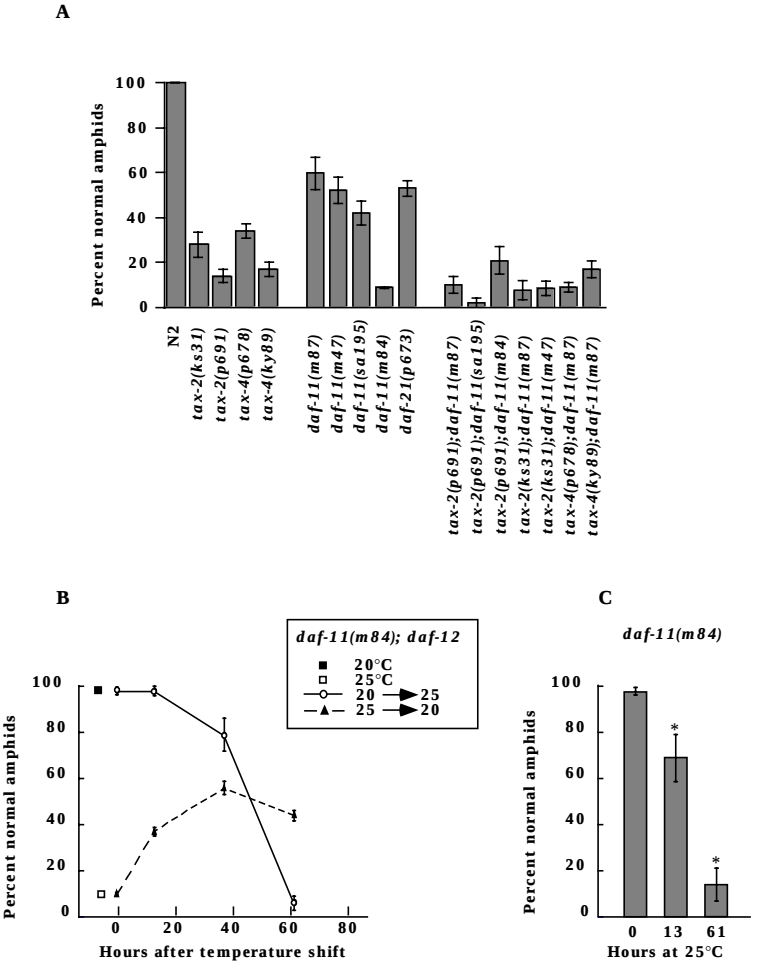

Fig. 5. *daf-11* is required in the adult to maintain normal axon morphology. (A) Per cent defective amphid axons in *tax-2, tax-4, daf-11* and *daf-21* single mutants and double mutant combinations. For each point, 200-700 DiO-stained worms cultured at 25°C were viewed. Error bars denote the 95% confidence level. An amphid was scored as defective if any amphid neuron had a defect. (B) *daf-11(m84); daf-12(m20)* animals were raised at the restrictive temperature (25°C) until adulthood, then shifted to the permissive (20°C) temperature, or vice versa. Amphid axon morphology was examined at several times after the temperature shift. *daf-12* did not affect axon morphology; it was included to suppress the *daf-11* dauer formation defect and allow animals to reach the adult stage. For the upshift, the value after 37 hours was significantly more defective than the control (P<0.001) and the value after 61 hours was more defective than at 37 hours (P<0.001). For the downshift, significant recovery was observed after 13 hours (P<0.001) and further recovery was observed after 37 hours (P<0.001). (C) To confirm that the *daf-12(m20)* mutation did not affect ASJ axons, *daf-11(m84)* animals were raised at the permissive temperature (20°C) until adulthood, then shifted to the restrictive temperature (25°C). Amphid axon morphology was examined after the temperature shift. Axons were significantly more defective at each time point than at the preceding one (asterisks, P<0.001).

trajectory into the nerve ring. However, an ASJ axon also extends posteriorly from the nerve ring into the ventral nerve cord. It is not clear whether this extension is an axon branch or whether it continues from the end of an unbranched ASJ axon (Fig. 6D).

The development of the abnormal ASJ axons was

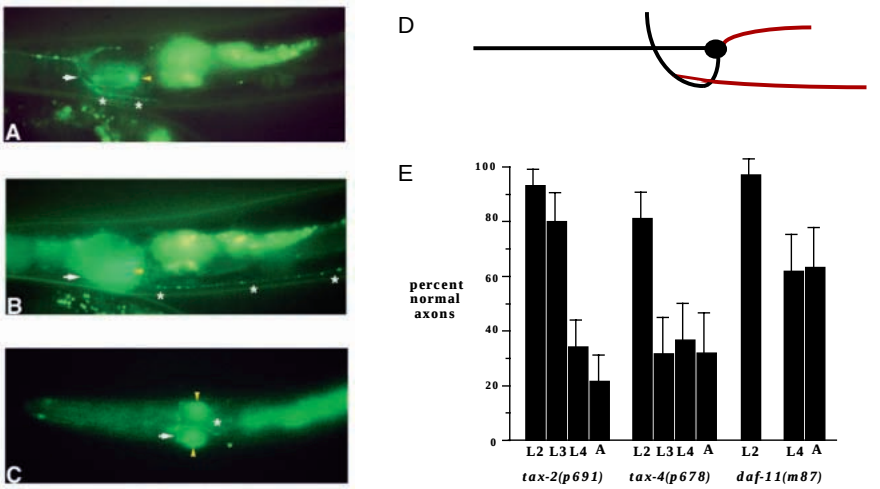

Fig. 6. *tax-2, tax-4,* and *daf-11* mutants exhibit ASJ axon outgrowth in late larval stages. (A,B) Two focal planes showing an ASJ neuron in a *daf-11(m87)* adult expressing a *gpa-9::GFP* transgene, lateral view. The ASJ cell body is denoted by the yellow arrowheads. The ASJ axon is present at its normal position in the nerve ring (white arrows), and also extends posteriorly in the ventral nerve cord (asterisks). Anterior is at left, and gut autofluorescence is visible posterior of the ASJ cell body. (C) Ventral view of a fourth larval stage *daf-11(m87)* mutant expressing a *gpa-9::GFP* transgene. The two ASJ cell bodies are denoted by yellow arrowheads. Both have normal axons in the nerve ring (white arrow), from which a posterior ventral process is beginning to emerge (asterisk). Anterior is at left, and gut autofluorescence is visible posterior of the ASJ neurons. (D) Summary diagram of wild-type ASJ axon morphology (in black), with anterior dendrite and U-shaped axon extending to the nerve ring, and typical mutant axon phenotypes (in red; see Figs 4 and 6). Mutant ASJ neurons usually have both an aberrant process and an ASJ axon that extends to the nerve ring. (E) Late appearance of mutant axon phenotypes in *tax-2, tax-4* and *daf-11* mutants. Animals bearing a *tax-2::GFP* transgene that is expressed in ASJ and five other neurons were scored for aberrant lateral or ventral posterior axons. Many of these defects were in ASJ, but some lateral axons arose from the AWB and ASI neurons. Since most *daf-11* animals became dauer larvae, L3 stage animals were not scored in this strain. Wild-type animals had 100% normal axons at all larval stages (data not shown). Fewer defects were observed with this transgene than were observed by dye-filling; this could represent a difference between the set of cells that are examined, the strains, or the efficiency of the axon visualization techniques. *n*=50-100 animals per data point; error bars indicate the 95% confidence interval.

examined using a *tax-2::GFP* reporter gene that is expressed in all larval stages in ASJ and five other pairs of sensory neurons. This reporter gene revealed that young *daf-11, tax-2* and *tax-4* had superficially normal ASJ axons that extended in the nerve ring (Fig. 6E and data not shown; similar results were observed in the rare *gpa-9::GFP* animals that expressed sufficient GFP during larval stages). The aberrant ASJ processes were first observed in a significant fraction of L3 *(tax-4)* or L4 *(tax-2)* animals. As expected from the adult phenotypes, the posterior ventral axons extended from sprouts that were first visible at the ventral part of the nerve ring (Fig. 6C). These results are consistent with the temperature-shift experiments that demonstrated that *tax-2* and *daf-11* are required late, but not early, for normal adult axon morphology.

tax-2 and *tax-4* can suppress or enhance dauer development

daf-11 shares axon and chemotaxis phenotypes with *tax-2* and *tax-4*, but these genes have distinct effects on dauer larva formation. Under sparse, well-fed conditions at 25°C, wild-type animals do not form any dauer larvae, strong *daf-11* mutants form >90% dauer larvae (Riddle et al., 1981), and *tax-2* and *tax-4* mutants displayed a weak dauer-constitutive phenotype with 1-2% dauer larvae (Fig. 7A). Interestingly, the weak *tax-2* and *tax-4* dauer formation phenotype was epistatic to the strong *daf-11* phenotype: double mutants formed only a few dauer larvae in the absence of dauer pheromone, a level comparable to *tax-2* or *tax-4* single mutants (Fig. 7A). Based on formal genetic analysis, these results indicate that *tax-2* and *tax-4* are downstream of and antagonistic to, *daf-11* in the dauer larva pathway. *tax-2* also suppressed the dauer-constitutive phenotype caused by a *daf-21* mutation, but *tax-2* and *tax-4* enhanced group 2 daf-c mutations in *daf-1, daf-4, daf-8* or *daf-14*. These double mutant combinations were dauer-constitutive even at low temperatures where the daf-c mutants would ordinarily form few dauers (see Methods).

Like entry into the dauer stage, recovery from the dauer stage is controlled by chemical cues, particularly the availability of food. The ASJ sensory neurons have a prominent role in dauer recovery, and *tax-2* and *tax-4* are expressed in these neurons (Bargmann and Horvitz, 1991b; Coburn and Bargmann, 1996; Komatsu et al., 1996). Wild-type dauer larvae recover and resume normal development within 12 hours when well-fed at 15°C. *daf-11* and *daf-21* mutants have a strong recovery defect (Vowels and Thomas, 1994), while *tax-2* and *tax-4* animals showed a slight defect and delay in dauer recovery (Fig. 7B). *tax-2; daf-11* and *tax-4; daf-11* animals formed dauer larvae when crowded, so it was possible to examine dauer larva recovery in the double mutants. The *tax-2* and *tax-4* dauer recovery phenotype was epistatic to the *daf-11* and *daf-21* phenotypes: most double mutant dauers recovered within 48 hours at 15°C, like the *tax-2* and *tax-4* single mutants (Fig. 7B). These results place *tax-2* and *tax-4* downstream of *daf-11* during dauer recovery.

To ask whether the ASJ neurons in *tax-2* mutants were able to promote dauer recovery as they do in wild-type animals (Bargmann and Horvitz, 1991b), the ASJ neurons were killed in *tax-2(p691)* dauer larvae, which were then tested for recovery in food. Killing ASJ did not inhibit dauer recovery in *tax-2* mutants: 8/12 *tax-2* animals in which ASJ had been killed recovered within 10 days after food was provided, while 7/12 control *tax-2* animals recovered in the same time. Thus, the ASJ neurons do not promote dauer recovery in *tax-2* mutants, suggesting that recovery proceeds by an alternative pathway. This alternative pathway may also exist in wild-type animals, since they demonstrate some ASJ-independent dauer recovery (Bargmann and Horvitz, 1991b).

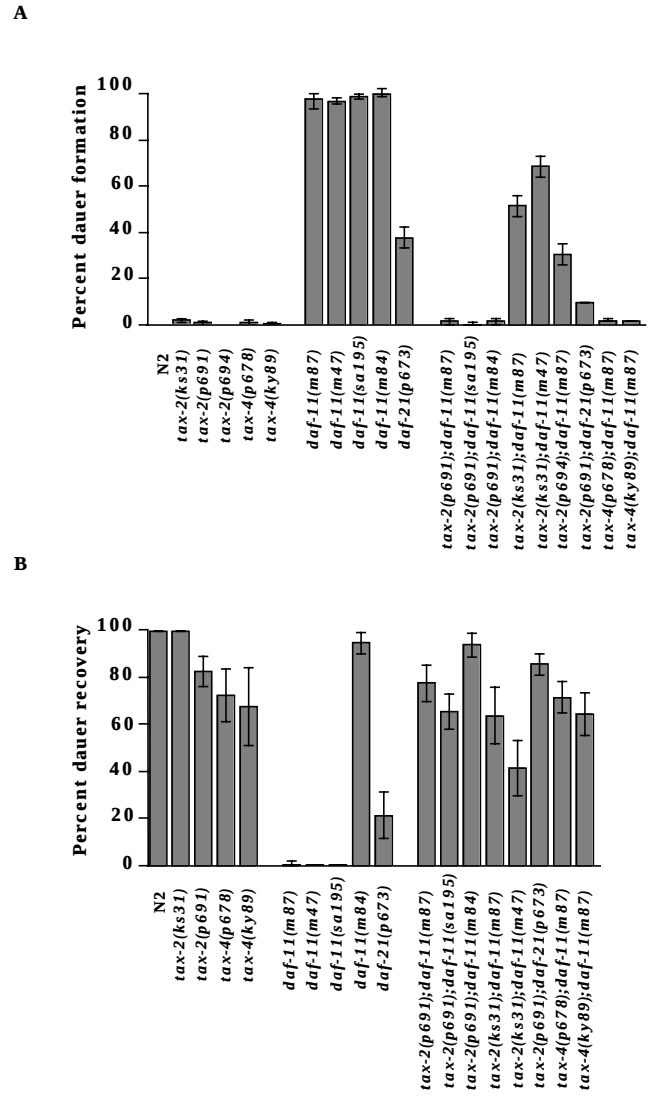

Fig. 7. *tax-2* and *tax-4* suppress the dauer-constitutive phenotypes of *daf-11* and *daf-21* mutants. (A) Per cent dauer larva formation of *tax-2, tax-4, daf-11* and *daf-21* single mutants and double mutant combinations. For each point, 200-700 animals were assayed (error bar represent the 95% confidence interval). Animals were cultured at 25°C. (B) Per cent dauer recovery of *tax-2, tax-4, daf-11* and *daf-21* single mutants and double mutant combinations. For each point, 100-200 animals were assayed (error bar represents the 95% confidence interval). Animals were cultured at 15°C.

DISCUSSION

daf-11, tax-2 and *tax-4* act in a postembryonic pathway that inhibits axon outgrowth

daf-11, *daf-21*, *tax-2* and *tax-4* all have similar effects on the development of sensory axons. The most common axon outgrowth defects in these mutants are aberrant posterior extensions of the ASJ sensory axons. The ASJ axons initially follow a superficially normal trajectory, but then invade regions of the animal from which they are normally excluded. Thus the normal activity of these genes prevents ectopic outgrowth of the ASJ axons.

The first overt defects in *tax-4* null mutants appeared in the third larval stage and *tax-2* defects appeared in the fourth larval stage. *daf-11* defects also appeared in these later stages. Although there may be subtle defects earlier, these results indicate that *tax-2, tax-4* and *daf-11* are specifically required to prevent late errors in axon outgrowth. TAX-4 has been proposed to be an α subunit and TAX-2 a β subunit of a common cyclic nucleotide-gated channel (Coburn and Bargmann, 1996; Komatsu et al., 1996); TAX-4 alone can form functional channels in vitro and possibly in vivo. These TAX-4 channels may provide limited activity in a *tax-2* mutant, thereby delaying its axon defects until the L4 stage.

Remarkably, lowering *daf-11* or *tax-2* activity in the adult stage caused ASJ axon sprouting and outgrowth. The ASJ neurons first send out their axons in mid-embryogenesis and function to regulate dauer formation well before the adult stage. Thus, chemosensory axons that form in the embryo have an unexpected ability to change their morphology at late developmental stages. Although some *C. elegans* neurons can send out or remodel their axons in larval stages (White et al., 1978, 1986), this is the first example of an axon that develops early but can reinitiate growth in the adult. Some recovery of normal ASJ morphology was provided by shifting adult animals to the permissive temperature, but full rescue of ASJ axon phenotypes required *tax-2* activity by the beginning of the fourth larval stage. These results suggest that pruning of aberrant axons is only efficient in earlier larval stages.

These results reveal a function for *tax-2* and *daf-11* in the maintenance of ASJ axon morphology. This axon maintenance process may be analogous to events that occur during critical periods of vertebrate development. The early events in vertebrate sensory axon guidance are mostly independent of sensory activity, but a loss or imbalance of sensory activity during a critical period can lead to substantial disruption of the final axon pattern (Harris, 1984; Shatz and Stryker, 1988). Even after functional sensory connections are formed, changes in activity can alter these connections (e.g. Brainard and Knudsen, 1993). Similarly, although the ASJ neurons have important functions during early larval stages, their axons are sensitive to *tax-2* and *daf-11* activity at much later times.

A late time of action and a slow transition between the normal to mutant states were observed for all phenotypes of *tax-2*, but the exact timing of the transition varied for different phenotypes. ASJ axons required *tax-2* activity both in the L4 and in the adult, adult ASE chemotaxis required *tax-2* activity in the adult, and adult AWC olfaction required *tax-2* activity in the adult and could be fully restored by *tax-2* activity in the adult.

These temperature-shift experiments demonstrate that *tax-2* activity is required in the adult for AWC olfaction and ASE chemotaxis. However, the late plasticity in ASJ development precludes a firm conclusion as to whether *tax-2* acts in AWC and ASE sensory transduction or in late developmental processes. The AWC olfactory neurons can resume full function when *tax-2* activity is provided only in the adult stage, and begin to recover within 3 hours of a temperature shift. Yet there is a long delay before AWC function changes fully, consistent either with persistence of functional TAX-2 protein or with an indirect effect of the protein on olfactory function. No axon defects have been observed in the AWC neurons of

tax-2 mutants, and many ASE neurons have normal axons, but these cells could have subtle defects in connectivity.

tax-2 and *tax-4* have multiple functions in dauer larva formation

tax-2 and *tax-4* are expressed in the ASI, ASG and ASJ neurons that control dauer larva formation, and these genes appear to play multiple roles in the dauer/non-dauer developmental decision. ASJ activity promotes dauer formation in the presence of pheromone, while ASI and ASG activity prevent dauer formation (Schackwitz et al., 1996; Bargmann and Horvitz, 1991b). *tax-2* and *tax-4* help prevent dauer larva formation, since *tax-2* and *tax-4* mutants display a weak dauer-constitutive phenotype and synergize with group 2 dauer-constitutive genes to form dauers at all temperatures. *daf-11* synergizes with group 2 genes in the same way (Thomas et al., 1993). *tax-2* and *tax-4* activity can also promote dauer larva formation, since group 1 mutations in *daf-11* and *daf-21* require *tax-2* and *tax-4* activity to generate a dauer-constitutive phenotype. The suppressed *daf-11; tax* double mutants do form dauer larvae in crowded conditions, demonstrating a residual response to dauer pheromone. The cilium-defective mutants that affect many sensory neurons have a combination of dauer-preventing and dauer-promoting activities that are reminiscent, although not identical, to those of *tax-2* and *tax-4* mutants. In particular, they can synergize with group 2 dauer-constitutive mutants and suppress group 1 dauer-constitutive mutants (Vowels and Thomas, 1992; Thomas et al., 1993).

daf-11 encodes a guanylyl cyclase (D. Birnby and J. Thomas, personal communication), which logically might activate a cyclic nucleotide-gated channel, yet paradoxically the dauer suppression results indicate that *tax-2* and *tax-4* antagonize *daf-11*. This result is most easily explained if *daf-11* and *tax-2/tax-4* are not affecting the identical set of neurons. ASJ is important for *daf-11* phenotypes (Schackwitz et al., 1996), but there are likely to be unidentified dauer-promoting neurons in addition to ASJ, and we speculate that *tax-2* and *tax-4* mutations suppress *daf-11* non-autonomously by inactivating such dauer-promoting neurons. The *C. elegans* genome has dozens of guanylyl cyclases, including numerous cyclases expressed in sensory neurons (Yu et al., 1997), but only a few cyclic nucleotide-gated channels (our unpublished observations). *tax-2* and *tax-4* are expressed in many sensory neurons and they probably affect cells that do not require *daf-11*.

Late neuronal plasticity in *C. elegans*

Three general models could be proposed to explain the congruence of developmental and behavioral defects in *tax-2*, *tax-4*, *daf-11* and *daf-21* mutants. First, the primary defect in these four mutants could be an axon guidance defect, and the behavioral defects could be consequences of mistakes in neuronal connectivity. Specifically, these genes could inhibit axon growth, like collapsins/semaphorins (Luo et al., 1993). Second, the primary defect in *tax-2*, *tax-4*, *daf-11* and *daf-21* could be a change in neuronal sensory activity that indirectly leads to a defect in axon outgrowth. Although activity has not been previously implicated in *C. elegans* development, normal electrical activity of the sensory neurons refines connectivity in vertebrate sensory systems (Shatz and Stryker, 1988; Brainard and Knudsen, 1993). Third, *tax-2*, *tax-4*, *daf-11* and *daf-21* could have two distinct functions, one of which is required in neuronal development and one of which is required for neuronal signalling. For example, some cell types might use these gene products strictly during axon outgrowth, while others might use the same gene products in chemosensory signal transduction.

The cyclic nucleotide-gated channel might also be an effector in other systems in which cyclic nucleotides influence neuronal development and plasticity. cAMP affects synaptic plasticity in *Drosophila* motor neurons (Zhong et al., 1992) and *Aplysia* sensory neurons (Schacher et al., 1993), and it can modify the response of *Xenopus* spinal neurons to axon guidance cues (Song et al., 1997). Interestingly, in *Drosophila* cAMP is important for larval plasticity of the synapse, but not in its initial development (Zhong et al., 1992). This late function might be analogous to the role of the cyclic nucleotide-gated channel in *C. elegans* neurons.

The chemosensory neurons are generated in the embryo and reach their mature morphology by hatching, when chemosensory behaviors can be assayed (Sulston et al., 1983). Yet their axon pattern can be remodeled into the adult stage, indicating that axon morphology is plastic throughout larval development. During this time, the animal is exposed to different chemosensory environments and generates different chemosensory behaviors. We speculate that the late neuronal plasticity revealed by *tax-2* and *daf-11* mutations allows activity and experience in the sensory nervous system to feed back on its connectivity.

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