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 (Dusenberry 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-penetration 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 most 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 gene (generously provided by Bert 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-unc 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 dauer 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).

**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 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 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

![Fig. 2](image-url)

**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=-.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.
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 chemosensory: 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 (25% 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 chemo sensory 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
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. 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) Percent 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).
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).
and 25°C. (B) Percent dauer recovery of combinations. For each point, 200-700 animals were assayed (error interval). Animals were cultured at 15°C. 200 animals were assayed (error bar represents the 95% confidence single mutants and double mutant combinations. For each point, 100-256

Fig. 7. *tax-2* and *tax-4* suppress the dauer-constitutive phenotypes of *daf-11* and *daf-21* mutants. (A) Percent 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 represents the 95% confidence interval). Animals were cultured at 25°C. (B) Percent 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 (Schachkwitz 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 ciliom-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. **ASI** is important for **daf-11** phenotypes (Schachkwitz et al., 1996), but there are likely to be unidentified dauer-promoting neurons in addition to **ASI**, 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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