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Development of *Drosophila* wing sensory neurons in mutants with missing or modified cell surface molecules

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SUMMARY

The neurons of the sensory receptors on the wing of *Drosophila melanogaster* have highly characteristic axon projections in the central nervous system (CNS). The morphology of these projections was studied in flies bearing mutations that affect cell surface molecules thought to be important in axon guidance. The animals used were mutant for the *fasciclinI (fasI), fasciclinII (fasII), fasciclinIII (fasIII)* and *neurally altered carbohydrate (nac)* genes. Axon populations were visualized by staining with DiI and light-reacting the dye with diaminobenzidine to yield permanent preparations.

The *fasI, fasII* and *fasIII* mutants as well as the *nac* mutant display altered axonal trajectories in the CNS. One phenotype seen in *fasII* mutants and in animals mutant for both *fasI* and *fasIII* was extra branching within the axon projection pattern. A second phenotype observed was a reduction or complete loss of one of the tracts, apparently due to the axons shifting to a neighboring tract. This was seen in the most extreme form in *nac* mutants and to a lesser degree in *fasIII* mutants.

To determine if the mutations discussed here affected axon guidance, wing discs were analyzed using the antibody 22C10 to label sensory neurons in the wing during metamorphosis. Both misrouting of axons and the appearance of ectopic neurons in the wing were observed. In the *fasI;fasIII*, the *fasII* and the *nac* mutants, there was misrouting of sensory axons in the developing wing. In addition, the *fasII* and *nac* mutants displayed ectopic sensory neurons in the wing. This implies that the cell surface molecules missing (*fasciclins*) or modified (by the *nac* gene product), in these mutants may play a role in both neurogenesis and axon guidance.

Key words: metamorphosis, carbohydrate, axonogenesis, *Drosophila*, cell surface, sensory neurons

INTRODUCTION

The establishment of correct neuronal connections during development of the nervous system requires information both to guide growing axons and to ensure proper connections. In both vertebrates and invertebrates, it has been found that necessary guidance cues mediating interactions between axons and the environment can be provided by cell surface molecules (reviewed in Jessell, 1990; Hortsch and Goodman, 1991). In *Drosophila melanogaster* genetic techniques have led to the identification of putative neuronal guidance molecules (Grenningloh et al., 1991). In the work presented here, the role of four proteins, those encoded by the *fasciclinI (fasI), fasciclinII (fasII), fasciclinIII (fasIII)* and *neurally altered carbohydrate (nac)* genes, was examined in the developing wing sensory system.

The *fasciclin* genes, *fasI*, *fasII* and *fasIII*, encode cell surface glycoproteins thought to be involved in the fasciculation of axon bundles during development (Grenningloh and Goodman, 1992; Grenningloh et al., 1990). Additionally, the *fasciclin* molecules have been shown to function as homophilic cell adhesion molecules mediating cell sorting in vitro (Snow et al., 1989; Elkins et al., 1990; Grenningloh et al., 1990). Null mutants of the *fasI* and *fasIII* genes show no gross morphologic abnormalities and are homozygous viable (Elkins et al., 1990b; Snow et al., 1989; T. Elkins, D. Ferres-Marco, C.S. Goodman, personal communication), while null *fasII* mutations are lethal (Grenningloh et al., 1991). The *nac* mutation affects an enzymatic process required for the addition of a carbohydrate moiety common to many proteins expressed on the surface of neurons and other tissues. One effect of the *nac* mutation is the loss of epitopes recognized by the antibodies to horseradish peroxidase (Katz et al, 1988; Snow et al., 1987). The axons of wing sensory neurons in animals mutant for these genes were examined both in their development within the wing and in their final adult projection pattern in the central nervous system (CNS).

The axons of the adult wing sensory neurons follow highly characteristic, easily identifiable and spatially separate pathways in the CNS, (Ghysen, 1978, 1980; Palka et al., 1979, 1986). The sequence of differentiation of neurons in the periphery and the establishment of axonal trajectories in the wing tissue are known (Murray et al., 1984; Jan et al., 1985), as is the developmental profile of the wing axonal projection pattern in the CNS during metamorphosis (Whitlock and Palka, 1989). Thus, the wing sensory

system allows for the easy recognition of abnormal development and final pattern of axonal trajectories in both the peripheral nervous system (PNS) and the CNS.

With the knowledge of both the adult structure and the developmental timing of the wild-type wing sensory system, the *fasI*, *fasII*, *fasIII* and *nac* mutants were examined for defects in development of the wing sensory system. The major findings of this study are the following: In the adult CNS, *fasI* mutants displayed only a slight increase in the amount of wing sensory axon branching in the CNS; *fasIII* mutants had both increased branching and altered axonal projections; adults simultaneously mutant in the *fasI* and *fasIII* genes, or mutant for *fasII* also displayed an increase in overall branching; *nac* mutants exhibited a pathway alteration similar to that of *fasIII* mutants.

In the periphery, misrouting of sensory axons in the wing was observed in animals mutant for both the *fasI* and *fasIII* genes, for the *fasII* gene, and for the *nac* gene. In addition to misrouting, ectopic sensory neurons were observed in the wings of the *fasII* and *nac* mutants.

MATERIALS AND METHODS

Animals

Wild-type animals were of the Sevelen and Canton-S strains. The *fasI*, *fasII*, and *fasIII* mutants and genetic control stocks were kindly provided by C.S. Goodman; *nac* stocks and genetic controls were kindly provided by F.Katz.

fasI^{-}

The *fasI* allele used (*fasI*^{TE77}) is a null mutation resulting from an insertion of a P-element containing the wild-type gene for the eye color *white (w)* and it is hereafter referred to as *fasI*$^{-}$. *fasI*$^{-}$ mutants are homozygous viable, displaying no gross morphological abnormalities although a behavioral abnormality has been reported (Zinn et al., 1988; Elkins et al., 1990). The *fasI* gene lies on the third chromosome at position 89D (Elkins et al., 1990b).

fasIII^{-}

The *fasIII* allele used (*E25,fasIII*$^{-}$) is a null mutation that resulted from an imprecise excision of a P-element inserted in or close to *fasIII* and is designated here *fasIII*$^{-}$. These animals are homozygous viable with no gross morphological defects (T. Elkins, D. Ferres-Marco, C.S. Goodman, pers. com.). The *fasIII* gene lies on the second chromosome at position 36E (Patel et al., 1987).

fasI^{-}*;fasIII*$^{-}$

Animals mutant for both the *fasI* and *fasIII* genes are homozygous viable, with no gross morphological defects (D. Ferres-Marco, C.S. Goodman, personal communication).

fasII^{e76}

The *fasII* allele used (*fasII*^{e76}) displays 5-10% of the wild-type level of FASII protein expression. The mutant was generated by the imprecise excision of a P-element insertion and is designated here *fasII*^{e76}. *fasII*^{e76} mutant animals show no morphological defects but they do display reduced viability. The *fasII* gene lies on the X chromosome at position 4B1-2 (Grenningloh et al., 1991).

fasII^{EB112}

This designates a null mutation for the *fasII* gene which is homozygous lethal. It was used in combination with the severe hypomorph *fasII*^{e76}. Transheterozygotes of these two mutations produce viable but sterile adults.

nac^{1}

This mutation is a hypomorph generated by ethyl methanesulphonate. The stock is marked with the recessive eye color *scarlet (st)*. The *nac*^{1} mutant is temperature sensitive: at the temperature that was used (25°C), there are no obvious morphological defects; at 18°C it causes female sterility and a scalloped wing phenotype. *nac* lies on the third chromosome at position 84F (Katz et al., 1988).

The following unpublished alleles of *nac* were also examined for neural defects: *nac*^{2} and *nac*^{3}, which are homozygous lethal due to mutations at loci other than *nac*, but are viable as transheterozygotes; *nac*^{4}, a homozygous viable allele (F.Katz and W. Moats, pers. com.). All *nac* alleles express the phenotypes associated with the original *nac*^{1} allele.

Control stocks

fasI^{+}

The genetic control for the *fasI*$^{-}$ mutants was an X-ray-induced revertant of the original P-element insertion, and is designated *fasI*$^{R18.1}$ (Elkins et al., 1990b). It was classified as a revertant based on its wild-type-like staining pattern with the anti-fasI antibody in the CNS.

fasIII^{+}

The control for the *fasIII*$^{-}$ mutants was an excision derivative of a P-element that created the *E25,fasIII*$^{-}$ mutation and was originally designated *E12,fasIII*$^{+}$. This excision yielded a wild-type-like staining pattern of the anti-fasIII antibody in the CNS (D. Ferres-Marco and C.S. Goodman, personal communication).

fasII^{e93}

The control for the *fasII* mutants was a precise excision derivative of the original P-element insertion which yielded 100% of wild-type level of fasII in Western blots (Grenningloh et al., 1991). The original designation for this excision derivative was *e93*.

st/st

The original isogenized third chromosome stock on which the *nac* mutations were induced was used as a genetic control for the *nac* mutants.

Staining of wing sensory neurons

The sensory neurons of the wing were stained using the fluorescent carbocyanine dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate, Molecular Probes, Inc., Eugene, OR; Honig and Hume, 1989) as described in Whitlock and Palka (1989). Briefly, DiI was solubilized in 95% ethanol and applied to a previously fixed wing blade with a broken micropipette. As the alcohol evaporated the dye adhered to the tissue. Staining was allowed to proceed in fixative for 3-5 days at room temperature. The CNS was then dissected, mounted in fluorocarbon oil (10S, Voltalef), coverslipped and placed under fluorescence for viewing and photography. Since DiI diffuses in the plasma membrane when applied to peripheral tissue, the only staining found in the CNS was that of the sensory axons. Overall, preparations suitable for analysis were obtained in 20-30% of the stained animals.

Permanent preparations

Permanent preparations were created by light-reacting the DiI-stained tissue with diaminobenzidene (as described in Sandell and Masland, 1988; Whitlock and Palka, 1991).

Quantification of data

The increased branching of wing sensory axons in the CNSs of the mutants was not immediately apparent. Only when the complete pattern of projection was reconstructed, using a camera lucida, were the branching defects obvious. These defects were quantified by counting the number of axon branches present in camera-lucida drawings of each preparation in a defined area surrounding the wing ovoid (see Fig. 1A, below). Only preparations that were darkly stained, intact and well cleared were included in this analysis. The number of branches counted for each individual preparation is referred to as the branching value. The average branching value of all specimens from a given genotype was plotted with the standard error of the mean. All mutant values were compared against the appropriate genetic control values and wild-type values in a Student *t*-test.

Antibody staining

The CNSs of wild-type pupae (12-24 hours after pupariation; AP) were stained using monoclonal antibodies made in mouse against the *fasI* (Hortsch and Goodman, 1990), *fasII* (Grenningloh et al., 1991) and *fasIII* (Patel et al., 1987) glycoproteins.

White pre-pupae were fixed at various times after pupariation in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.3) at room temperature for at least four hours. The CNSs were then dissected in fixative, rinsed in PBS, and placed in a blocking solution of 5% neonatal calf serum and 0.3% saponin in PBS. The preparations were incubated overnight in the primary antibody at 4°C. The dilution for the primary antibodies was 1:2 for anti-fasI, 1:6 for anti-fasII, and 1:5 for anti-fasIII. The secondary antibody was a fluorescently labeled anti-mouse IgG conjugated to Cy3 (Jackson Labs). The preparations were then rinsed and mounted in 80% glycerol in PBS with 4% propylgallate to prevent quenching. For permanent preparations, the tissue was incubated in biotinylated anti-mouse secondary antibody (Vector Labs) and processed with the Vectastain Elite Avidin-Biotin HRP kit (Vector Labs).

Neurons and their processes were visualized using the 22C10 antibody (Fujita et al., 1982) at a dilution of 1:50. The 22C10 antibody was used, rather than the more common anti-horseradish peroxidase (anti-HRP; Jan and Jan, 1982), because the neurons of *nac* mutants do not stain with anti-HRP (Katz et al., 1988).

RESULTS

Adult sensory neuron projections in the CNS

Wild type

The axons from the wing sensory neurons have characteristic projection patterns in the CNS, which are illustrated in Fig. 1A (Ghysen, 1978, 1980; Palka et al., 1979; Whitlock and Palka, 1992). The majority of wing sensory axons terminate in the thoracic nervous system. The abdominal ganglion receives no projection from the wing sensory system (Ghysen, 1978); a subclass of wing sensory neurons terminates in the suboesophageal ganglion of the brain (Ghysen et al., 1983).

In the CNS, the axons from the sensory neurons are grouped in four spatially distinct bundles (see Ghysen, 1978, 1980; Palka et al., 1979, 1986): The axon bundle originating from the sensory receptors on the radius projects along a dorsal tract that extends both anteriorly (dorsal anterior tract; dat, Fig. 1A) to the suboesophageal ganglion, and posteriorly (dorsal posterior tract; dpt, Fig. 1A) to the metathoracic ganglion. Two other posteriorly projecting axon bundles originate primarily from sensory receptors of the third vein (L3, Fig. 3A) and fall into two developmentally distinct classes based on their time of birth: axons from the earlier-born sensory receptors project along a medial tract (mt, Fig. 1A) in the ventral neuropil while those born later project laterally in the ventral neuropil (lateral tract; lt, Fig. 1A; Palka et al., 1986). The axon bundle originating from the neurons of the wing margin (L1, Fig. 3A) forms a compact oval projection called the bristle ovoid (bo, Fig. 1A, Palka et al., 1979), which lies between the pro- and mesothoracic neuromeres.

fasI^{-}

In *fasI*$^{-}$ mutants the pattern of wing sensory projections was like that of wing sensory axons in the wild-type CNS (Fig. 1B). However, the mutant did show a slight increase in overall branching. This increase was significantly different both from the relevant genetic control ($P = <0.001$, df=*17*), and the wild-type ($P=<0.025$, df=*14*; Fig. 2, Table 1). The mutant animals also displayed abnormal fast phototaxis behavior (K. Whitlock, unpublished)

fasIII^{-}

The pattern of wing axons seen in the CNS of this mutant was dramatically altered compared to that of wild-type animals (Fig. 1C). Axons of the medial tract (solid arrow) were often misrouted into the lateral tract, which often appeared thicker (open arrow). This misrouting occurred after the medial axon projections had been initiated, and the crossover of axons into the lateral tract occurred in an area that is a branch point in wild-type animals. The penetrance of this phenotype (percentage of individuals of this genotype that show this phenotype) was 55% (Table 1). The average branching value for the neuron population in this mutant was greater than that of the *fasIII*$^{+}$ control ($P=<0.01$, df=*10*) and that of the wild-type ($P=<0.025$, df=*13*) animals (Figs 1C, 2).

fasI^{-}*;fasIII*$^{-}$

Animals simultaneously mutant for both *fasI* and *fasIII* displayed a misrouting of the medial axons into the lateral tract similar to that observed in the *fasIII*$^{-}$ mutant (Fig. 3D, arrow). Yet, this misrouting did not occur as often or to the extent that was observed in animals mutant for *fasIII*$^{-}$ alone (Fig. 1C). In these *fasI*$^{-}$*;fasIII*$^{-}$ mutants, the branching value was approximately twice that of the wild-type value (Fig. 2, Table 1) and significantly different from both that of the genetic control ($P=<0.001$, df=*13*) and that of the wild type ($P=<0.001$, df=*14;* Fig. 2, Table 1).

fasII^{e76}

The axon pattern of wing sensory neurons in the CNS of the males of the *fasII*^{e76} mutant was indistinguishable from that of wild type. However, the branching value in females (Fig. 2, Table 1) was significantly different from both that of the genetic control ($P=<0.001$, df=*9*) and that of the wild-type animals ($P=<0.001$, df=*14*). While the branching value was only computed for the area surrounding the bristle ovoid (Figs 1E, 2), extra branching was apparent in all areas of wing sensory neuron termination (Fig. 1E, open

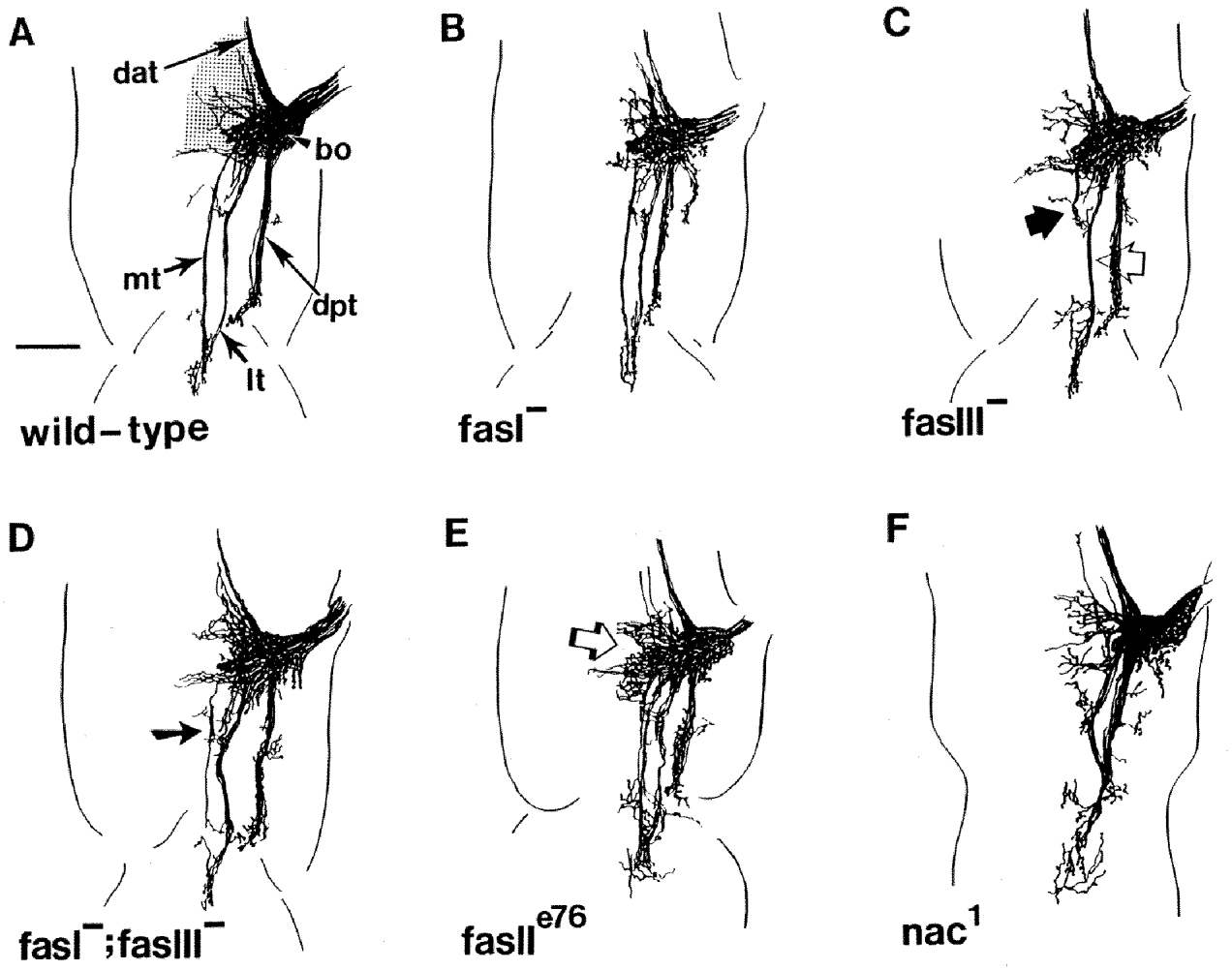

Fig. 1. Camera-lucida drawings of adult wing sensory axon projections in the CNS of both wild-type and mutant animals. The drawings depict only the thoracic nervous system, so that the dorsal anterior tract (dat), which extends into the subesophageal ganglion of the brain, is not fully shown. (A) Wild-type CNS. In the wild-type CNS, axons originating from the proximal wing extend in the dorsal anterior tract (dat) and the dorsal posterior tract (dpt), indicated by **long arrows**. The axons originating from the large campaniform sensilla on the wing extend in the ventral neuropil in the medial tract (mt) and the lateral tract (lt), indicated by **short arrows.** The axons originating from the anterior margin extend into the bristle ovoid (bo). The stippled area indicates the region where axon branches were counted to obtain 'branching value'. (B) *fasI*⁻. This mutant has a wild-type pattern of wing sensory axon projection in the CNS, but there is a slight increase in axonal branching (Fig. 2, Table 1). (C) *fasIII*⁻. In this mutant, there is an alteration in the pattern of axonal tracts (**closed arrow**). The axons of the mt tract start to grow along their normal tract, but cross into the lt tract where they are not normally found (**open arrow**). This phenotype is variable (see Table 1). (D) *fasI*⁻*;fasIII*⁻. This mutant also has an altered pattern of axonal projections in the CNS. There is misrouting of fibers from the mt (**solid arrow**) to the lt. This misrouting never involves all axons of the mt and is of low penetrance (Table 1). There is a dramatic increase in branching (Fig. 2, Table 2). (E) CNS of *fasII*^{e76} female that shows a wild-type pattern of axon tracts. There is dramatic increase in axonal branching (**open arrow**) (Table 2). (F) *nac*^{1}. In this mutant, there are no axons in the mt, and the lt axon bundle appears thicker. This phenotype was most prevalent in the original allele. The branching was not calculated for this mutant due to the morphological alterations caused by the total loss of the mt tract. Scale bar: 80 μm. dat, dorsal anterior tract; dpt, dorsal posterior tract; mt, medial tract; lt, lateral tract; bo, bristle ovoid.

arrow). Thus, a reduced level of the fasII glycoprotein affects all wing sensory axons.

By visual inspection, there also appeared to be a greater number of varicosities. The phenotype of animals that were transheterozygotes for the hypomorphic and the null allele (*e76/EB112*) did not display a more severe wing sensory axon phenotype in the CNS than that seen in the homozygous hypomorph. This would suggest that the *fasII*^{e76} homozygote is a phenotypic null for excess axon branching.

nac^{1}

This mutant displayed severe misrouting in the projection of the wing sensory axons into the CNS. The axons that normally project into the medial tract were not apparent in 80% of the animals examined, and the axon bundle in the lateral tract appeared thicker (Fig. 1F). Branching value was not calculated for the *nac*^{1} mutants because the alteration of the bristle ovoid area made it difficult to delineate an appropriate area for counting branches.

Mutants homozygous for any of the three other *nac* alle-

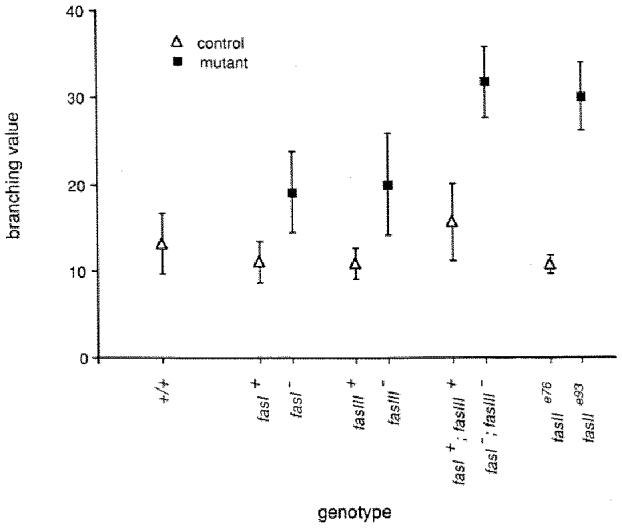

Fig. 2. This graph depicts the branching value of the axons, calculated by counting the number of axonal branch points within the area delineated in Fig. 1, for each genotype except the *nac*[1] mutant. The ordinate represents the number of branches; the abscissa lists the genotype where the data for the mutant are grouped by pairs with the corresponding genetic controls. Values for mutants are represented by closed squares (■) and wild-type and genetic controls by open triangles (△).

les, *nac*[2], *nac*[3] and *nac*[4], were examined and found to have defects similar to those seen in the *nac*[1] mutants. Thus, the defect observed in the original allele, *nac*[1], was probably due to a mutation at *nac*.

Control stocks

No tract choice defects or significant differences from wild-type branching values were detected (P=>0.05) in the control stocks (Fig. 2 and Table 1).

Development of wing sensory neurons in the PNS

Developing wings from the mutant strains were stained with the 22C10 antibody at 6 and 24 hours AP and compared to control and wild-type wings stained at identical stages. Six hour wings were examined for both time of neurogenesis and the time of axonogenesis. The 24 hour time point allowed one to determine whether the normal axon paths from the neuronal somata to the base of the wing had been followed.

At 6 hours AP, all wing imaginal discs examined from the mutants appeared wild-type in the number of neurons present and the extent of their axonogenesis. At 24 hours AP, in contrast, differences between the mutants and wild-type pupal wings were readily apparent in *fasI*⁻*;fasIII*⁻, *fasII*^{e76}, and *nac*[1] (Fig. 3, Table 2) mutants.

fasI⁻*;fasIII*⁻

24 hour wings of animals mutant for both *fasI* and *fasIII* displayed misrouting at the distal tip of the wing (Fig. 3B, dark box). A subset of sensory axons grew along the posterior edge of the wing rather than following the normal route along the anterior wing margin. In several of these preparations showing misrouted sensory axons, the axons left the posterior margin and turned up the fourth vein, a region normally devoid of axons. This phenotype was not sexually dimorphic and its penetrance was 25% (Table 2).

fasII^{e76}

24 hour wings of *fasII*^{e76} mutants displayed ectopic neurons on the posterior margin of the wing (Fig. 3C), an area that normally has no neurons (Fig. 3A, light box). These ectopic neurons extended axons both proximally and distally along the posterior wing margin, but the axons failed to join with the main wing nerve. In addition there was occasional misrouting of axons at the distal tip of the wing (data not shown). The presence of ectopic neurons was more frequent in the females (80%) than in the males (50%;

Table 1. The mutant phenotypes of adult wing sensory axons in the central nervous systems of the *fasI*⁻, *fasIII*⁻, *fasI*⁻*;fasIII*⁻, *fasII*^{e76} and *nac*[1] mutants

Genotype	*n*	Phenotype	Branching value	Penetrance
fasI⁻	8	increased branching of wing sensory axons	19.1	60%
fasI⁺	10	wild-type	11.3	
fasIII⁻	6	increased branching of wing sensory axons	20.0	60%
		medial axons cross to lateral tract		55%
fasIII⁺	5	wild-type	10.8	
fasI⁻*;fasIII*⁻	8	increased branching of wing sensory axons	31.8	100%
		medial axons cross to lateral tract		25%
fasI⁺*;fasIII*⁺	7	wild-type	15.5	
fasII^{e76}	8	increased branching of wing sensory axons*	23.5	100% (males)
			32.3	100% (females)
fasII^{e93}	3	wild-type	10.7	
nac[1]	9	most to all medial tract axons travel in the lateral tract	ND	80%
st/st	4	wild-type	10.0	
+/+	8	wild-type	13.1	

The branching value is an average value for all the preparations examined of a given genotype.
*The branching values for *fasII*^{e76} was greater in females in than males.
(ND) The branching value for the *nac*[1] mutants was not calculated due to the altered morphology of the central axon projections.

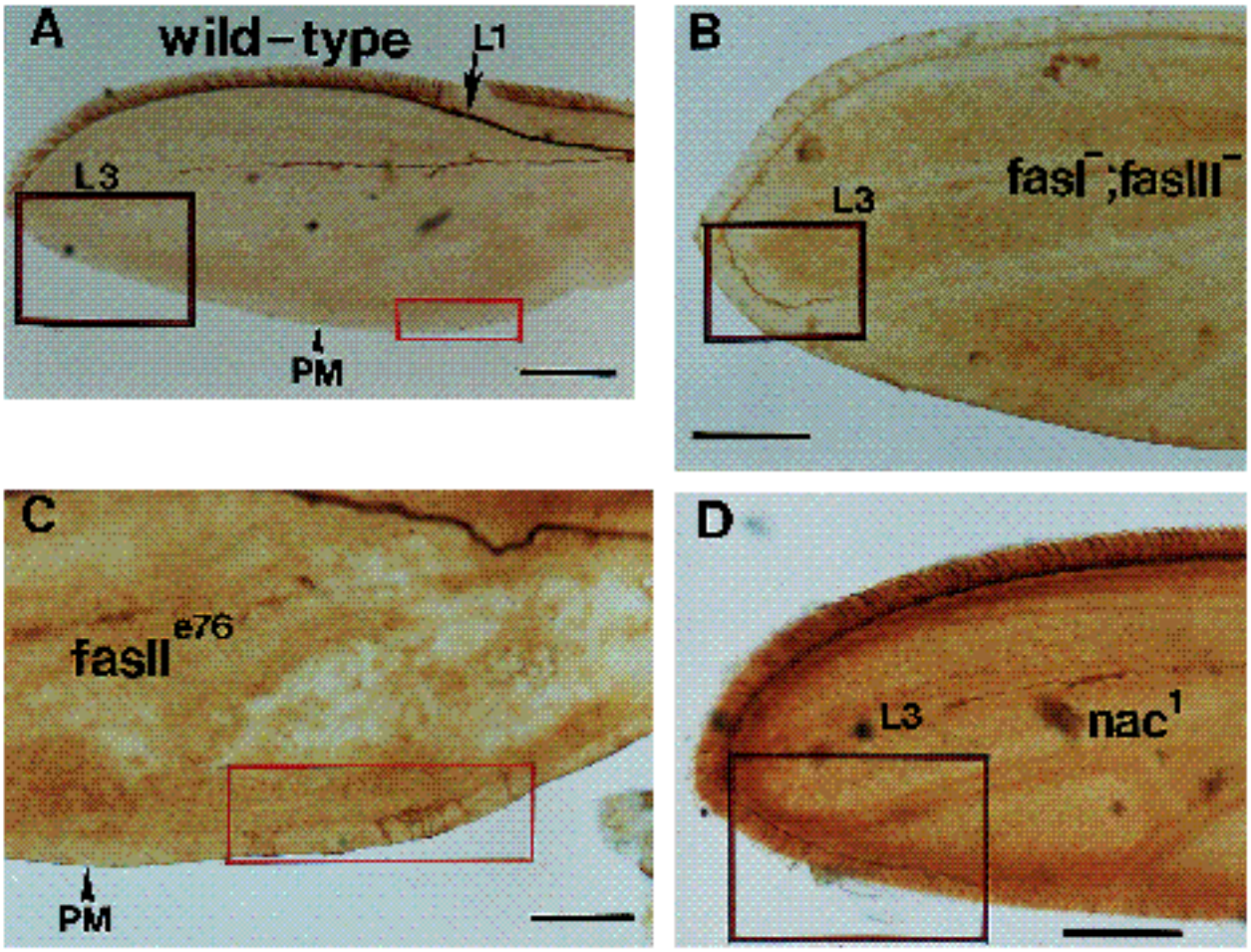

Fig. 3. Wild-type and mutant phenotypes in 24 hour AP wings. The proximal end of the wing lies to the right and the anterior margin of the wing toward the top of the page. (A) Wild-type wing. The veins in which the axons travel are the first (L1) and third (L3) veins of the wing; PM indicates the posterior margin of the wing where neurons are not normally found in wild-type wings. The bold box and the light box indicate areas of the posterior margin of the wing where sensory neurons or their outgrowing axons are not normally found. The corresponding areas in the mutant wings are noted with boxes of the same type. (B) *fasI⁻;fasIII⁻* mutant wing. The box indicates the misrouting of sensory axons along the distal posterior margin of the wing. These axons have started to grow into the fourth vein of the wing, an area usually devoid of neurons. (C) *fasII^{e76}* mutant wing. The box indicates the appearance of ectopic sensory neurons on the proximal posterior margin of the wing. (D) *nac^{1}* mutant wing. The box indicates a bundle of axons growing along the posterior margin of the wing in the distal wing. Scale bars: A, 100 μm; B,D, 50 μm; C, 25 μm. L1, vein one; L3, vein three; PM, posterior margin.

Table 2. Altered phenotypes of wing sensory axons in the developing wing of *fasI⁻;fasIII⁻*, *fasII^{e76}*, and *nac^{1}* mutants at 6 and 24 hours after pupariation

	6 hours AP		24 hours AP		
Genotype	*n*	Phenotype	*n*	Phenotype	Penetrance
fasI⁻;fasIII⁻	40	wild-type	35	axon misrouting	25%
fasI⁺;fasIII⁺	16	wild-type	40	wild-type	
fasII^{e76}	22	wild-type	16 17	axon misrouting and extra neurons	50% (males) 80% (females)
fasII^{e93}	28	wild-type	24	wild-type	
nac^{1}	23	wild-type	19	axon misrouting and extra neurons	25%
st/st	10	wild-type	25	wild-type	

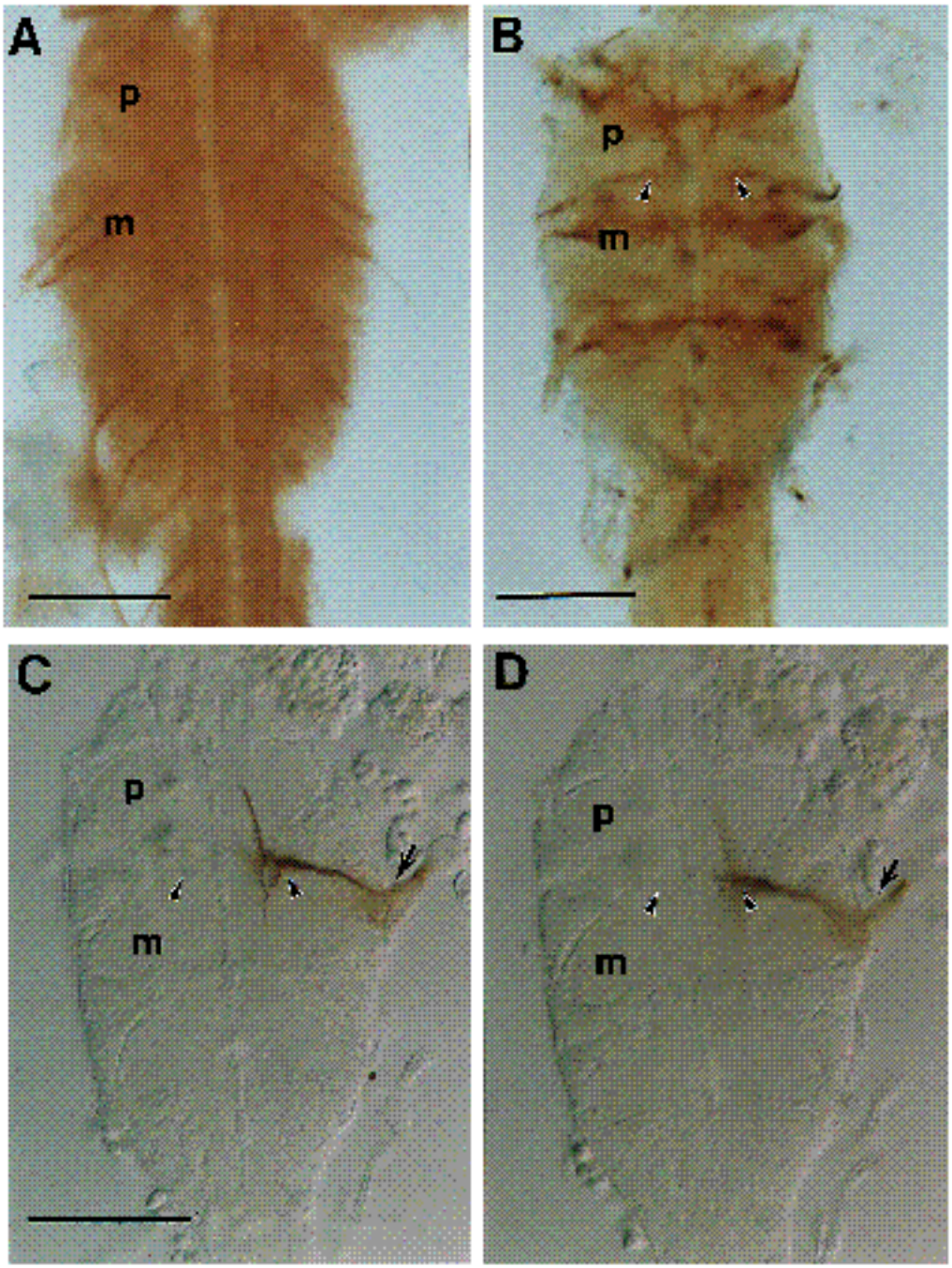

Fig. 4. Pupal thoracic nervous systems of wild-type animals stained with antibodies to *fasII* (A) and *fasIII* (B) glycoproteins. Nervous systems are stained at 20 hours AP, a time when wing sensory axons are entering the CNS. The anti-fasciclinII (A), stained primarily longitudinal tracts and some peripheral nerve bundles, while the anti-fasIII (B) stained primarily commissural tracts in the CNS. (C,D) DiI, intensified with DAB, stained wing sensory axons in the pupal CNS at 20 hours AP. **Arrow** on right indicates site of wing nerve entry, which lies between the pro- (p) and meso- (m) thoracic neuromeres. (C) Dorsal focal plane depicting the axons of the dorsal anterior tract (dat) and dorsal posterior tract (dpt). (D) Ventral focal plane depicting axons entering the medial tract (mt) in the CNS. **Arrowheads** indicate area of fasIII immunoreactivity seen in B. Scale bars: 100 μm. p, prothoracic neuromere; m, mesothoracic neuromere.

Table 2). The number of ectopic neurons present was also sexually dimorphic: when ectopic neurons did occur, females displayed a greater number than did males.

nac^{1}

The phenotype of 24 hours AP of *nac*^{1} mutants was similar to that of the *fasI*$^{-}$;*fasIII*$^{-}$ mutants, but was more extreme in that the misrouted axons traveled further along the posterior margin of the wing (Fig. 3D). However, they never traveled far enough to join the main wing nerve near the base of the wing (see Fig. 1A, LI). This misrouting at the distal tip of the wing was often accompanied by the appearance of several extra neurons around the area posterior to the junction between the third vein and the wing margin (Table 2).

Controls

All of the controls displayed the characteristic wild-type pattern of sensory neurons at 6 and 24 hours AP (Table 2).

Fasciclin antibody staining in wild-type wings

Wild-type wings from 6 and 24 hours AP were stained with antibodies to the *fasI*, *fasII* and *fasIII* glycoproteins. All three antibodies labelled faintly the neurons of the anterior wing margin at 6 hours AP; the anti-fasI antibody also labelled faintly the large campaniform sensilla on the third

vein. The anti-fasII antibody showed epithelial labelling at 6 and 24 hours AP. No labelling of neurons by any of the antibodies was seen at 24 hours AP (data not shown).

Fasciclin antibody staining in the wild-type central nervous system

The *fasI*, *fasII* and *fasIII* genes code for glycoproteins that are expressed on specific subsets of the axon scaffold of the CNS during embryonic development (for a review see Grenningloh et al., 1990). However, it was not known if they were expressed during metamorphosis.

Anti-fasciclin antibodies stained the wild-type CNS during pupal development at a time when the wing sensory axons are establishing their adult projection pattern (12-24 hours AP; Whitlock and Palka, 1992). The anti-fasI antibody did not stain specific tracts in the nervous system but stained the optic lobes intensely (not shown). The antibody to fasII stained a small subset of longitudinal tracts running through the thoracic nervous system and into the brain (Fig. 4A), as well as a subset of peripheral axons. The antibody to fasIII strongly stained a small subset of commissural tacts in the pro-, meso- and metathoracic ganglia (Fig. 4B). Commissures were also stained in the abdominal ganglia and the brain, but less intensely than were those in the thoracic nervous system (data not shown). In addition, some longitudinal tracts were stained and a small scaffold-like pattern was observed in the abdominal ganglia (data not shown). Based on morphological landmarks in the CNS, it appears that part of the mesothoracic accessory neuromere is stained with anti-fasIII antibody. This is an area of known wing sensory axon termination and is indicated in Fig. 4B.

DISCUSSION

Subtle defects in axon development are evident in all mutants

In this study, I have shown that mutations in the *fasciclin* genes, *fasI*, *fasII*, *fasIII*, and the *nac* gene disrupt development and adult morphology of wing sensory neurons. The proteins encoded by these genes are broadly expressed cell surface adhesion molecules (fasI, fasII and fasIII) and an enzyme involved in the glycosylation of different proteins during development (nac). The phenotypes described here, which result from the disruption of these proteins, suggest that they play a subtle role in neurogenesis and axonogenesis in the PNS and axon termination in the CNS.

While extra branching in the projection of wing sensory axons into the CNS was the only morphological phenotype recognized in the adult of *fasI* mutants, it has been shown previously that mutations in *fasI*, while having no phenotype when alone, take on a severe phenotype when combined with other mutations (e.g., *abelson*; Elkins et al., 1990b). The loss of either fasI or fasIII has no effect on the developing sensory axons in the wing. Yet, I found that the combined loss of the *fasI* and *fasIII* gene products led to defects in the growth of wing sensory axons in the periphery. These data indicate that the *fasI* product (an extrinsic membrane glycoprotein) and the *fasIII* product (an integral membrane glycoprotein) in combination help to delineate pathways for axonal growth in the developing wing sensory system.

Although hypomorphic *fasII* mutants did not display gross abnormalities in CNS structure (Grenningloh et al., 1991), they did show increased branching of the wing sensory axons and misrouting in the wing (Whitlock and Palka, 1991 and this study). When the hypomorphic allele was placed over a null allele, there was no increase in the severity of the phenotype, suggesting that the observed phenotype is close to that of a phenotypic null.

nac and *fasIII* mutants have in common a phenotype of axon misrouting in the CNS

The *nac* mutation produces an alteration in a carbohydrate moiety shared by proteins expressed on the surface of neurons and other tissues (Katz et al., 1988). Surprisingly, the loss of *nac* function affected only a subset of the developing wing sensory neurons. The misrouting of wing sensory axons in the *nac* mutant was dramatic in that when the medial axon bundle was missing, it was never inititated. This is unlike the *fasIII* mutant where the medial tract was always started before crossing over to the lateral tract.

Unlike the *nac* mutation, the *fasIII* mutation removes a cell surface glycoprotein; yet both mutants displayed a similar defect in the CNS. This shared phenotype may indicate that the lateral tract has not only greater adhesivity, but also a greater redundancy of cues and thus may be buffered from alterations in these cell surface molecules. Another explanation could be that the axons of the medial tract are developmentally delayed in these mutants and choose to travel in the later developing lateral tract. This, however, is unlikely because I have found that the medial tract axons are present at their normal developmental time.

The misrouting of the medial axon bundle was not observed as often in animals mutant for both *fasI* and *fasIII* as it was in flies mutant only for *fasIII* (Table 1). A possible explanation for this less severe phenotype in the double mutant could relate to the relative 'attractiveness' of the medial and lateral tracts. Perhaps fasIII is expressed on the medial tract and fasI the lateral tract, both in conjunction with other cell surface molecules. When only fasIII is removed from one tract, there is a clear difference in 'attractiveness' for the incoming axons. But when both fasI and fasIII are removed there is now a balance of 'attractiveness' causing axons to misroute less often. This is supported by the pattern of anti-fasI and anti-fasIII immunoreactivity during metamorphosis (see below).

Ectopic sensory neurons: a possible role for *fasII* in neurogenesis?

The occurrence of ectopic neurons in *fasII* mutants suggests that cell surface molecules, which are traditionally believed to be involved in axon guidance, might also be involved in neurogenesis. On the posterior margin of the wing, there are normally uninnervated hairs, which arise from one round of mitosis 16-20 hours after pupariation (Hartenstein and Posakony, 1989). Reduction of the *fasII* product might allow for extra divisions in the precursors of these normally uninnervated bristles to produce sensory structures.

The posterior margin seems to be especially sensitive to failures in the mechanism(s) that prevent the occurrence of

extra neurons and the straying of axons into this area, normally devoid of neurons and axons (such as seen in *nac* and *fasI;fasIII* mutants). Indeed, mutations in the *fasII* gene, as well as in *Notch* (A. Giangrande, personal communication), lead to the production of ectopic neurons and the straying of axons into this area, indicating a developmental link between the processes controlling neurogenesis and axon guidance.

Fasciclin glycoproteins are expressed in the CNS during metamorphosis

In the *Drosophila* embryo, the fasciclin glycoproteins are expressed in specific subsets of the axonal scaffold in *Drosophila*, in addition to many non-neuronal cells (Grenningloh et al., 1990).

Until now it was not known if the fasciclin glycoproteins were expressed in the pupal nervous system. I have found that the anti-fasciclin antibodies label at a time when wing sensory axons are growing into the CNS, and do so in an area where wing sensory projections terminate, as well as in other areas in the CNS. Whether it is the actual wing sensory axons that are stained with the anti-fasIII antibody is not known, but it is possible that the defects seen in the mutants are due to the loss of *fasIII* expression in this area of the CNS.

Genetic versus experimental removal of fasciclins

The results obtained in this study are at odds with those obtained in *Schistocerca.* In this hemimetabolous insect, Chromophore Assisted Laser Inactivation (Jay and Keshishian, 1990; Booth et al., 1991) of the fasciclin glycoproteins on developing sensory neurons resulted in axon defasciculation in leg sensory axons (fasI) and defects in axonogenesis (fasII). In contrast, only subtle defects were found in *fasI* and *fasII* mutants in both the data presented here and in Elkins et al. (1990b) and Grenningloh et al. (1991). Although the laser ablation is believed to affect only molecules labelled with the chromophore (Jay et al., 1988), the possibility that it affects neighboring molecules has not been entirely eliminated. In addition, it is possible that these molecules are used for slightly different purposes in these two evolutionarily diverse groups of insects.

The effects of the $fasI^{-}$, $fasII^{e76}$, $fasIII^{-}$ and nac^{1} mutations on both neurogenesis and guidance indicate that these molecules play multiple roles in neuronal development. Because the axon tracts in the CNS for the most part remain unaltered and none of the phenotypes described here are 100% penetrant, it appears that these molecules are involved in the 'fine tuning' of the development of neurons both in the PNS and CNS.

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