

# Genetic Analysis of Stomatogastric Nervous System Development in *Drosophila* Using Enhancer Trap Lines

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The stomatogastric nervous system (SNS) of *Drosophila melanogaster* is a small, simply organized neural circuitry which innervates the anterior enteric system. It is responsible for regulating the passage of food through the pharynx and esophagus and into the midgut. Here we show that the development of the SNS is amenable to genetic dissection. We screened lines from a P-element mutagenesis, selecting those with *lacZ* reporter gene expression and/or a phenotype in the SNS, associated glia, and garland cells. We report a collection of expression patterns and mutant phenotypes among lines found to have a mutation in genes required for the establishment of the larval SNS. Our results indicate that SNS development depends on pattern organizer genes including components of the Ras/Raf pathway. © 1997 Academic Press

## INTRODUCTION

How does an embryo orchestrate a mass of undifferentiated cells into an intricately wired nervous system? In *Drosophila melanogaster*, it is known that there is a process of neural determination, which specifies certain cells from among others to follow a neural fate (Campos-Ortega, 1993). These cells then serve as neural precursors and give rise to the complement of cells which will form the mature nervous system (Goodman and Doe, 1993). After migrating to their required locations, these neural cells will assume their programmed role, either as glial support cells or as neurons. Neuronal cells will then go through a process of extending axons to innervate their specific targets. Each of these stages employs groups of genes, interacting along various signaling pathways, to instruct neural cells and their neighbors as to their appropriate behavior. This includes the members of proneural and neurogenic genes, for neural determination (Artavanis-Tsakonas and Simpson, 1991; Heitzler *et al.*, 1996), neural precursor genes and neural identity genes for implementing differentiation and specific fates, respectively, of individual neural precursors (Jan and Jan, 1993; Vaessin *et al.*, 1991), and genes required to guide both migrating cells and the growth cones of extending axons to

their correct targets (reviewed in Tessier-Lavigne and Goodman, 1996).

This seemingly complete understanding of the process of neurogenesis is, however, a picture that has developed by a fill-in-the-dots style of reconstruction. Many of the details of specific molecular pathways and interactions are still unknown. To gather more information about the mechanisms of neurogenesis, we chose to analyze the stomatogastric nervous system (SNS).

The SNS innervates the anterior enteric system. It consists of four small ganglia and their interconnecting nerves, which simplifies the analysis of mutant phenotypes. The morphological process of SNS development in the wild-type embryo has been previously characterized (Hartenstein *et al.*, 1994; González Gaitán and Jäckle, 1995). In addition, the genetic pathways that are known to direct neurogenesis in the peripheral and central nervous systems (PNS and CNS) have been shown to function in directing SNS development (González Gaitán and Jäckle, 1995). Here we present a genetic screen for genes which affect different phases of embryonic SNS development.

We have also included SNS-associated glial cells and the garland cells in our studies. The garland cells form a ring around the anterior-ventral side of the proventriculus. Structural and physiological studies have shown these cells to be involved in intense endocytosis and exocytosis (Aggarwal and King, 1967; Kosaka and Ikeda, 1983; Narita *et al.*, 1989; Schulze *et al.*, 1995) and they have been proposed to

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function as nephrocytes, removing waste from the hemolymph by endocytosis (Aggarwal and King, 1967; Rizki, 1978). Yet, it has been noted that the garland cells express a number of neural-specific markers including *prospero*, the neural antigen *hrp*, *fasciclin II* (*fas II*), and *syntaxin* (Grenningloh et al., 1991; Matsuzaki et al., 1992; Schulze et al., 1995; Sun and Salvaterra, 1995), which suggests that they have a neural character and that their endocytic and exocytic activity is in fact synaptic activity involved in regulating the proventriculus. We report here how we have used the current understanding of SNS, garland cell, and SNS-associated glial development as a diagnostic tool to screen for genes which function in the development of the nervous system. Both novel genes as well as characterized genes were recovered by the screen, and the results show that pattern organizer genes, including the pair rule gene *hairy* (Ingham et al., 1985) and members of the Ras/Raf pathway, neural precursor, and glial genes, are required for development of the SNS.

## MATERIALS AND METHODS

### *Drosophila* Stocks and Egg Collections

All stocks are maintained according to protocols described in Roberts (1986) and Ashburner (1989). The food medium used is a mixture composed of dry yeast, soya flour, corn flour, beet sugar syrup, malt, propionic acid, Nipagin, and ethanol. Egg collections were done in cages using apple juice agar plates supplemented with a live yeast paste as the support for egg deposition. Genotypes are as described in Lindsley and Zimm (1992).

### Screening the P-Element Insertion Lines

An initial round of screening was performed looking for embryonic expression of  $\beta$ -galactosidase ( $\beta$ -gal) from the *lacZ* gene in the integrated P-element. More than 900 autosomal, lethal insertion lines from the collection generated in the lab of A. Spradling (Spradling et al., 1995) were screened for  $\beta$ -gal activity with X-gal as described in Ashburner (1989). Those lines showing expression in some stage of the SNS or in the garland cells were then re-screened by staining with the monoclonal antibody Mab22C10 (Fujita et al., 1981) to look for defects in the SNS as well as with an anti- $\beta$ -gal antibody to verify the  $\beta$ -gal pattern. A total of 70 lines were initially selected from the second and third chromosomes combined, from which 18 lines were chosen for further testing.

### Whole-Mount *in Situ* Hybridization and Antibody Staining

Whole-mount *in situ* hybridization to embryos as well as to polytene chromosomes was done as described in Hartmann and Jäckle (1995). Embryonic antibody stainings were done as described in González Gaitán and Jäckle (1997). To identify the homozygous mutant embryos blue balancers were used as described (González Gaitán and Jäckle, 1995). Antibody concentrations used were rabbit anti- $\beta$ -gal 1:1000 (Cappel); mouse anti- $\beta$ -gal 1:200 (Promega); Mab22C10 1:50 (Fujita et al., 1981); anti-Repo 1:50 (Halter et al., 1995); anti-Fas II 1:30 (Grenningloh et al., 1991); anti-Ac 1:3 (Skeath

and Carroll, 1992); anti-Crb 1:20 (Tepass et al., 1990); anti-Fkh 1:50 (Weigel et al., 1989); and anti-Kr 1:500 (Gaul and Jackle, 1987).

### Mounting the Embryos

To observe a given staining, embryos were embedded in araldite as described in González Gaitán and Jäckle (1997). Once in the araldite, the embryos were mounted into glass capillaries (Hilgenberg capillaries, 65 mm long, outer diameter 0.25 mm, wall width 0.025 mm) by allowing the embryos to be sucked up by capillary action. Mounting into the glass capillaries facilitated ease of viewing in all angles of the embryos under the microscope.

### Plasmid Rescue

Genomic DNA was isolated as described in Ashburner (1989) with the following modifications. The flies were homogenized in a total of 3 ml homogenization buffer (10 mM Tris-Cl, pH 8; 60 mM NaCl; 10 mM EDTA; 0.15 mM spermidine) in a chilled Dounce homogenizer. Following addition of SDS and proteinase K to the nuclear juice, the samples were incubated at 55°C for 1 hr. This was then extracted with 1 vol phenol/chloroform followed by extraction with 24:1 chloroform:isoamyl alcohol. The DNA was precipitated and the pellet resuspended in 400  $\mu$ l TE, pH 8, to which was then added 1  $\mu$ l DNase-free RNase and 2  $\mu$ l 0.5 M EDTA, pH 8. After 30 min at RT this was phenol and chloroform extracted as before and precipitated with 100% ethanol for 30 min at -80°C, and the pellet washed in cold 70% ethanol and resuspended in 100  $\mu$ l TE, pH 8.

Between 10 and 50  $\mu$ g of DNA was digested with *Xba*I or *Xba*I/*Nhe*I, the reaction phenol extracted, and the DNA precipitated as above. Pellets were resuspended in 24  $\mu$ l TE, which was then divided into six ligation reactions to maximize kinetics. The ligation reaction contained 0.5 mM ATP, 10 mM DTT, 0.04 mg/ml BSA, 1 $\times$  ligation buffer (20 mM Tris, pH 7.6, 10 mM MgCl<sub>2</sub>), and 3 U ligase in a final volume of 200  $\mu$ l. The ligation was done at 16°C for 16 hr followed by phenol extraction and precipitation as before. The pellets of all six reactions were combined in a total of 16  $\mu$ l TE final volume. Two microliters ligated plasmid was then transformed by electroporation, grown 1 hr at 37°C in 600  $\mu$ l LB + 0.4% glucose, plated 4  $\times$  150  $\mu$ l onto kanamycin plates (100 mg/ml), and grown at 37°C.

Genomic inserts obtained by plasmid rescue were liberated from the P-element plasmid by excision with *Hind*III and subsequently subcloned into the SK+ Bluescript vector (Stratagene) by a standard cloning protocol (Sambrook et al., 1989).

### Genomic and cDNA Screening

A  $\lambda$ FIX II genomic library, a  $\lambda$ Zap cDNA (Stratagene), and a cDNA library prepared by Dr. Nick Brown (Brown and Kafatos, 1988) were screened as described in Sambrook et al. (1989). Hybridization was done at 65°C in a hybridization solution containing 5 $\times$  SSPE, 5 $\times$  Denhardt's, 0.1% SDS, 10 mM Na pyrophosphate, 50 mM Na phosphate buffer, pH 7, and 0.1 mg/ml salmon sperm DNA. Washes were done at 65°C in 0.2 $\times$  SSPE/0.1% SDS. The plasmid rescue fragments and various genomic pieces for genomic walking were used as probes and were labeled with [ $\alpha$ -<sup>32</sup>P]CTP by random priming using the Pharmacia Quick Prime kit.

### $\Delta 2$ -3-Induced Transposition of the P-Element

To verify that the lethality and phenotype seen in the line arise from the P-element insertion, the P-element was removed from the genome by  $\Delta 2$ -3 transposase-mediated transposition (Robertson and Engels, 1989). For second chromosome P-element insertions, *P, cn/Cyo; ry* females from the enhancer trap line were crossed to *Cyo/Sp;  $\Delta 2$ -3, Sb e/TM6* males. The *Cyo/P, cn;  $\Delta 2$ -3, Sb e/ry* F1 males were back-crossed to *P, cn/Cyo; ry* females in individual vials containing four males and four females, to trace possible clustered jump-out events. To look for revertants of the lethality caused by the original insertion, the F2 generation was screened for *P, cn/\*, cn; ry* flies (phenotypically  $Cy^+$ ), where \* signifies the chromosome from which the P-element has been removed. To look for imprecise excision events that may have produced deletions and thereby stronger mutations, single \*, *cn/Cyo; ry* F2 males (phenotypically  $Cy, cn, ry$ ) were individually back-crossed to several *Cyo/P, cn; ry* females and the F3 was screened for the absence of *P, cn/\*, cn; ry* progeny (phenotypically  $Cy^+$ ) and for *Cy/\*, cn; ry* males and virgin females, which were mated to establish stocks.

For third chromosome P-element insertions, *P, ry/TM3, Sb ry* females from the enhancer trap line were crossed to  $\Delta 2$ -3, *Sb e/TM6, Dr* males. The  $\Delta 2$ -3, *Sb e/P, ry* F1 males were back-crossed to *P, ry/TM3, Sb ry* females in individual vials containing four males and four females. To look for revertants the F2 generation was screened for \*, *ry/P, ry* flies (phenotypically  $Sb^+$ ). Imprecise excision events were detected by individually back-crossing single, F2 \*, *ry/TM3, Sb ry* males with several *P, ry/TM3, Sb ry* females. The F3 were screened for the absence of \*, *ry/P, ry* progeny (phenotypically  $Sb^+$ ). \*, *ry/TM3, Sb ry* males and virgin females (phenotypically  $ry, Sb$ ) were mated to establish stocks.

## RESULTS

We begin with a description of SNS development (Figs. 1 and 2), outlining five distinct phases of the process which can be assessed for aberrations in a given mutant background. Following this, we present 10 P-element lines which reveal a number of genetic components required for SNS, glial, or garland cell development.

### SNS Development in Wild Type

During the first phase of neurogenesis, the SNS anlage corresponds in position to Victoria Foe's mitotic domain 23 (Foe, 1989; this paper). This anlage eventually becomes localized to the dorsal lip of the stomodeal opening by stage 10 (staging according to Campos-Ortega and Hartenstein, 1985), at which point proneural genes begin to be expressed in all cells of the domain, producing a coherent, proneural expressing cluster (Fig. 1A). Once the proneural cluster has formed, a few SNS precursor cells will delaminate from the anterior-most area of the anlage (Hartenstein *et al.*, 1994).

As with the stereotyped procedure of neural determination that is understood in the CNS or PNS (Campos-Ortega, 1993), proneural expression is then antagonized by expression of the neurogenic genes to single out specific cells among the cluster of proneural gene-expressing cells (González Gaitán and Jäckle, 1995). However, in this case the singling out serves a purpose other than to select which of

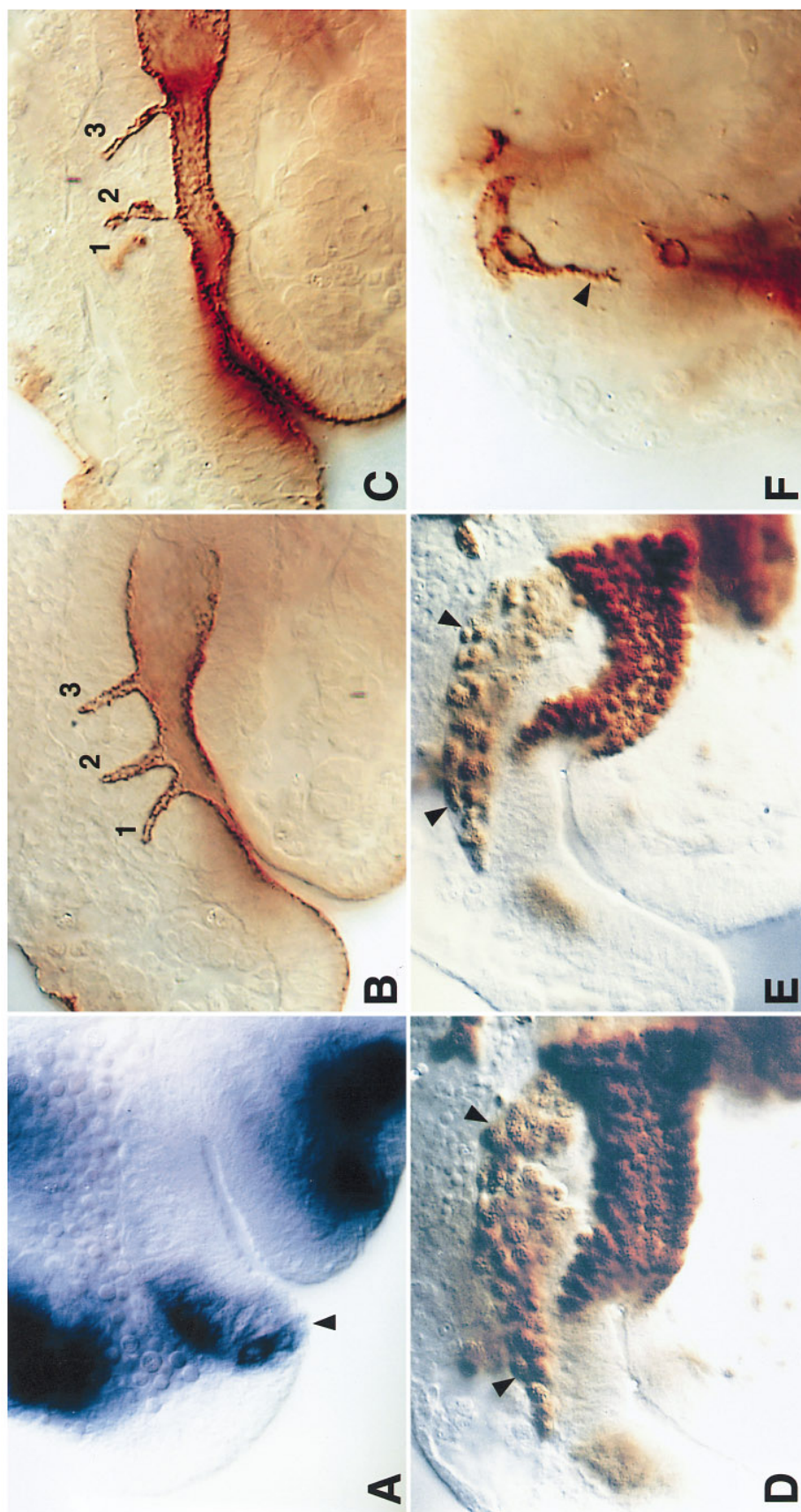
the cells will follow a neural fate. In the CNS or PNS one cell is singled out per cluster of proneural gene-expressing cells and this cell serves as a stem cell-like neural precursor (reviewed in Goodman and Doe, 1993). Contrary to this, three cells are singled out from within the single proneural cluster which is the SNS anlage and these cells then initiate a distinct feature of SNS development. Serving as tip cells, they direct the second phase of SNS development, whereby an invagination process occurs forming three distinct epithelial folds with a single proneural-expressing cell at each tip (Fig. 1B; for details see González Gaitán and Jäckle, 1995).

Once the invagination process is complete, proneural gene expression appears *de novo* in all of the cells contained within the three invaginations, and a third phase of SNS development begins, during which the three invaginations pinch off from the epithelium to form separate vesicles (Fig. 1C). A fourth phase can be categorized as the period of cell migration, when the cells of the three vesicles localize to their appropriate destinations where they will eventually organize into the mature embryonic SNS (Figs. 1D and 1E). How they find their way to these positions is as yet unknown.

The final, morphologically distinct phase of SNS development is the process whereby these neural cells generate the axonal scaffold which occurs in a manner reminiscent of the CNS (Goodman and Doe, 1993). Neurons from the individual ganglia send out pioneer axons which will meet up with outgrowing axons from the other ganglia and eventually establish the interconnecting nerves (González Gaitán, unpublished results). Other neurons send out axons to establish the nerves that innervate the dorsal pharyngeal muscles, the midgut, and the CNS (Fig. 1F). Once these foundation tracts have been laid, the growth cones of the remaining cells of the various ganglia navigate along them to reach their final targets. The mature embryonic SNS consists of only four ganglia—the frontal ganglion, the esophageal ganglion 1, the esophageal ganglion 2, and the proventricular ganglion—and their associated nerve tracts (González Gaitán and Jäckle, 1995; Figs. 2A–2C; overview in Fig. 2D).

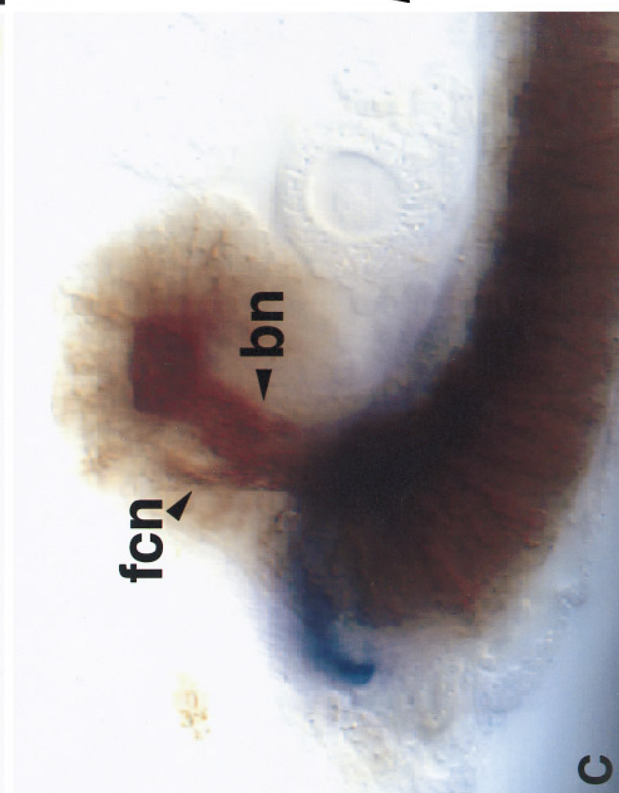
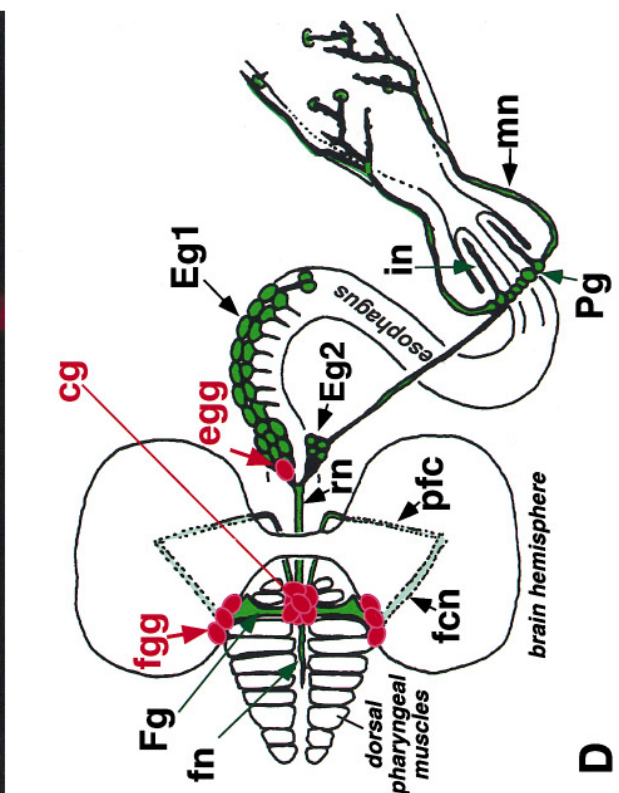
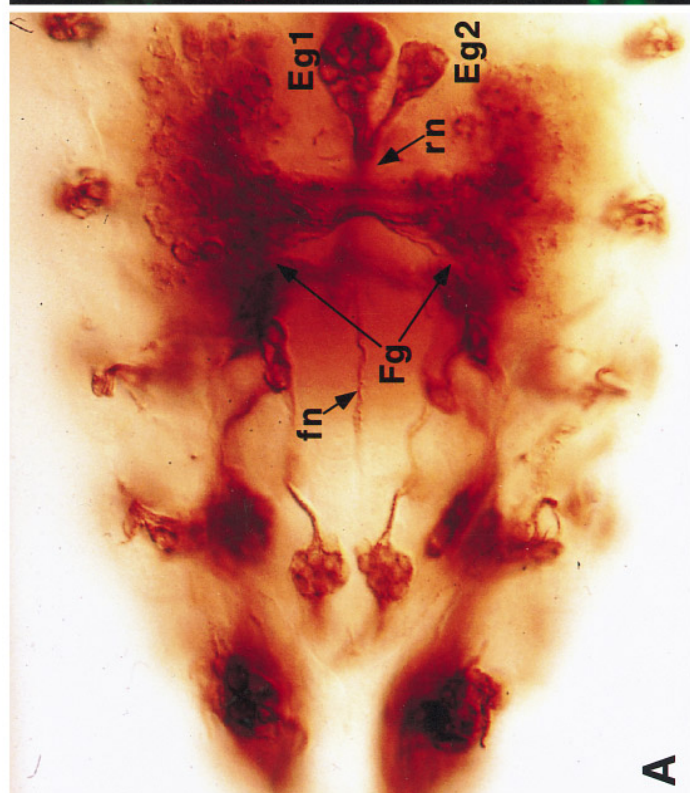
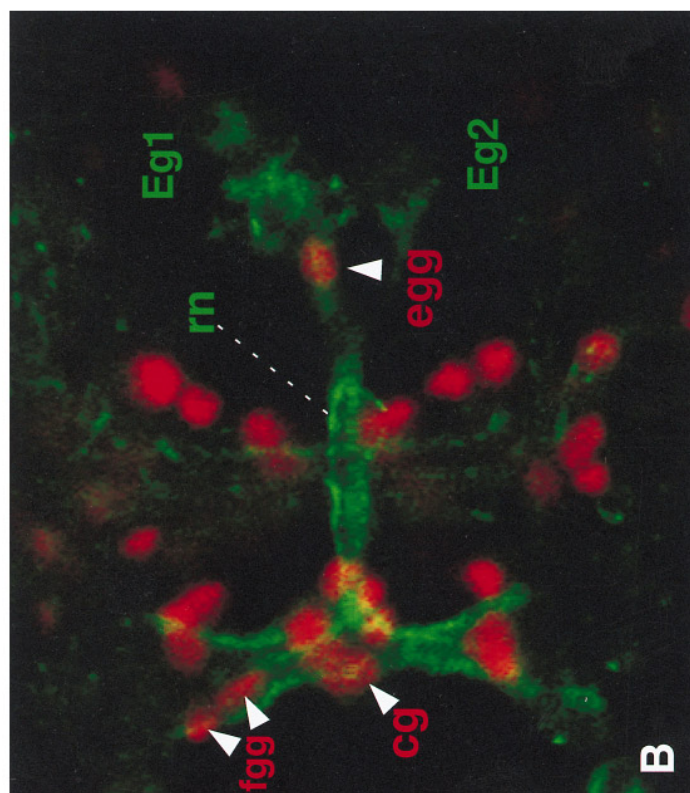
We have also identified SNS-specific glia, which may aid both in directing the migrating neural cells to their final destinations and in growing SNS axons to their targets (González Gaitán, unpublished results). The glia arise from a set of delaminating cells adjacent to the invaginations at approximately stage 11. These then become situated such that they prefigure the pathways to be established by the pioneer nerves for the recurrent nerve and frontal connectives. The glia of the mature SNS are found as three groups of cells (Fig. 2B, schematized in Fig. 2D). One group is associated with the frontal ganglion (the frontal ganglion glia), one group is found in the frontal commissure, at the base of the frontal nerve (commisural glia), and a third group is located at the fork in the recurrent nerve off of which the two esophageal ganglia extend (esophageal ganglia glia).

In summary, morphological and molecular features of SNS development can be subdivided into five phases, namely, initial proneural gene expression within the anlage,



**FIG. 1.** The five phases of wild-type (WT) embryonic SNS development. Phase one of SNS development begins with proneural gene expression in the SNS anlage. (A) A stage 10 embryo showing lethal of scute expression encompassing the anlage. Invagination of the anlage occurs in the second phase. (B) A stage 12 embryo stained with anti-Crumbs shows the three complete invaginations (1, 2, 3). The third phase involves the pinching off of the invaginations to produce three epithelial vesicles. (C) A stage 13 embryo stained with anti-Crumbs shows the vesicles pinching off from the surface, where (1) is already separated, (2) is pinching off, and (3) is about to pinch off. During stages 13 (D) and 14 (E) anti-Forkhead labeling allows visualization of the fourth phase during which cells dissociate from the vesicles and migrate to their intended positions. The axonal tracts are established during the fifth phase. MAb22C10 labeling of a stage 15 embryo (F) shows the axons growing ventrally from neurons of the frontal ganglion (Fig) to form the frontal connective (fcn). All images are lateral perspectives. See text for antibody details. The embryos in this and the following figures are arranged such that anterior is left and, in lateral views, dorsal is always up.





the invagination process, vesicle formation, migration to the appropriate locale, and the establishment of the neuronal tracts interconnecting the four ganglia. We used this description as a basis to screen for mutations which affect specific stages of development in the system.

### Genes Affecting SNS Development

We screened embryos from more than 900 lines containing single, lethal, autosomal P-element insertions (Spradling *et al.*, 1995). Our criteria for selecting a line for further analysis consisted of two main points:  $\beta$ -gal expression from the inserted *lacZ*-containing P-element in any stage of the embryonic SNS or garland cell development and/or a P-element-induced embryonic SNS phenotype.

Table 1 summarizes both the  $\beta$ -gal expression and the defects seen in the lines to be discussed.  $\beta$ -Gal patterns included expression in the SNS anlage, in the invaginations, in several ganglia, and in glia. Expression was also found in the garland cells of several of these lines.

To visualize defects in the developing SNS of putative mutants, we used anti-Crumbs (anti-Crb) antibodies to score early defects. Anti-Crb antibodies recognize a protein in the apical membrane of ectodermal epithelial cells (Tepass *et al.*, 1990) providing visualization of invaginating epithelia and therefore of the earliest morphological defects of the developing SNS: a disruption of the invagination process. Later aberrations can be visualized using the antibody Mab22C10, which recognizes an antigen in the axonal cytoskeleton (Estes *et al.*, 1996; Fujita *et al.*, 1981), as well as the antibody against Fasciclin II (anti-Fas II), which recognizes a subset of axonal tracts (Grenningloh *et al.*, 1991). The defects detected included various manifestations of defasciculation, loss of cells or nerve tracts, and falsely positioned ganglia. Fasciculation seems to be the most sensitive feature of the system, since we saw some form of defasciculation in most of the lines with a detectable phenotype.

### Expression in the SNS Anlage

Embryos with the insertion *l(3)1620* have  $\beta$ -gal expression which can first be detected in the early SNS anlage. It is already expressed in Victoria Foe's domain 23 (Fig. 3A) and continues to be expressed in the SNS anlage throughout the invaginations until the time that the vesicles pinch off from the epithelium (Fig. 3B). Embryos homozygous for the P-element insertion consistently have extra neural cells in the PNS where there are often extra cells in the dorsal clus-

ter of neurons and the pentachordotonal organs (Fig. 3C; a description of the PNS can be found in Dambly-Chaudière and Ghysen, 1987). Likewise, there are extra neural cells in the ganglia of the SNS and correspondingly broader fascicles as a result (Fig. 3D). The P-insertion is found cytologically at 66E on the third chromosome, as determined by *in situ* hybridization to polytene chromosomes with a *lacZ* probe (not shown). We performed plasmid rescue experiments to clone the genomic region flanking the P-insertion (Wilson *et al.*, 1989) and obtained a 220-base-pair genomic fragment. This plasmid rescue fragment was used to obtain a cDNA, which was found to cross-hybridize to *hairy (h)* DNA (Ish-Horowicz *et al.*, 1985). The cytological location and the isolated *h* cDNA are consistent with the  $\beta$ -gal pattern which appears in seven stripes in the blastoderm embryo, typical of the *h* expression pattern (Fig. 3A; Ingham *et al.*, 1985). However, there is no pair rule phenotype associated with the P-element insertion which must be due to a fortuitous hit of the P-element affecting only the function of the gene required for neural development. As a result we are able to perceive the role of *h* in neural development without the interference of secondary traits stemming from the pair rule requirements.

The insertion *l(3)5124* has an embryonic  $\beta$ -gal pattern found in the anlage of the SNS until early stage 11 (Fig. 4A), in the garland cells (Fig. 4B), and in the commissural glia of the stage 16 SNS (Fig. 4C). The mature SNS is quite strongly disrupted in these embryos, often displaying projections sent out randomly from the ganglia and apparently fewer cells in the loosely associated esophageal ganglia (Fig. 4D). There are three insertions in this line, one of which was found to be in the gene *couch potato (cpo)* (Berkeley Drosophila Genome Project and Flybase, 1995), a neural precursor gene (Vaessin *et al.*, 1991) known to code for an RNA binding protein (Bellen *et al.*, 1992). The misrouting of axons and loss of organization are typical phenotypes for neuronal precursor gene mutations (Vaessin *et al.*, 1991; Doe *et al.*, 1991). However, it is also possible that the phenotype arises from a defect from one of the other two insertions. Further analysis of this line should reveal the identity of the gene responsible for the phenotype seen and provide information on how the activity of this gene functions in either differentiation or identity determination of SNS cells.

### Defects in Cell Migration and Axonal Outgrowth

The insertion *l(3)2526* expresses  $\beta$ -gal in the early delaminating SNS precursors cells which arise from a region just

**FIG. 2.** The WT SNS at stage 16 of embryogenesis. (A) Dorsal view of the SNS in embryos stained with Mab22C10 in which the frontal nerve (fn), frontal ganglion (Fg), recurrent nerve (rn), and esophageal ganglia 1 and 2 (Eg1 and Eg2) are all visible. The proventricular ganglion (Pg) and proventricular nerve (pn) are out of focus. (B) Dorsal view of the SNS double stained with Mab22C10 (green) and anti-Repo (red) to show the SNS-associated glia. cg, commissural glia; fgg, frontal ganglion glia; egg, esophageal ganglion glia. (C) Lateral view of a BP102 staining showing the frontal connective (fcn) of the SNS where it joins the CNS in the subesophageal ganglion. bn, brain connective. (D) A schematic diagram of the SNS with its associated glia. in, internal nerves; mn, midgut nerves. For more details, see González Gaitán and Jäckle (1995).

**TABLE 1**  
Summary of Data from the Analyzed P Lines

P-line	$\beta$ -Gal expression	Phenotype	Gene	Chromosome location
<i>l(3)1620</i>	Mitotic domain 23 SNS anlage	Extra neural cells in the embryonic SNS	<i>hairy</i>	66E
<i>l(3)5124</i>	SNS invaginations SNS anlage and delaminating cells cg Garland cells	Fused invaginations Mispositioned ganglia	<i>couch potato</i>	90D1-2 85E9-10 96B19-20
<i>l(3)2526</i> <i>l(3)10419</i>	Early delaminating cells Cells delaminating from vesicles cg Garland cells	rn defasciculation Disorganized Eg1/Eg2 Lack of fcw	<i>prospero</i>	86D
<i>l(3)1728</i>	Fg	Mild defasciculation Eg1/Eg2 not separated rn does not fork	Unknown	85D7-8
<i>l(3)6683</i>	Fg and Pg	Severe defasciculation of rn with two independent nerves between the Fg and either Eg1 or Eg2	Unknown	62E6-7
<i>l(3)5484.2</i>	fgg, cg, and egg	Mispositioned ganglia Defasciculation Condensed SNS	<i>pointed</i>	94F
<i>l(3)7825</i>	SNS invaginations cg	Mispositioned ganglia Defasciculation Condensed SNS	<i>pointed</i>	94F
<i>l(2)5671</i>	Tip cells of invaginations	Embryonic lethality Adult rough eyes	<i>Star</i>	21E1-2
<i>l(2)6694</i>	Garland cells SNS ganglia	Pupal lethal	$\alpha$ - <i>adaptin</i>	21C1-2

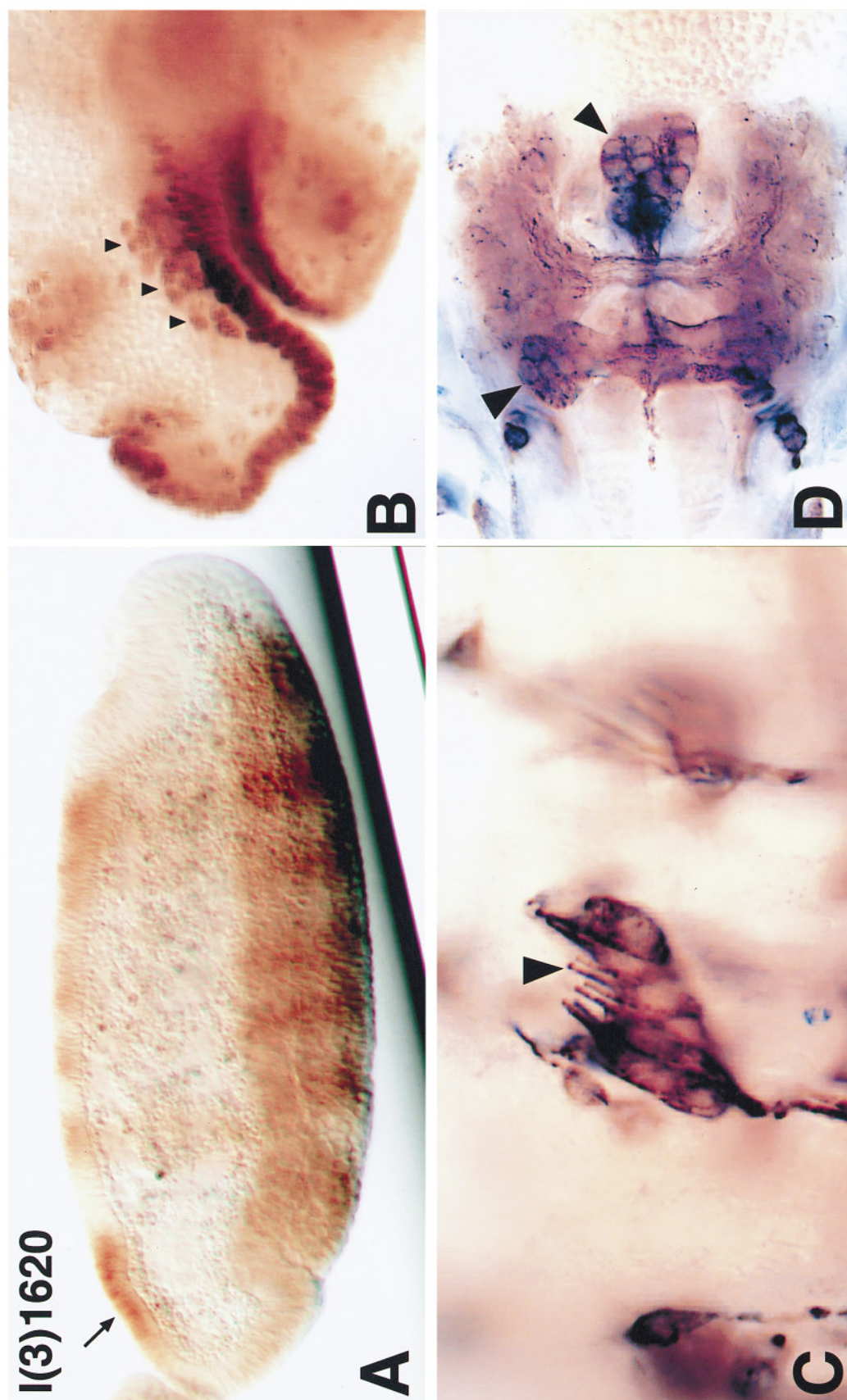
anterior to the SNS anlage at approximately stage 10 (Fig. 4E).  $\beta$ -Gal is also found in cells delaminating dorsally from the independent vesicles, in a large number of neuroblasts in the CNS and PNS (not shown) and in the garland cells (Fig. 4F). Later expression in the SNS is seen in the commissural glial cells (Fig. 4G). The frontal connectives in homozygous *l(3)2526* embryos are often severely reduced or absent altogether (compare Fig. 2C with Fig. 4H), and there is defasciculation, particularly in the recurrent nerve (not shown). *In situ* hybridization with a *lacZ* probe to polytene chromosomes of *l(3)2526* placed the P-insertion at 86D on the third chromosome, the region of the *prospero* (*pros*) gene (Doe *et al.*, 1991). Complementation analysis with a *pros* allele confirmed *l(3)2526* to be an allele of the *pros* gene. Another insertion, *l(3)10419*, also proved to be an allele of *pros*. *l(3)10419* embryos also have  $\beta$ -gal expression in the early, delaminating cells of the SNS (Fig. 4I), in the garland cells (Fig. 4J), in the commissural glia (Fig. 4K), and in the neuroblasts of the PNS and CNS (not shown). However, when homozygous this insertion causes a more severe defasciculation of the recurrent nerve than *l(3)2526*, as well as a compacting of the esophageal ganglia (Fig. 4L). As with *cpo*, *pros* is also a neuronal precursor gene (Vaessin *et al.*, 1991).

It is known to be involved in generalized differentiation of the precursor cells, but not in establishing their individual identities (Vaessin *et al.*, 1991), and has been shown to regulate the expression of other neuronal precursor genes as well as later neuronal marker genes (Doe *et al.*, 1991; Spana and Doe, 1995).

$\beta$ -Gal expression in *l(3)1728* embryos is found in several tissues throughout embryonic development. It is seen in the PNS, the CNS, the gut, and epidermis (Figs. 4M and 4N). In the SNS it is seen from stage 15 to the end of embryogenesis in the frontal ganglion (Fig. 4O), but is no longer seen in first instar larva. In addition to mild defasciculation, in homozygous *l(3)1728* embryos a consistent phenotype arises whereby the two esophageal ganglia fuse, failing to form the forked recurrent nerve with clearly separate ganglia at the end (compare Fig. 2A with Fig. 4P). A single P-element insertion was localized to 85D7-8 in this line and was proven to be responsible for the lethality of the line by successful reversion of the lethality after  $\Delta$ 2-3 transposase-induced removal of the P-element. The gene disrupted by the insertion remains to be identified.

*l(3)6683* embryos show  $\beta$ -gal expression in most neural tissue. In addition to the garland cells, it is seen late in





**FIG. 3.** h expression and requirement in the SNS as revealed by the I(3)1620 enhancer trap line. (A) The  $\beta$ -gal expression pattern in a stage 6 embryo shows the h typical pair rule stripes and anterior, cephalic spot (arrow) which corresponds to Victoria Foe's mitotic domain 23 and the earliest detectable stage of the SNS anlage. (B)  $\beta$ -Gal expression in a stage 12 embryo is found in the three SNS invaginations (arrowheads) as well as in the clypeolabrum and esophagus. Mab22C10 staining reveals extra neurons in the pentachordotonal sensory organs of the PNS (C; arrowhead) and the Fig. Eg1, and Eg2 of the SNS (D; arrowheads). Abbreviations in this and following figures are as in Fig. 2.



the SNS in the frontal ganglion (Fig. 5A) as well as the proventricular ganglion (not shown). Anti-Crb staining revealed a mild disruption in the invaginations at stage 12 of homozygous embryos (Fig. 5B). However, the  $\beta$ -gal expression in the frontal ganglion corresponds well with a later phenotype seen in homozygous embryos: the recurrent nerve is severely defasciculated such that the nerves extending between the frontal ganglion and the esophageal ganglion 1 are completely independent from those connecting the frontal ganglion with the esophageal ganglion 2 (Fig. 5C). A single P-element insertion in this line is localized at 62E6-7 (Berkeley Drosophila Genome Project and Flybase, 1995). The identity of the disrupted gene, however, remains to be determined. Further work will establish what type of molecule this gene makes and how it functions in fasciculation in the SNS.

The insertion *l(3)5484.2* has an embryonic  $\beta$ -gal pattern which strongly resembles the pattern of glia, including glia of the SNS (Fig. 5D). We confirmed that it is in fact a glial pattern by double staining with anti- $\beta$ -gal and anti-Repo (not shown), an antibody against a homeobox-containing protein expressed in most embryonic glial cells (Halter *et al.*, 1995; Xiong *et al.*, 1994). Staining homozygous embryos with Mab22C10 showed that the SNS is often considerably reduced in size and defasciculated (Fig. 5F). Polytene chromosome *in situ* hybridization localized both the P-insertion and a 16-kb genomic plasmid rescue fragment, to the third chromosome at 94F.

Both the expression pattern and phenotype of *l(3)5484.2* resemble that of the gene *pointed* (*pnt*) and, in fact, both this and another P-insertion, *l(3)7825*, turned out to be alleles of *pnt* (O'Neill *et al.*, 1994; own observations). In addition, a cDNA isolated using fragments of the *l(3)5484.2* 16-kb genomic plasmid rescue piece (see above) revealed 100% sequence homology to the *pnt* coding region. This cDNA shows an *in situ* pattern in the glia of the SNS (not shown). Although *l(3)7825* is also an allele of *pnt*, the  $\beta$ -gal pattern of these embryos is considerably different from the glial pattern of *l(3)5484.2*. The embryonic  $\beta$ -gal pattern in *l(3)7825* is seen in the SNS anlage and all three invaginations (Fig. 5G) as well as in the commissural glia (Fig. 5H). Outside of the SNS it is found in the CNS, PNS, visceral mesoderm, and malpighian tubules (not shown). The differential  $\beta$ -gal expression in the two lines may be due to the

influence of different enhancers on the different P-elements. However, that both lines show expression in the glia is consistent with the SNS glial pattern of the *pnt* cDNA.

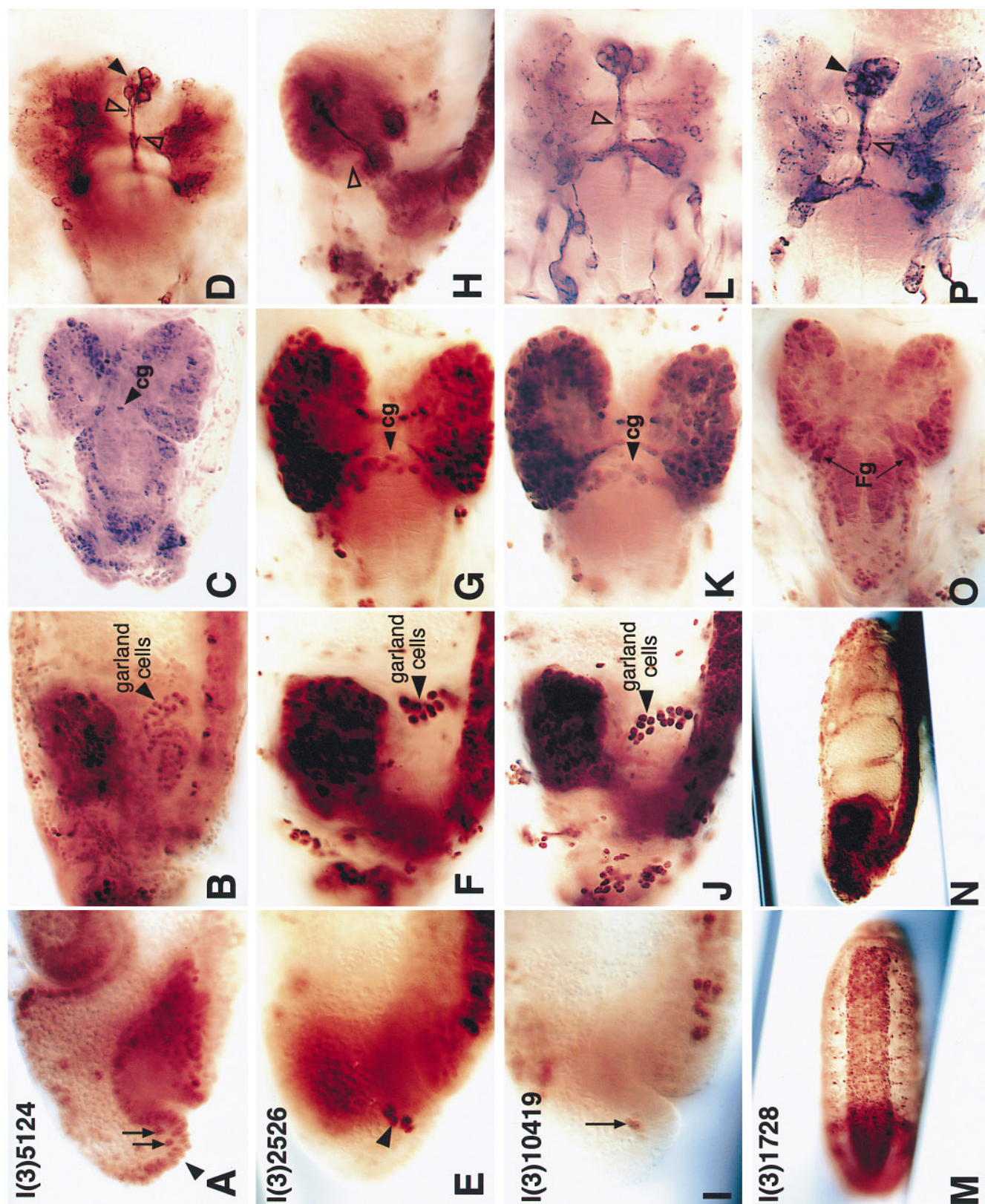
The phenotype of homozygous *l(3)7825* embryos, however, is similar to that of *l(3)5484.2*, having a condensed and defasciculated SNS (Fig. 5I). Staining *l(3)5484.2* homozygous embryos with anti-Crb antibodies provided an explanation for the diminished SNS in these *pnt* mutants, by revealing a dramatic reduction in the invaginations and a failure of vesicle formation (Fig. 5E). This is consistent with the  $\beta$ -gal expression in the invaginations, as seen in *l(3)7825*, while the lack of organization in the remnant SNS structures would be consistent with a role for *pnt* in the SNS glia.

Homozygous embryos of *l(2)5671* show a  $\beta$ -gal pattern in the tip cells of the SNS invaginations, during stages 10 to 12 (Fig. 5L) as well as the commissural glia cells at stage 16 (Fig. 5M) and midline cells of the CNS (Fig. 5N). While lethality occurs in homozygous offspring during embryonic stages, heterozygous *l(2)5671* adults show a dominant rough eye phenotype. A single P-element insertion at 21E1-2 on the third chromosome, also determined by genomic Southern blots and *in situ* hybridization to polytene chromosomes (Flybase and own observations), is responsible for the homozygous lethality and phenotype seen, since excision of the P-element using a  $\Delta 2-3$  transposase results in viability (see Materials and Methods). *l(2)5671* is a mutation in the *Star* (*S*) locus (Heberlein *et al.*, 1993) as it does not complement *S<sup>54</sup>* (Kolodkin *et al.*, 1994; own observations). *S* is known to be a member of the Raf/Ras signal transduction pathway which functions during glial cell development (Klamt *et al.*, 1991) and eye development (Heberlein *et al.*, 1993; Kolodkin *et al.*, 1994). That it is also expressed in the SNS tip cells implicates this Raf/Ras pathway in the process of invagination during SNS development.

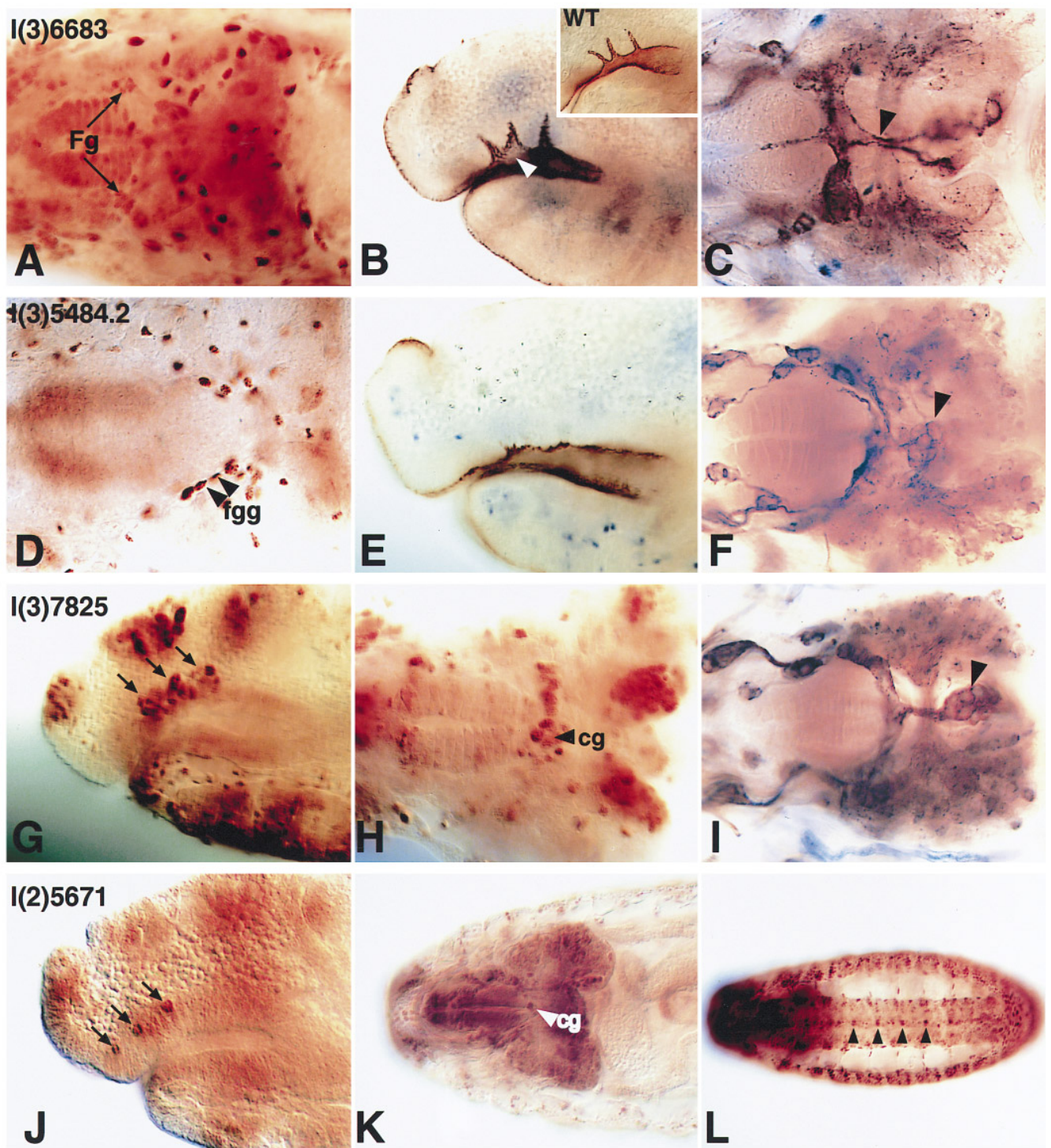
### A *Drosophila* $\alpha$ -adaptin Mutation

The insertion *l(2)6694* produces embryonic  $\beta$ -gal expression in CNS glia and in the garland cells from stage 11 on (Fig. 6A–6C). Expression in the CNS glia was confirmed by double anti-Repo/anti- $\beta$ -gal immunostaining (not shown), but no expression in the PNS glia could be detected. During larval stages, expression can be seen in the SNS ganglia (not

**FIG. 4.** Lethal enhancer trap lines with  $\beta$ -gal expression and phenotypes in the SNS. (A–D) *l(3)5124*, an insertion in *couch potato*. (A–C) Anti- $\beta$ -gal immunostaining of *l(3)5124* embryos showing expression in the SNS anlage (arrowhead) and early delaminated cells (arrows) at stage 10 (A) and both in the garland cells (B) and commissural glia (cg) at stage 15 (C). (D) *l(3)5124* homozygous mutant embryo immunostained with Mab22C10 antibodies during stage 16. There are axonal projections off the rn from the Fg and the Eg2 (open arrowheads) and fewer cells in the esophageal ganglia (solid arrowhead). (E–L) *l(3)2526* and *l(3)10419* insertions in *prospero*. (E–G)  $\beta$ -Gal expression in *l(3)2526* and (I–K) *l(3)10419* embryos can be localized in the early delaminated cells at stage 10 (E, I), the garland cells at stage 16 (F, J), and the cg at stage 16 (G, K), respectively. (H) *l(3)2526* and (L) *l(3)10419* homozygous mutant embryos immunostained with Mab22C10 antibodies during stage 16. Note a loss of the fcn (H; arrowhead) and defasciculation along the rn (L; arrowhead). (M–P) *l(3)1728*, an insertion in a novel gene. (M–O)  $\beta$ -Gal expression in *l(3)1728* during stage 16. Note expression in the CNS, the epidermis (M, N), and the gut (N), as well as in the Fg (O). (P) Homozygous mutant *l(3)1728* embryo immunostained with Mab22C10 antibodies during stage 16 showing defasciculation along the rn (open arrowhead) and the condensed Eg1 and Eg2 (solid arrowhead). (A,B,E,F,H,I,J,N) Lateral views; the rest are dorsal views.

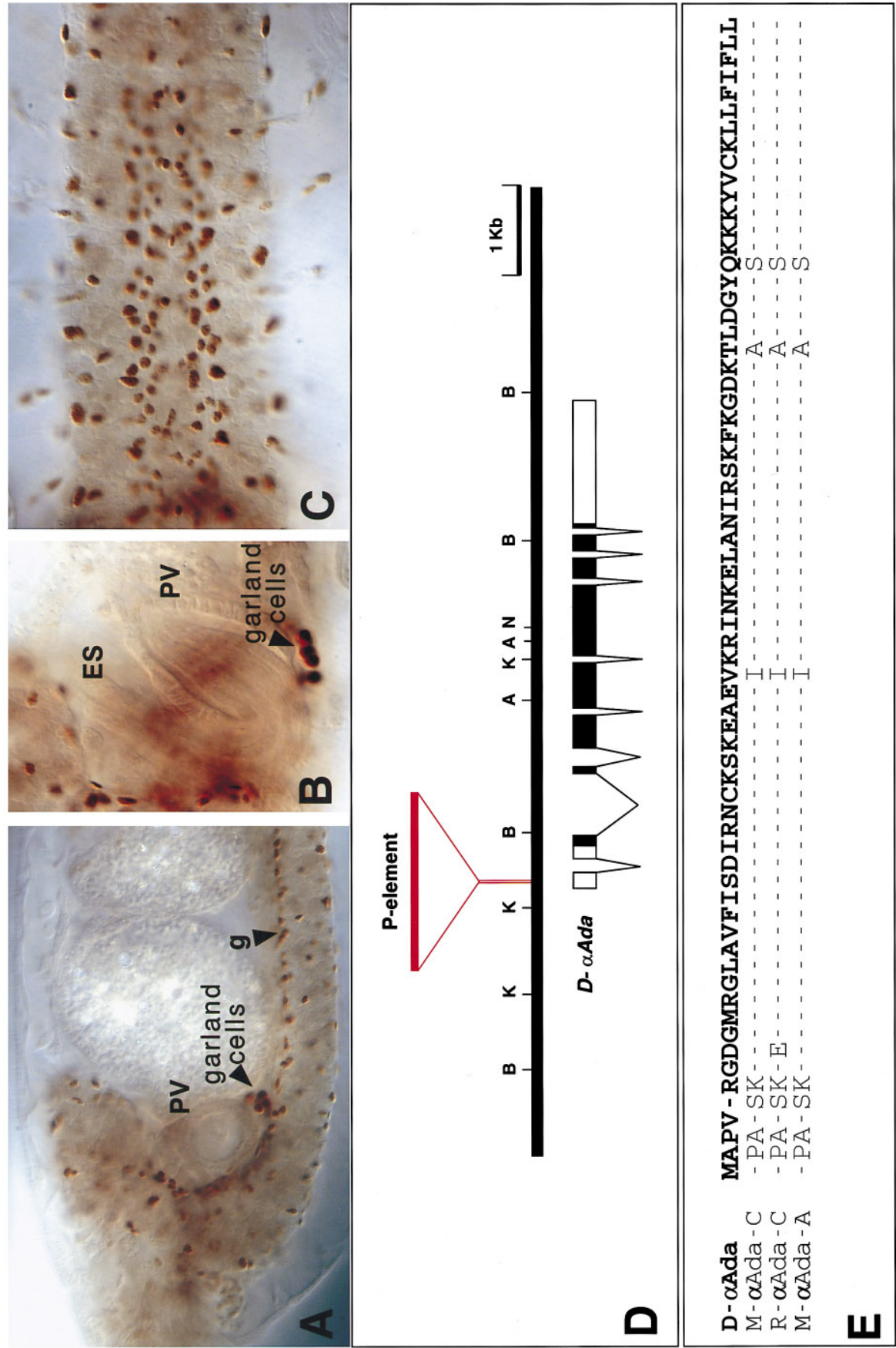






**FIG. 5.** Lethal enhancer trap lines with  $\beta$ -gal expression and phenotypes in the SNS. (A–C) *l(3)6683* insertion in a novel gene. (A) Anti- $\beta$ -gal immunostaining of *l(3)6683* embryo at stage 16 showing expression in the cg and fgg. (B) Anti-Crb immunostaining of *l(3)6683* embryo at stage 12 showing mild defects in the invaginations (arrowhead) vs wild-type (inset) at the same stage. (C) *l(3)6683* homozygous mutant embryo immunostained with Mab22C10 antibodies during stage 16 shows defasciculation of the rn (arrowhead). (D–I) *l(3)5484.2* and *l(3)7825* insertions in *pointed*. (D) Anti- $\beta$ -gal immunostaining of *l(3)5484.2* embryo at stage 16 showing expression in the fgg (only the marked side is in focus). (E) Anti-Crb immunostaining of *l(3)5484.2* embryo at stage 12 showing reduced invaginations. (F) *l(3)5484.2* homozygous mutant embryo immunostained with Mab22C10 antibodies during stage 16 shows a reduction and condensed arrangement of the ganglia (arrowhead). (G,H) Anti- $\beta$ -gal immunostaining of *l(3)7825* embryos showing expression in the three invaginations at stage 12 (G; arrows) and the cg at stage 15 (H; arrowhead). (I) *l(3)7825* homozygous mutant embryo immunostained with Mab22C10 antibodies during stage 16 shows the fused Eg1 and Eg2 (arrowhead). (J–L) *l(2)5671* insertion in *Star*. Anti- $\beta$ -gal immunostaining of *l(2)5671* embryos showing expression restricted to the three tip cells during stage 11 (J; arrows), in the cg by stage 16 (K), as well as in ventral midline cells of the CNS (L; stage 15, arrowheads). (A,C,D,F,H,I,K,L) Dorsal views; the rest are lateral views.





**FIG. 6.** The l(2)6694 P-element insertion tags a *Drosophila*  $\alpha$ -adaptin gene. (A–C) Anti- $\beta$ -gal immunostaining of l(2)6694 embryos showing reporter gene expression. (A) Lateral view showing expression in the garland cells associated to the proventriculus (PV) and in glial cells (g) of the ventral cord. (B) Higher magnification (2 $\times$ ) of a dorsolateral view of the same embryo showing the garland cells, ES, esophagus. (C) Ventral view showing the pattern of CNS glial cells. (D) Genomic organization around the P-element. A single P-element is inserted in the first exon of a 4.8-kb transcript bearing eight introns as determined by genomic Southern blot analysis and sequencing relevant portions of the cDNA and genomic fragment. B, BgIII; K, KpnI; A, ApaI; N, NheI. (E) Sequence of the N-terminal region of the protein and comparison with the vertebrate homologs. D- $\alpha$ Ada, *Drosophila*  $\alpha$ -adaptin; M- $\alpha$ Ada-C, mouse  $\alpha$ -adaptin-C; R- $\alpha$ Ada-C, rat  $\alpha$ -adaptin-C; M- $\alpha$ Ada-A, mouse  $\alpha$ -adaptin-A.



shown). *l(2)6694* is a pupal lethal mutation that does not show any phenotype in the morphology of the embryonic nervous system. A single P-element insertion in 21C1-2, as determined by genomic Southern and *in situ* hybridization to polytene chromosomes (Flybase and own observations), is responsible for its lethality, since excision of the P-element using a  $\Delta 2-3$  transposase results in viability. Following plasmid rescue, a 4.5-kb genomic fragment mapping to the 21C1-2 region was used as a probe for whole-mount *in situ* hybridization to embryos and revealed a transcription unit expressed ubiquitously in the embryonic nervous system, in the garland cells, and in the larval SNS (not shown). Using this probe, a 4.8-kb  $\lambda$ zap cDNA (Stratagene) was isolated (Fig. 6D) which also maps to 21C and is expressed with a pattern identical to the plasmid rescue fragment. Partial sequence data reveal that the protein shares homology with vertebrate  $\alpha$ -Adaptins (Fig. 6E).  $\alpha$ -Adaptins form part of the AP-2 complex that initiates receptor-mediated endocytosis, a process essential for vesicle recycling during synaptic transmission (De Camilli and Takei, 1996; Robinson, 1994).

## DISCUSSION

By screening for reporter gene expression and defects in the SNS of embryos containing single P-element insertions, we have demonstrated that this system is an effective tool to dissect the genetic pathways directing its development. We isolated several P-element insertion lines which are alleles of genes known to be required for proper neural development, and assessment of the role of these genes during SNS development provides further insight into various features of the system.

### *Clarifying a Role for hairy in the SNS Anlage*

Until now, the anlage of the SNS was known in its earliest state as a cluster of cells just anterior to the stomodeal opening which express both proneural genes as well as the genes *forkhead* and *Krüppel* (González Gaitán and Jäckle, 1995). The line *l(3)1620* demonstrates that the SNS anlage is already determined much earlier in development and that the gene *h* is expressed in the anlage and can be used as a marker for the earliest stages of SNS development. In addition, it is published that *h* is a transcriptional repressor of the proneural gene *achaete* (*ac*) (Van Doren *et al.*, 1994), and loss-of-function *h* mutations result in ectopic sensory organs in the adult flies (Mohr, 1922; Van Doren *et al.*, 1994). We see in the line *l(3)1620* that there are also supernumerary neural cells already in the embryonic stage of neural development. As a repressor of *ac*, *h* could play a role in delineating the boundaries of *ac* expression in proneural clusters and/or in the process of inhibiting *ac* expression in those cells within a proneural cluster which are to be prevented from following a neural fate. That there are extra neural cells in the embryonic PNS could comply with either of these possibilities. However, that there are extra neural

cells in the SNS implies that *h* is functioning in the delineation of the size of a proneural cluster, since singling out of individual neural precursors does not occur in the SNS. This is in agreement with the results published by Skeath *et al.* (1992), demonstrating that pair rule genes (including *h*) regulate the anterior-posterior position of proneural clusters. It also supports the suggestion made by Van Doren *et al.* (1994) that *h* is acting as a global regulator in restricting *ac* expression to the proneural clusters in imaginal discs. In this case, *h* is expressed in the SNS anlage prior to proneural gene expression there and is likely aiding in the establishment of the size of the proneural cluster.

### *The Invaginating SNS*

Both *pnt*, which is expressed early in the invaginations and late in the SNS glia, and *S*, which is expressed in the tip cells of the invaginations, are members of the *spitz* group of genes (Mayer and Nusslein-Volhard, 1988) which are involved in signaling along the Ras/Raf signal transduction pathway (Schweitzer *et al.*, 1995a). What role is the Ras/Raf pathway performing during SNS development? On one hand, expression in the embryonic SNS glia is consistent with the essential function of the Ras/Raf pathway during glial development and glia/neuron interactions during CNS neurogenesis (Klambt, 1993; see below). On the other hand, a novel role for this signaling pathway, instructing cells to perform a morphogenetic movement during the invagination process, is revealed in the SNS.

At the start of the Ras/Raf signaling cascade, the secreted *S* protein has been suggested to facilitate the processing of the precursor of the ligand, Spitz (Schweitzer *et al.*, 1995b). *Pnt*, on the other hand, is an ETS-like transcription factor functioning at the end of the transduction process in the cell receiving the signal (O'Neill *et al.*, 1994). The requirement and expression of *S* and *pnt* lead us to speculate that the Ras/Raf pathway is involved in the process of SNS invagination, where a signal emanating from the three tip cells is targeted to the other SNS primordial cells. These cells perform cell shape changes, which might be induced in response to the Spitz signal, resulting in the invagination of the epithelium. The analysis of the role of other integral components of the Ras/Raf pathway will shed more light on the role of this pathway in the control of morphogenetic movements during development.

### *Guidance of Cell Migration and Axonal Projection*

Although examples of defects in the process of vesicle formation were not recovered in the screen, we found several examples of aberrations in the cell migration and/or axonal projection involved in establishing the complete SNS.  $\beta$ -Gal of the line *l(3)6683* is found in cells of the frontal ganglion and it would appear that these cells are responsible for directing the fasciculation of the recurrent nerve. In the mutant condition, the extending nerves may fail to produce the appropriate signals and so fail to adhere or be attracted to the other extending axons which make up the recurrent

nerve. Thus, once having established an initial pathway between the frontal ganglion and either the esophageal ganglion 1 or esophageal ganglion 2, axonal tracts of one ganglion or the other will grow only along the initial pathway formed, even if the route it takes is abnormal.

In the line *l(3)1728*,  $\beta$ -gal expression is also found in the frontal ganglion and seems to be required for proper fasciculation of the recurrent nerve. However, in this situation there are aberrant axonal extensions off of an otherwise fasciculated recurrent nerve, and the two esophageal ganglia become closely associated to each other rather than separated. Although *lacZ* reporter gene expression associated with these ganglia is not apparent in this line, this may be the result of only a subset of the enhancers having been trapped by this particular insertion. There is definitely a failure in spatial coordination in the system, which could normally be controlled by a signal sent from the frontal ganglion or by cross-talk between the neurons of the esophageal ganglia 1 and 2. Current analysis of the genes affected in *l(3)6683* and *l(3)1728* is addressing what kind of molecules these novel genes code for and how they function in SNS development.

A role for *pnt* in glia cells can be deduced from the phenotype detected in both *l(3)5484.2* and *l(3)7825*. While the reduction in cell number in *pnt* mutant embryos arises from an early defect in the invagination process (see above), the disorganized SNS seen in these mutants could be explained by a glial defect, such that the cells of the remnant ganglia are unable to spread out to their final positions because the guidance usually provided by the SNS glia is missing. The cell bodies can be seen extending ectopic projections, and perhaps in the absence of the necessary signals, the cells do not know which direction to head in and are unable to move from where they are. The situation is reminiscent of the phenotype seen in the CNS of *pnt* mutants. *pnt* is expressed in both the glial support cells of the VUM cluster of neurons and the midline glial cells, and both groups of cells are involved in the formation of the commissural pathways in the developing CNS (Klamt, 1993). In the SNS, *pnt* appears to function in a similar process of instruction from glial cells to neurons.

Line *l(3)5124* often displays projections sent out randomly from the ganglia (Fig. 4D), as if searching for guidance cues. It is possible that the apparent disorientation seen in the cells of the SNS in this line may be due to the fact that the identity of these cells was not properly determined. It has been proposed that *cpo* may control the processing of RNA molecules that are required for neural specification (Bellen et al., 1992). In *cpo* mutants, a defective neural identity of SNS cells may be impairing the interpretation of cues defined by local guidance molecules during formation of the system, which results in the fasciculation phenotype. Further characterization of the role of *cpo* in SNS development may provide some interesting answers as to what happens in the neuroblasts following the initial decision to follow a neural fate effected by proneural genes.

The lack of frontal connectives in the lines *l(3)2526* and *l(3)10419* comes as no surprise because it is known that

*pros* mutants also lack their longitudinal connectives (Doe et al., 1991). This lack of connectives may be due to defective glial functioning in both systems, as *pros* is expressed in the commissural glia, as well as the longitudinal glia of the CNS, and is known to play a role in glial development (Doe et al., 1991; Jacobs, 1993). In addition to its role in glial development, *pros* is expressed and functions in neural precursors. It is known that following asymmetric division of a neuroblast, the *pros* transcript and protein are asymmetrically localized to the GMC and excluded from the neuroblast (Hirata et al., 1995; Knoblich et al., 1995; Spana et al., 1995). Yet, in the SNS, those cells separating from the vesicles which are expressing *pros* will undergo no further rounds of division (Hartenstein et al., 1994) and therefore do not require an asymmetrical distribution of *pros*. It seems most likely that *pros* functions as a neural precursor gene (Vaessin et al., 1991) in the SNS, PNS, and CNS, but that in the SNS the lack of further cell division following neural determination eliminates the need for a mechanism to distinguish between offspring.

### A Gene Involved in Synaptic Function

The line *l(2)6694* represents the first known mutant allele for the gene encoding  $\alpha$ -adaptin. The difference between the glial-specific  $\beta$ -gal pattern in *l(2)6694* and the ubiquitous cDNA expression pattern in the CNS may arise because only a subset of the enhancers were trapped by the P-element insertion. During larval stages  $\alpha$ -adaptin is also expressed in the mature SNS ganglia. The  $\alpha$ -Adaptin protein forms part of a heterotetramere, the AP2 adapter complex, that is involved in the initiation and internalization of coated pits and vesicles during endocytosis and, in particular, it has been suggested by *in vitro* studies that  $\alpha$ -adaptin plays a role in the physiology of the differentiated synapse by initiating synaptic vesicle recycling (De Camilli and Takei, 1996; Robinson, 1994). With an  $\alpha$ -adaptin mutant in hand, we are now able to address this proposed function of AP2 during the synaptic vesicle cycle *in vivo* and gain insight into the role of endocytosis at the synapse.

Several of the lines we have thus far selected are alleles of genes already characterized and known to be required for various aspects of neurogenesis. We can now use these lines as markers as well as for further genetic dissections of the different stages of SNS development. The example of how assessing the role of *h* in the SNS compared to the PNS enables us to discern between two possibilities that were less easily discernible in the PNS alone reinforces the strength of using the SNS to study mechanisms of neurogenesis. It allows us to view the processes of neurogenesis from a different perspective and, when combined with knowledge gleaned from studies of the PNS and CNS, serves to give a more complete picture of neurogenesis.

### ACKNOWLEDGMENTS

The original embryonic X-gal staining of the individual lines was done together as a communal effort by several members of the

Jäckle lab, to whom we are indebted. We are also grateful to Rocío Fernández de la Fuente for excellent technical assistance and Anna LaRosée for useful comments on the manuscript. We thank C. Goodman, J. Urban, P. Carrera, E. Knust, J. Modollet, and A. Spradling for fly stocks and reagents. This work was supported by a doctoral fellowship from Boehringer Ingelheim Fonds to J.P.F. as well as funding from the Max Planck Society and the Fonds der Chemischen Industrie (H.J.).

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Received for publication February 10, 1997

Accepted April 11, 1997