NEURON-GLIA INTERACTIONS IN THE NERVOUS SYSTEM OF *DROSOPHILA* EMBRYOS

BY

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ABSTRACT

Several cell lineages derived from the mesectoderm occupy and contact axons in the midline of the developing *Drosophila* CNS. Which of these midline cell lineages contribute to commissural axon morphogenesis? In the absence of the midline cells as in mutant embryos of the *single-minded* gene, the longitudinal axons collapse at the midline and commissural axons are absent. Despite the similarity in axon tract phenotype, the midline cells in *slit* mutant embryos survive but are displaced. Correct cytoarchitecture of the midline cells is therefore dependent on the activity of Sli protein which is in turn necessary for commissure formation. In mutant embryos displaying a fused commissure phenotype (*rhomboid* and *Star*), the anterior and middle midline glia cells failed to migrate and died by apoptosis after commissure development. In these mutants the number of cells in midline neuronal lineages was reduced before defects in midline glia were apparent.

In wildtype embryos approximately 50% of cells in three midline glia lineages died by apoptosis after commissure separation as shown by ultrastructural and enhancer trap analysis. Midline glia lineages died by apoptosis as shown morphologically and by their survival in embryos deficient in the cell death gene *reaper*. Quantitative analysis revealed variable survival of cells in the anterior, middle and posterior midline glial lineages during embryogenesis suggesting heterogeneity among these cells. The presence of extra anterior, middle and posterior midline glial lineages relative to wildtype numbers in *reaper* mutant embryos suggested that cell death regulates either midline glial proliferation or cell fate determination during wildtype

embryogenesis. Alterations in axon-glia contact correlated with changes in midline glia survival.

What happens to apoptotic cells in the *Drosophila* embryonic central nervous system? A variety of glia in the nervous system were capable of phagocytic activity including midline glia, longitudinal tract glia, nerve root glia and subperineurial glia, revealed by electron microscopy. However, the majority of apoptotic cells in the central nervous system were engulfed by subperineurial glia. In the absence of phagocytic haemocytes in embryos mutant for the *Bicaudal-d* gene, most apoptotic cells were retained in subperineurial glia at the outer edges of the central nervous system. Apoptotic cells were expelled from the central nervous system of *Bicaudal-d* mutant embryos suggesting that phagocytic haemocytes participate in the removal of apoptotic cells from the central nervous system but are not essential for this process.

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LIST OF ABBREVIATIONS

CNS- Central nervous system

DAB- Diaminobenzedine

DER- Drosophila Egfr

Egfr- epidermal growth factor receptor

Egfr^{elp}-- a dominant allele of the epidermal growth factor receptor

flb- a loss of function allele of the epidermal growth factor receptor

GMC- ganglion mother cell

HS-*rho*- the *rhomboid* gene placed under control of a heat shock promoter

LRR- leucine rich repeat

MEC- mesectodermal cell

MG- midline glia

- MGA- Anterior midline glia
- MGM- Middle midline glia
- MGP- Posterior midline glia

MNB- median neuroblast

MP- midline precursor

NGS- normal goat serum

NEE- neuroectodermal element

PBS- phosphate buffered saline

PBT- phosphate buffered saline contaning Triton-X

TGF-β- Transforming Growth Factor beta

UMI- unpaired medial interneuron

VE- ventral ectoderm

VNC- ventral nerve cord

VUM- ventral unpaired median neuroblast

THIS THESIS IS DEDICATED TO MY HUSBAND ALAN KARCZ AND TO MY SON AARON KARCZ

INTRODUCTION

This introduction focuses on the mesectoderm and the cells comprising it, the midline cells in the central nervous system (CNS) of *Drosophila* embryos. Cells located at the midline of the CNS in vertebrate and invertebrate embryos influence the establishment of axon tracts (Klämbt et al., 1991; Bovalenta and Dodd, 1991) and of adjacent tissues (Kim and Crews, 1993; Yamada et al., 1991).

The *Drosophila* midline cells are developmentally analogous to the floorplate cells at the ventral midline of the vertebrate neural tube (Dodd and Jessell, 1988; Bernhardt et al., 1992). Both *Drosophila* midline cells and floorplate cells are among the first cells in the CNS to differentiate (Klar et al., 1992) implying an early role. Both are adjacent to the mesoderm, have an early and unique pattern of determination and provide a substrate for commissural axons.

The importance of the midline cells and floorplate in commissural axon pathway formation has been demonstrated by studying genetic mutations resulting in an absence of these cells in embryos of *Drosophila* (Crews et al., 1988; Thomas et al., 1988; Rothberg et al., 1990), mice (Bovalenta and Dodd, 1991) and Zebrafish (Bernhardt et al., 1992). The similarities between the midline cells in vertebrate and invertebrate embryos suggest that similar mechanisms and molecules may govern their development. Indeed, the guidance of axons to their targets occurs by diffusible chemotropic factors (*netrin*-1 and *netrin*-2) secreted by target cells in both vertebrates (Kennedy et al., 1994) and invertebrates (Serafini et al., 1994). The netrins are homologous to UNC-6 which is a laminin-related protein that guides axon migration in *Caenorhabditis elegans* (Serafini et

al., 1994; Ishii et al., 1992; Hedgecock et al., 1990). The expression of *D-netrin* by midline cells in *Drosophila* embryos provides exciting evidence that the midline can function as an attractant for commissural axons (Mitchell et al., 1994).

Investigation of midline cell function has been facilitated by the availability of mutations in genes expressed in these cells (Mayer and Nüsslein-Volhard, 1988; Klämbt et al., 1991). In addition, a variety of experimental tools exist to identify and study the development of midline cells including antibodies, enhancer traps (Klämbt et al., 1991) and *in vivo* dye labelling (Bossing and Technau, 1994).

The identification of transcription factors and signalling proteins has begun to provide a molecular characterization of the mechanisms that establish the mesectoderm boundaries and that determine the identity of its cells. Once the mesectoderm is specified, how do midline cells acquire their identity? How is midline cell development regulated and how do these cells interact with the CNS axons? These questions will be addressed in this introduction with an emphasis on cell lineage specification and the mechanisms regulating heterogeneity in cell lineages.

(1) History of the midline cells: From grasshopper to *Drosophila*

(1a) Morphology of the grasshopper CNS

Initial studies of *Drosophila* CNS neurons were based on descriptions of neuronal specificity in the grasshopper, *Schistocerca americana* (Thomas et al., 1984; Goodman et al., 1985). The grasshopper CNS is arranged as a segmental chain of cephalic, thoracic and abdominal ganglia, with a gradient of development

that ascends from anterior to posterior. The embryonic axon scaffold in each ganglion, first described by Bate and Grunewald (1981), is composed of two bilateral bundles of axons called the longitudinal connectives and two commissural connectives. Two bundles of axons make up the anterior commissure and one bundle of axons comprises the posterior commissure, both of which connect the two sides of the longitudinal connectives. The intersegmental and segmental nerves connect the CNS to the peripheral nervous system. Neuroblasts separate from the epidermis and generate specific neurons to form a segmental unit of the nervous system, a ganglion (Bate, 1976). The most obvious signs of the CNS segmental pattern are the axons generated from early differentiating neurons. This was recognized as the basic design of the arthropod CNS and studies focussed on how the pattern was constructed during development.

The segmentally repeated group of neuroblasts derived from the neuroepithelium gives rise to a bilaterally symmetric population of neurons in each embryonic ganglion. Each neuroblast divides asymmetrically and produces a ganglion mother cell (GMC) and the latter divides symmetrically to generate pairs of postmitotic sibling neurons (Bate and Grunewald, 1981; Doe et al., 1985). Studies of neuronal diversity revealed that the pattern of neuroblasts varied between segments due to the addition or deletion of neuronal precursor cells (Doe and Goodman, 1985a). Neuroblast diversity was created by cell interactions and positional cues while the fate of NB progeny was influenced by their lineage (Doe and Goodman, 1985b). There are also glial precursors (GP) that give rise to various glia in the CNS, although these cells have not been well characterised (Doe and Goodman, 1985a). In each segment there are two bilaterally symmetric

populations of 30 neuroblasts (Bate, 1976), an unpaired median neuroblast and seven midline progenitors (Bate and Grunewald, 1981; Doe and Goodman, 1985a).

The midline progenitors and their progeny are located at or near the midline of the CNS. The nuclei of the midline progenitors are dorsally situated and their cytoplasm extends to the ventral surface of the CNS (Doe and Goodman, 1985a). Bate and Grunewald (1981) identified seven midline progenitors called midline progenitors 1-7 (MP1-7) and one neuroblast called the median neuroblast (MNB). Unlike the CNS neuroblasts, MP1-7 divide once symmetrically to produce a pair of sibling neurons (Doe and Goodman, 1985). However, the MNB divides asymmetrically from a stem cell to produce at least 90 neurons in each segment (Bate and Grunewald, 1981; Goodman and Spitzer, 1979; Thompson and Siegler, 1993; Condron and Zinn, 1994).

Therefore, the lineage and morphology of the midline progenitors is distinct from that of all neurons in the insect CNS. What mechanism creates the distinction between the midline cells and CNS neurons and are other developmental mechanisms unique to these cells?

These early studies also revealed that there are segment-specific differences in the pattern of midline precursors (Doe and Goodman, 1985a). There were seven midline precursor in all CNS segments except four. Variations in midline precursor included their absence in some segments and their duplication in others, with consistent patterns between embryos. In segment A8 (abdominal) there was an additional midline precursor called MP0 which produces two neurons that send axons posteriorly along the MP1/MP2 fascicle to innervate the hindgut. In segment A11, MPs are not present at all. This segment-

specific variability was proposed to reflect molecular heterogeneities specified by homeotic genes such as *Antennapedia* (Levine et al., 1983) and contribute to the structural and functional differences between segments.

The generation of both neurons and glia from the MNB has recently been shown by *in vivo* dye labelling (Condron and Zinn, 1994) and this heterogeneity is controlled by the segment polarity gene *engrailed* (Condron et al., 1994). In the absence of *engrailed* expression extra midline neurons are generated at the expense of midline glia and this conversion occurs at the precursor level (Condron et al., 1994).

Thompson and Siegler (1993) show that variations in the MNB lineages result from temporal and segment-specific cell death. The periods of cell death correlate with embryonic molts and therefore may be regulated by hormones. Therefore, the heterogeneity in both MNB precursors and their progeny is generated by two distinct mechanisms during embryogenesis. Are these mechanisms mutually exclusive?

(1b) Does the *Drosophila* embryonic CNS contain cells analogous to the Grasshopper cells?

Details of the midline precursor and neurogenesis in grasshopper embryos were used as a foundation to study cells in the CNS of less morphologically accessible but genetically manipulatable systems such as *Drosophila*. Early studies suggested that the *Drosophila* CNS was a miniature version of the grasshopper CNS (Thomas et al., 1984). Although grasshopper and *Drosophila* are separated by 300 million years of evolution, their central nervous systems were proposed to be constructed from a common arthropod plan (Thomas et al., 1984). Similarities between *Drosophila* and grasshoppers extend to the structure of the CNS axon scaffold and to common neurons with similar fasciculation choices. Early neurons in *Drosophila*, such as MP1, dMP2, pCC, aCC, RP1 and RP2 also pioneer the first longitudinal, commissural and peripheral axonal pathways (Thomas et al., 1984; Goodman et al., 1984; Bastiani et al., 1985; Jacobs and Goodman, 1989b).

Using dye injections of neurons, Thomas et al., (1984) showed that the midline precursor characterised in the grasshopper also exist in *Drosophila*, the moth *Manduca sexta* and the crayfish *Procambarus*. Thomas et al., (1984) noted: "Each species has specific differences in the precise number of precursors just as within a species there are specific differences between segments." Therefore, segmental variability may also occur in the midline precursor of *Drosophila* embryos.

Neurons homologous to those pioneering the axon tracts in grasshopper embryos were identified in *Drosophila* embryos by serial electron microscopy (Jacobs and Goodman, 1989b). Examination of their axon trajectories and fasciculation targets revealed a striking conservation in cellular specificity between the two insect nervous systems. Differences in fasciculation choices

were due to the small size and rapid development of the *Drosophila* embryonic CNS.

(2a) The mesectoderm and its lineages in *Drosophila* embryos

Mesectodermal cells form a distinct mitotic domain (Foe, 1989) and proliferate while still part of the ectoderm (Bossing and Technau, 1994). During the cellular blastoderm stage the mesectoderm separates the presumptive mesoderm from the presumptive neurogenic region on either side of the embryo (Figure 1A and Figure 2) (Crews et al., 1988; Thomas et al., 1988; Klämbt et al., 1991). At this stage the midline precursors comprise the mesectoderm and are arranged as a bilateral strip of four cells per ventral nerve cord segment. During gastrulation the mesoderm invaginates and the bilateral row of midline precursor meet at the ventral midline to form a two cell wide row of eight midline precursor in each ventral nerve cord segment (Figure 1A and 1B; Nambu et al., 1990; Klämbt et al., 1991). At this stage in development these cells can be identified by their position and shape and by expression of the *single-minded* (*sim*) gene.

Eight midline precursors give rise to four different classes of cells in each ventral nerve cord segment shown by ultrastructural and enhancer trap analysis (Figure 1B; Jacobs and Goodman, 1989b; Klämbt et al., 1991). Early studies showed that from anterior to posterior in a segment there are three midline glia (MG) precursors that divide once to produce six MG referred to by final position as MGA, MGM and MGP (anterior, middle and posterior, respectively; Jacobs and Goodman, 1989a). The fourth precursor called MP1 divides once to produce a bilateral pair of MP1 neurons (Thomas et al., 1984). Two to three precursors

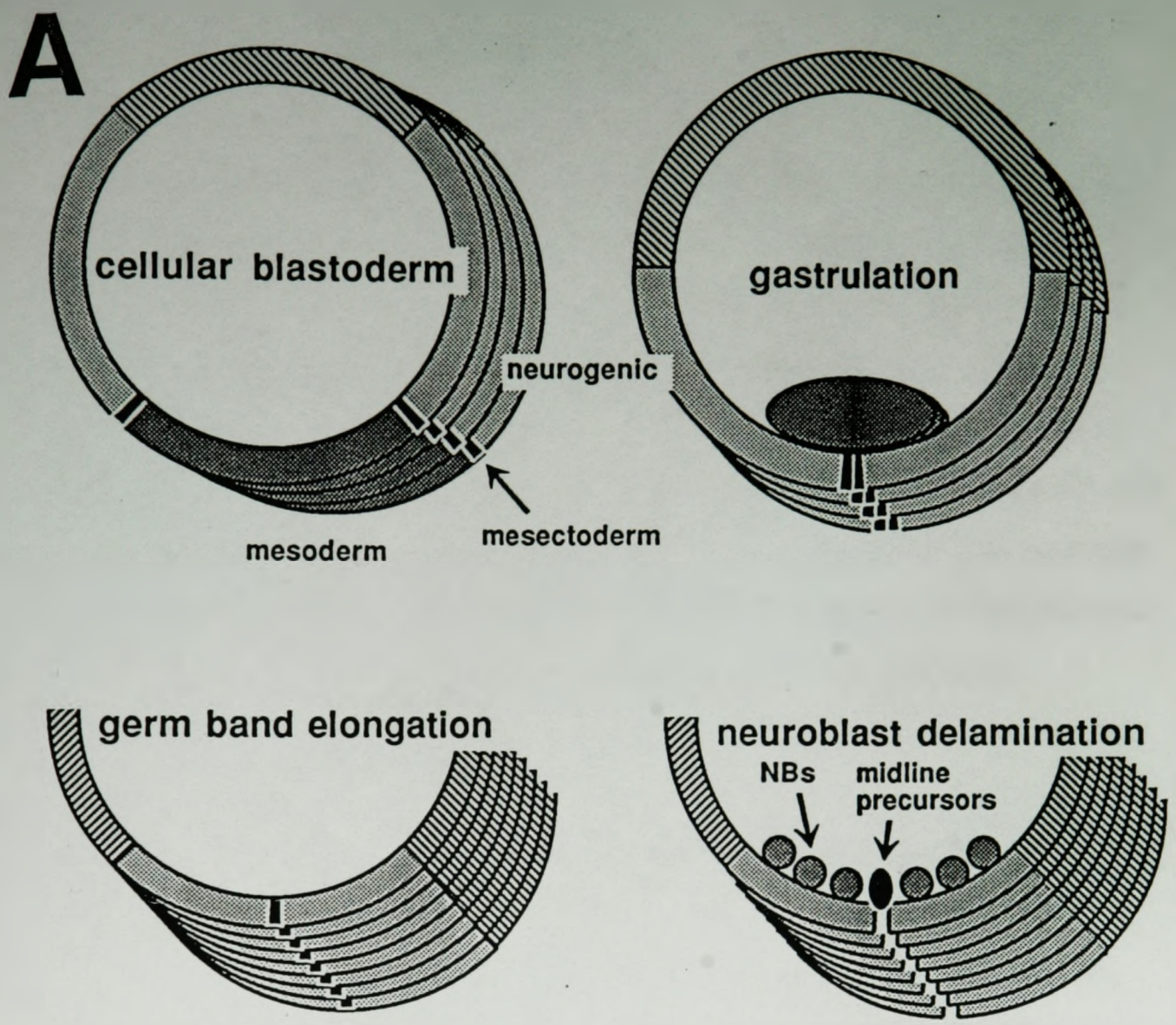
produce six ventral unpaired median neuron (Goodman et al., 1984). The final precursor in each segment produces the MNB and possibly its support cells. Therefore, the midline lineages were recognised as an ideal system because they were composed of an easily identifiable set of cells with an invariant fate.

The nuclei of the MG are located at the dorsal midline of the ventral nerve cord and process from these cells ensheath the anterior and posterior commissures (Jacobs and Goodman, 1989a). After axon tract establishment, the MGA nuclei are anterior to the anterior commissure, the MGM nuclei are between the anterior and posterior commissures while the MGP nuclei are posterior to the posterior commissure (Jacobs and Goodman, 1989a; Klämbt et al., 1991). During early development glia are distinguished from neurons by their morphology and proximity to presumptive axon tracts. Later in development glia increase their surface area and ensheath commissural axons and their cytoplasm becomes more electron lucent than that of neurons (Jacobs and Goodman, 1989a).

Axons from the MP1 neurons extend posteriorly in ventral nerve cord segments and pioneer the longitudinal axon tracts (Jacobs and Goodman, 1989b; Grenningloh et al., 1991). After axon tract establishment, the MP1 neurons assume a ventrolateral position to the midline. The ventral unpaired median neuron neurons are located ventral to the posterior commissure and extend axons dorsally between the anterior and posterior commissures to contact the MGA (Jacobs and Goodman, 1989b). The MNB and its progeny are located posterior to the posterior commissure (Klämbt et al., 1991).

Using a new method for single cell labelling, Bossing and Technau (1994) have demonstrated that there is variability in the number of midline progenitors

Figure 1. Development of the ventral midline and its cells in *Drosophila* embryos. (A) Development of the mesectoderm is shown schematically in cross-sections. At the cellular blastoderm stage the mesectoderm separates the mesoderm from the neurogenic region on either side of the embryo. During gastrulation the two mesectodermal regions meet at the ventral midline as the mesoderm invaginates. The midline precursors delaminate from the ventral ectoderm with the neuroblasts. (B) The neuronal and glial midline lineages are shown schematically as they would occur in a ventral nerve cord segment (From Klämbt et al., 1991).


A
cellular blastoderm
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and their progeny in ventral nerve cord segments. This method involves the *in vivo* labelling of individual cells in the embryo with a lipophilic fluorescent tracer (DiI) that surrounds the cell in its original position and is also transferred to the progeny of these cells. According to lineage detection using DiI labelling, five different classes of midline clones were identified. All clones were of the same identity as those previously characterised with the exception of a novel cell type called the unpaired median interneurons (UMI). It was proposed that positional information within a segment is insufficient for specification of midline precursor and therefore the involvement of cell-cell interactions may govern the determination of midline precursor identity.

(2b) Interactions between midline cells and the CNS axon tracts

Based on electron microscopic and enhancer trap studies, a model was proposed to describe interactions between the midline cells and the CNS axon commissures (Klämbt et al., 1991).

The first CNS growth cones are detected at stage 12/5 (8.5 hours of development) immunocytochemically with the monoclonal antibody BP102. These growth cones grow toward the ventral unpaired median neuron neurons and then migrate anteriorly around them (into the space that the MP1 just migrated from) to contact their contralateral homologs and form the posterior commissure.

The growth cones that pioneer the anterior commissure migrate toward the posterior edge of the MGA and cross the midline during stage 12/3. At this stage the anterior and posterior commissures are not yet separated from each other. At

stage 12/0, the axons of the ventral unpaired median neuron neurons are located between the anterior and posterior commissures while their cell bodies are more ventrally located (Jacobs and Goodman, 1989b). Separation of the commissures correlates with the posterior migration of the MGM over the MGA and along the ventral unpaired median neuron neurons and the medial migration of the RP1 neurons. The MGM and RP1 both assume final positions between the two commissures. Electron microscopy showed that the ventral unpaired median neuron growth cones contact the MGM between the two commissures. The MGM migrate posteriorly over top of the MGA and move along the ventral unpaired median neuron axons to separate the commissures. The MGP are not involved in commissure separation and migrate anteriorly into the previous segment. Through these migrations the MG nuclei assume their positions as anterior, middle and posterior with respect to the commissural axons.

(2c) Genetic test of the cellular basis of commissure formation

The model described above predicted that different cells and therefore different signals were involved in the morphogenesis of the two commissures. This model proposes key roles for the MGA, MGM and ventral unpaired median neuron neurons in the formation of the anterior commissure. The loss of one of these cell types should result in a predictable phenotype and therefore provide a reliable test for the model. The ideal method with which to test this model would be to individually ablate each cell type and assay for the predicted axon phenotype. However, embryonic cell ablation methods are not completely characterised in *Drosophila* embryos. This confined researchers to test this model

by studying the cellular defects in mutant embryos with malformed CNS axon tracts.

Three groups of axon tract phenotypes have been described for mutations in midline genes (Klämbt et al., 1991). These phenotypic groups include; (1) collapsed longitudinal axon tracts resulting from mutations in the *single-minded* (*sim*) and *slit* (*sli*) genes, (2) no posterior commissure formation as in mutations in the *orthodenticle* (*otd*) gene and (3) commissures that were incompletely separated (fused commissures) common to members of the *spitz* group including *spitz* (*spi*), *Star* (*S*) and *rhomboid* (*rho*) and certain alleles of the *Drosophila* epidermal growth factor receptor (DER) (Raz and Shilo, 1992).

To determine which cells were affected in each mutant group, midline lineages were analysed using enhancer traps as cellular markers in wholemount embryos (Klämbt et al, 1991). Mutations in the genes *sim* and *sli* result in an absence of commissures and a collapse of the longitudinal axon tracts at the midline (Mayer and Nüsslein-Volhard, 1988; Thomas et al., 1988; Rothberg et al., 1988). In *sim* mutants the midline cells do not proliferate or extend into the nerve cell layer as determined using a P[*sim/lacZ*] reporter construct (Nambu et al., 1991). The *sim* expressing cells do not differentiate into neurons and glia and die.

In *sli* mutant embryos it was determined that all midline cells were ventrally displaced and did not differentiate. These conclusions were based on wholemount embryo analysis using antibodies and enhancer traps as cellular markers (Rothberg et al., 1990). Klämbt et al., (1991) states that the death of midline cells in *sim* and their displacement and lack of further differentiation in *sli* begins at stage 12/3, a time at which the commissures begin to form. As the

midline cells in *sli* mutant embryos were ventrally displaced at stage 12/3, the axon tracts collapsed at the midline.

In embryos mutant for the *otd* gene (a homeobox-containing gene), the posterior commissure is absent from ventral nerve cord segments while the presence of the anterior commissure is variable (Finkelstein et al., 1990; Klämbt et al., 1991). Enhancer trap analysis revealed that the ventral unpaired median neuron neurons and possibly the MNB degenerated between stages 12/5 and 12/3 (Finkelstein et al., 1990; Klämbt et al., 1991). This correlation between the absent ventral unpaired median neuron neurons and posterior commissure supported the role of the ventral unpaired median neuron neurons in posterior commissure formation as predicted by the model. It was also suggested that the MG may have been indirectly affected in *otd* mutants as the MGM failed to migrate posteriorly over the MGA.

Mutations in the *spitz* group genes and temperature sensitive alleles of the epidermal growth factor receptor (Egfr) result in an incomplete separation of the anterior and posterior commissures (Mayer and Nüsslein-Volhard, 1988; Klämbt et al., 1991; Raz and Shilo, 1992). Klämbt et al., (1991) reported that the commissures in *spitz* group mutants develop properly until the migration of the MGA. Enhancer trap analysis revealed that the MG were absent from early stages in *rho* mutant embryos. In *S* mutant embryos the MG were initially present but failed to migrate and died during stage 14. In *spi* mutant embryos, the MG also did not migrate but remained at the dorsal surface of the VNC. Therefore, the failure of the MGA to migrate over the MGM correlated with the fused commissure phenotype. All other midline neuronal lineages were thought to have

not been perturbed in *spi* group mutants. In the absence of the function of the gene faint little ball (*flb*), the MG die (Raz and Shilo, 1992).

3. Molecular mechanisms governing midline cell development

(3a) Boundaries and fields in embryos: Delineation of the mesectoderm by dorsal-ventral patterning genes

The terms boundaries and fields have been used in developmental biology to conceptualize a group of cells with similar properties (Ingham and Arias, 1992). The use of molecular probes has allowed researchers to study the cellular and molecular basis of `fields'. Lawrence (1992), states that insect bodies are constructed piecemeal: The pieces consist of many cells and they meet at boundaries that are fixed in position. How are cells allocated to one region of the embryo (a `field') and how do boundaries arise? One well characterized example is the mechanism of dorsal-ventral patterning in *Drosophila* embryos.

In the late blastoderm embryo, dorsal-ventral patterning genes commit a group of cells on either side of the mesoderm to become lateral neurogenic ectoderm (N-ECT) (Figure 2) (see Ferguson and Anderson, 1991 and Chasan and Anderson, 1994 for reviews). The lateral N-ECT includes the mesectoderm (MEC), the ventral ectoderm (VE) and the lateral ectoderm (LE) (Figure 2). Distinction of the lateral N-ECT from surrounding tissues is reflected by expression of the *achaete-scute* (ASC) complex and *rhomboid* gene (Romani et al., 1987; Kosman et al., 1991; Rao et al., 1991 see Kim and Crews 1993).

A gradient of the maternal protein Dorsal establishes separate territories of gene expression within the N-ECT (Kosman et al., 1991). The Dorsal protein gradient is the result of differential nuclear localization of the protein. Equal amounts of Dorsal protein are synthesized throughout the embryo, however all of the protein is in the nuclei on the ventral side while the protein is completely cytoplasmic on the dorsal side. The protein is only active when translocated into the nucleus and acts as a DNA-binding transcription factor. Stimulation of the Toll receptor results in the nuclear transport of the Dorsal protein in ventral cells where it can influence gene expression.

The Dorsal protein gradient activates the expression of two zygotic genes, *twist* (*twi*) which contains a helix-loop-helix domain and *snail* (*sna*) which contains a zing finger motif (Anderson, 1987). The nuclear Dorsal protein is present in highest concentrations in the presumptive mesoderm while Twist protein is expressed in the mesoderm and the adjacent mesectoderm (Thisse et al., 1988). Expression of *sna* is restricted to the presumptive mesoderm (Boulay et al., 1987).

In *sna* mutant embryos, an increase in the number of cells expressing the genes *single-minded* (*sim*) and *rhomboid* (*rho*) implies that ventral cells now assume a more dorsal fate; in this way cells that would normally become mesoderm now become mesectoderm (Nambu et al., 1990; Rao et al., 1991). In double mutant embryos of *snail* and *twist* there is an absence of *rho*-expressing cells indicating a reduction in neurogenic ectoderm. In *dorsal* mutant embryos there is no transcription of *twist* and *snail* (Thisse et al., 1991). These observations were the first to suggest that transcriptional regulators were involved in mesectodermal determination.

The *dorsal* gradient directly regulates the expression of tissue specific genes. *dorsal* represses the activities of *zerknullt* (*zen*) and *decapentaplegic* (*dpp*) in ventral regions and in embryos from mothers that lack dl activity, *zen* and *dpp* are transcribed around the embryo. Both *dorsal* and *twi* bind to a region in the promoter of the *rho* gene called the neuroectodermal element (NEE) (Ip et al., 1991). Mutations in the dl and *twi* NEE sites abolish *rho* expression. It has also been found that the *sim* promoter contains *sna*-binding sites (Kasai et al., 1992).

Therefore, Sna defines the border between the mesoderm and mesectoderm by binding to the NEE and repressing *rho* and *sim* expression in the mesoderm. The differential activation or repression of transcription by Dorsal restricts *twi* and *sna* to ventral cells and *zen* and *dpp* to dorsal cells. However, it is the combined action of Dorsal and Twist that establish the sharp Sna borders as mutations in *dorsal*- and *twist*-binding sites drastically reduce the levels of expression while mutations in either site independently has minimal effects on *sna* expression (Ip et al., 1992). The temporal and spatial regulation of gene expression is one way in which boundaries and `fields' can be specified. Does this mechanism occur during midline cell development?

(3b) Genetic history of genes expressed in the midline

Mutations in the genes *spi*, S, *sim*, *pointed* (*pnt*) and *rho* are referred to as the *spitz* group and cause similar alterations in ventral ectodermal derivatives of the *Drosophila* embryo. The genes S, *spi*, *rho* and *pnt* were identified in a large-scale screen for embryonic lethal mutations affecting the pattern of the larval cuticle (Jürgens et al., 1984; Nüsslein-Volhard et al., 1984). The *sim* allele was identified in a screen of chromosomal deletions for mutations affecting the pattern of embryonic neurons and was classified as a *spitz* group member (Thomas et al., 1988). Mutations in these five *spitz* group loci result in a reduction of the ventral denticle bands and a fusion of the left and right parts of the head skeleton producing a pointed (*spitz*) appearance (Mayer and Nüsslein-Volhard, 1988).

There is a reduction in the distances between the anterior and posterior commissures in embryos mutant for *spitz* group genes. Characterisations of the mutant CNS showed an altered spatial arrangement in a subset of neurons expressing the protein even-skipped.

The correlation between the epidermal and CNS phenotypes suggested that *spitz* group genes act early in development before the neuroblasts delaminate from the ventral ectoderm. The expression of *spitz* group genes before neuroblast delamination supports this theory. Mayer and Nüsslein-Volhard (1988), postulated that the epidermal phenotype could be accounted for if the midline cells also gave rise to epidermal cells as well as neuroblasts. The mechanisms underlying this problem were not discovered until recently when molecular markers were employed to assay for gene expression in the early epidermis.

In two simultaneous reports it was shown that *spitz* group gene function (Kim and Crews, 1993), as well as the activity of an allele of the *Drosophila*

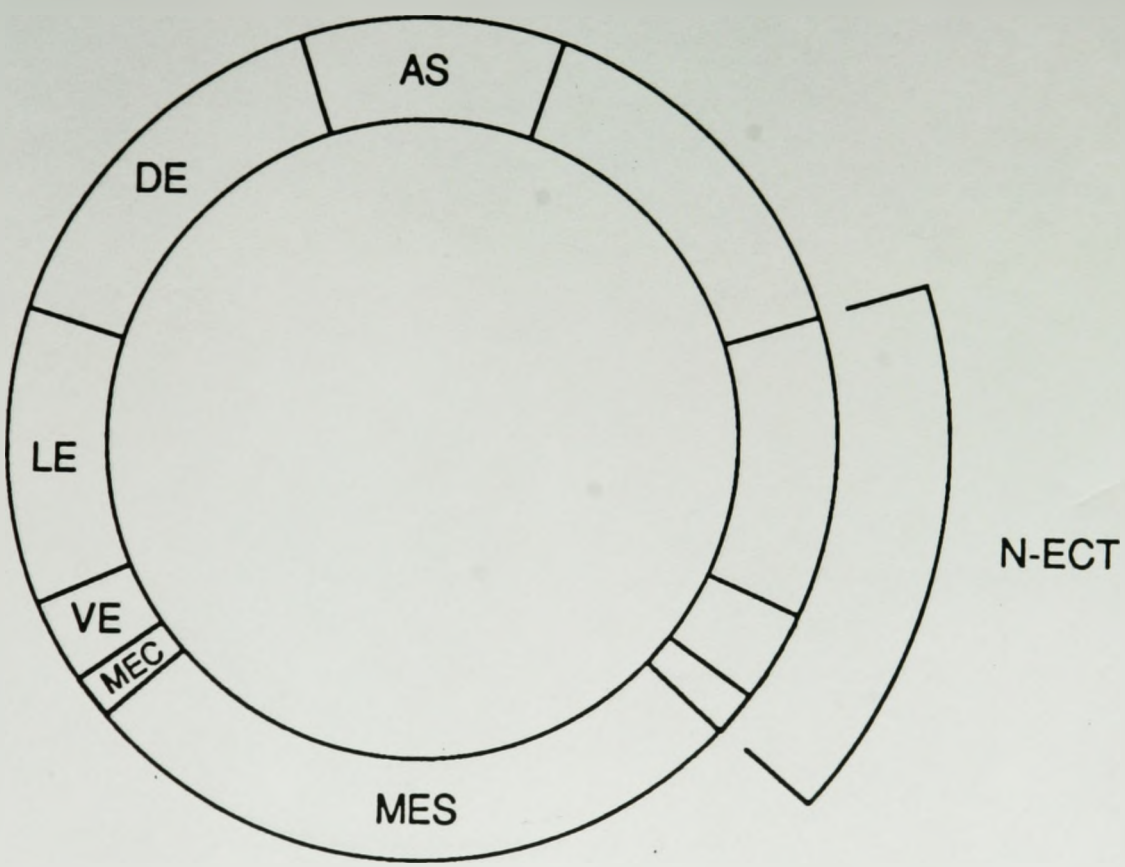
epidermal growth factor receptor (*flb*) (Raz and Shilo, 1993), are required for gene expression and formation of VE cell precursors. VE cells give rise to the ventral denticles at stage 17 when the cuticle is secreted. Loss of *otd* gene expression (a VE marker) in the ventral epidermis of *spitz* group and *flb* mutant embryos revealed that these genes function early in embryogenesis (stage 9). In the absence of *flb* function, ventral ectodermal cells express markers normally restricted to the lateral ectoderm (Raz and Shilo, 1993). The results suggest that the activity of *flb* and possibly the *spitz* group genes are required to establish the identity of cells in the ventral ectoderm during stages 8 and 9 of embryogenesis.

Expression of the *single-minded* gene is restricted to the midline cells (Thomas et al., 1988; Nambu et al., 1990) suggesting that VE cell fate may be dependent on CNS midline cell function (Kim and Crews, 1993). It was proposed that the midline cells may send an inductive signal to establish VE identity or that they could be required for the VE precursors to respond to dorsal-ventral patterning gene activity.

Do the *spitz* group genes and *flb* function in a similar pathway to determine VE cell fates? In a weak *flb* background, lack of zygotic *spi* and *S* function result in a cuticle phenotype of severe *flb* mutants. This synergistic phenotype suggests that *spi* and *S* may participate in VE cell fate determination through the *Egfr* signalling pathway (Raz and Shilo, 1993).

These studies confirm initial observations that the cuticle phenotypes of *spitz* group mutants reveal a common function among these genes. Reduction in the number of VE cells in mutant embryos explains the absence of the centralmost ventral denticles in the *spitz* group as originally noted by Mayer and Nusslein-Volhard (1988).

Figure 2. A blastoderm fate map is schematically shown in cross-section. At this stage the mesoderm (MES) is the most ventral region while the lateral neurogenic ectoderm (N-ECT) is composed of the lateral ectoderm (LE), the ventral ectoderm (VE) and the mesectoderm (MEC). The dorsal epidermis (DE) and amnioserosa (AS) occur in more dorsal regions of the embryo (From Kim and Crews, 1993).



Blastoderm fate map

(3c) Expression and characterization of midline genes

Molecular characterisations have revealed several interesting aspects about the structure and function of various genes expressed in midline cells. These details are necessary to understand how each gene contributes to a putative signalling pathway as described in the following section.

single-minded (*sim*): This gene encodes a nuclear protein that belongs to the basic helix-loop-helix (bHLH) family of transcription factors (Nambu et al., 1991). Expression of *sim* is restricted to the MEC and begins at the completion of gastrulation. The expression of *sim* is therefore a useful early marker for MEC specification. The *sim* gene controls the expression of many midline genes and is therefore considered a master regulator of MEC gene expression (Nambu et al., 1991). After axon tract establishment and the differentiation of the midline neurons (stage 14), expression of the *sim* gene becomes restricted to the MG.

slit (*sli*): The *sli* gene encodes a secreted protein containing multiple domains proposed to be involved in cell-cell communication and cell adhesion (Rothberg et al., 1988; Rothberg et al., 1990). The *sli* gene is initially expressed in all the MEC beginning at the germ-band-extended stage and becomes restricted to the MG during stage 13. Immunoelectron microscopy revealed that sli protein is localized to the MG and to the longitudinal and commissural axon bundles (Rothberg et al., 1990).

spitz (*spi*): The *spi* gene encodes a protein containing a putative signal sequence, one EGF domain, a potential transmembrane domain and a dibasic amino acid sequence which may be a proteolytic cleavage site (Rutledge et al., 1992). The spi protein was found to be similar to TGF-β *spi* transcripts and protein are ubiquitous in embryonic tissues with increased expression in the procephalic region, the ventral midline and mesodermal layer. Expression is first detected at stage 6 when gastrulation begins. There are multiple transcripts due to alternate splicing. *spi* is involved in dorsal-ventral axis formation, glial migration, sensory organ determination and muscle development (Rutledge et al., 1992; Klämbt et al., 1991).

rhomboid (*rho*): The *rho* gene encodes a putative integral membrane protein with a PEST sequence, (found in other proteins with short half lives) and a leucine zipper motif in the putative cytoplasmic region (Bier et al., 1990; Rogers et al., 1986). *rho* is initially expressed at the cellular blastoderm stage in two longitudinal ventrolateral domains 7-8 cells wide which progressively decrease to a 1 cell wide strip of cells after mesoderm invagination. Expression is also observed in precursors to the chordotonal organs and in 1-2 cells wide in the ectoderm at the anterior portion of each segment.

Star (*S*): The *Star* gene encodes a novel protein with a putative type II transmembrane domain and two PEST sequences (Kolodkin et al., 1994). The cytoplasmic domain of *Star* is composed of alternating positive and negative charges similar to those found in the leucine zippers in c-fos and N-myc. *Star* transcripts are first detected in a longitudinal ventrolateral domain that is 7-9 cells

wide in the early blastoderm stage. Subsequently *S* expression becomes progressively restricted to 1-2 cells wide at the ventral midline by germband elongation. *S* is also expressed in dorsoventral epidermal stripes and in the optic lobe anlagen of the embryonic brain. During germband retraction *S* expression is restricted to the MG. Post-embryonically, *S* is expressed in the photoreceptor cells and in the third instar larval eye discs at the morphogenetic furrow.

pointed (*pnt*): The *pnt* gene encodes two putative transcription factors (P1 and P2) of the ETS family (Klämbt 1993; Karim et al., 1990). Expression of the two proteins is restricted to the glia cells during embryonic development. P1 transcripts are expressed in two broad stripes during the cellular blastoderm stage in the lateral neurogenic region. During gastrulation there is a gradient of P1 transcript expression from the dorsal edge of the neurogenic region to lower levels toward the mesectodermal anlage. During gastrulation P1 transcript expression gradually restricts from 10-11 cells in the lateral neurectoderm to 3 cells adjacent to the mesectodermal cells in a fashion similar to that of *rho* and S. During stage 12, P1 expression is first observed in the CNS in the longitudinal glia. Ectopic expression of P1 in all midline cells forces cells to enter the glial differentiation pathway (Klaes et al., 1994).

Before the cellular blastoderm stage, P2 transcripts are found at the anterior tip of the embryo. During gastrulation P2 is expressed in the mesoderm until stage 14. During stage 12, P2 is expressed in the midline glia cells until the end of embryogenesis.

(3d) Common phenotypes among *spitz* group mutants: Does this imply a common signalling pathway?

A role for *spitz* group genes during embryonic development was first revealed by the studies of Mayer and Nusslein-Volhard (1988) as introduced above. Similarities in the phenotypes of mutations among *spitz* group gene members in cells of the CNS midline (Klämbt et al., 1991), in specific sensory organs of the peripheral nervous system (PNS) (Bier et al., 1990) and in derivatives of the ventral ectoderm (Mayer and Nüsslein-Volhard, 1988; Kim and Crews, 1993) suggested that these genes may interact in a common developmental pathway.

Star and *rho* are co-expressed in the R2, R5 and R8 cells in the eye. Both *Star* (Kolodkin et al., 1994) and *Rhomboid* (Freeman et al., 1992) are co-localized in the eye disc. Overexpression of *Star* or *rhomboid* results in supernumerary photoreceptor cells. The similar mutant phenotypes of *S* and *rho* suggest that they could function in a similar pathway during photoreceptor development, however there is no direct evidence for this.

Kolodkin et al., (1994) provide genetic evidence that *Star* interacts with the *Egfr* and *sevenless* during photoreceptor differentiation. A synergistic interaction was detected between *Star* and the gain-of-function allele $Egfr^{Elp}$ in the eye. This means that the eye phenotype of a *S*,+/*Elp*,+ fly possessed a more severe phenotype than the eye phenotypes of either *Elp*/+ or *S*/+ flies alone. Therefore, the $Egfr^{Elp}$ mutation interacts dominantly with loss-of-function *Star* alleles. Interactions in the eye between *Star* and loss-of-function mutations in $Egfr^{flb}$ were revealed by the synergistic effect of combined loss-of function

Egfr^{flb} alleles and *S*/+ alleles. It was therefore proposed that *Star* functions with the *Egfr* in determining early R cell development.

The Rhomboid protein is localized to the developing ommatidia in the third instar eye imaginal disc and functions downstream from the *rough* homeobox containing gene (Freeman et al., 1992). Ectopic expression of the *rho* gene causes non-neuronal mystery cells to be transformed into photoreceptor cells; a phenotype similar to that resulting from a lack of function of the *argos* gene (Freeman et al., 1992a; see below). The mystery cells represent a pool of uncommitted cells. However, loss of *rho* function in the eye results in the subtle phenotype of disruption of the ommatidial array. Given the dramatic effects of ectopic *rho* expression it was proposed that *rho* has an overlapping function with other genes involved in photoreceptor cell differentiation.

Interactions between *spitz* group genes in the developing wing

rhomboid is expressed in wing vein primordia and in developing veins and its' localized expression in these tissues directs the cells to become veins. Ectopic *rho* expression results in extra wing veins. Homozygous flies for the *rho*^{ve} allele have no veins and *rho* is not expressed in the mutant wing discs (Sturtevant et al., 1993).

It is postulated that Rho acts as an accessory protein to amplify signalling in the *Egfr*/*Ras* pathway. This information comes from the results of genetic interactions between *rho* alleles and mutations in components of the *Egfr* signalling pathway (Sturtevant et al., 1993) and from evidence that *rho* acts upstream of *Egfr* in oogenesis (Ruohola-Baker et al., 1993). In *Drosophila*, researchers can assay for genes of a common pathway by testing for dosage-

sensitive interactions between mutations in these genes. The wing vein phenotype assayed in this genetic system is sensitive to the dosage of other genes when they are in the same pathway. When one copy of a gene is lost in this system, the mutant phenotype becomes worse. The combination of reduction in functionl *rho* alleles (rho^{ve}) and loss-of-function *Egfr* alleles ($Egfr^{top}$) enhanced the loss of vein phenotype. The combination of gain-of-function alleles of rho (HS-*rho*) and *Egfr* ($Egfr^{Elp}$) resulted in an enhanced wing vein phenotype. Further, the gain-of-function allele of *Egfr* ($Egfr^{Elp}$) compensates for a loss of *rho* function (loss of the rho^{ve} allele). This result would also suggest that *rho* functions upstream from *Egfr*. Therefore, these dosage-sensitive interactions between *rho* alleles and mutations in *Egfr* alleles suggest that *rho* functions by hyperactivating *Egfr* signalling.

Sturtevant et al., (1993) also observed interactions between *rho* and *S* and *rho* and *spi*. The strongest interaction was between *S* and *rho* where heterozygous *S* enhanced the *rho* loss of vein phenotype and suppressed the extra vein phenotype of HS-*rho*. Therefore, *S* acts downstream from *rhomboid* in wing vein development. A role for *spi* was also conferred since there was a loss of vein phenotype in combination of heterozygous *spi* with rho^{ve}, although the phenotype was not as severe as in combinations with heterozygous *S*.

The putative ligand (Spitz) and receptor (Egfr) are ubiquitously expressed in wing discs and therefore *rho* is hypothesized to spatially restrict Egfr activity during wing vein formation (Figure 3; Sturtevant et al., 1993). This explains why the ubiquitous expression of *rho* from a heat shock construct results in dominant phenotypes resembling activation of the *Egfr* signalling pathway in cells where it normally would not be active.

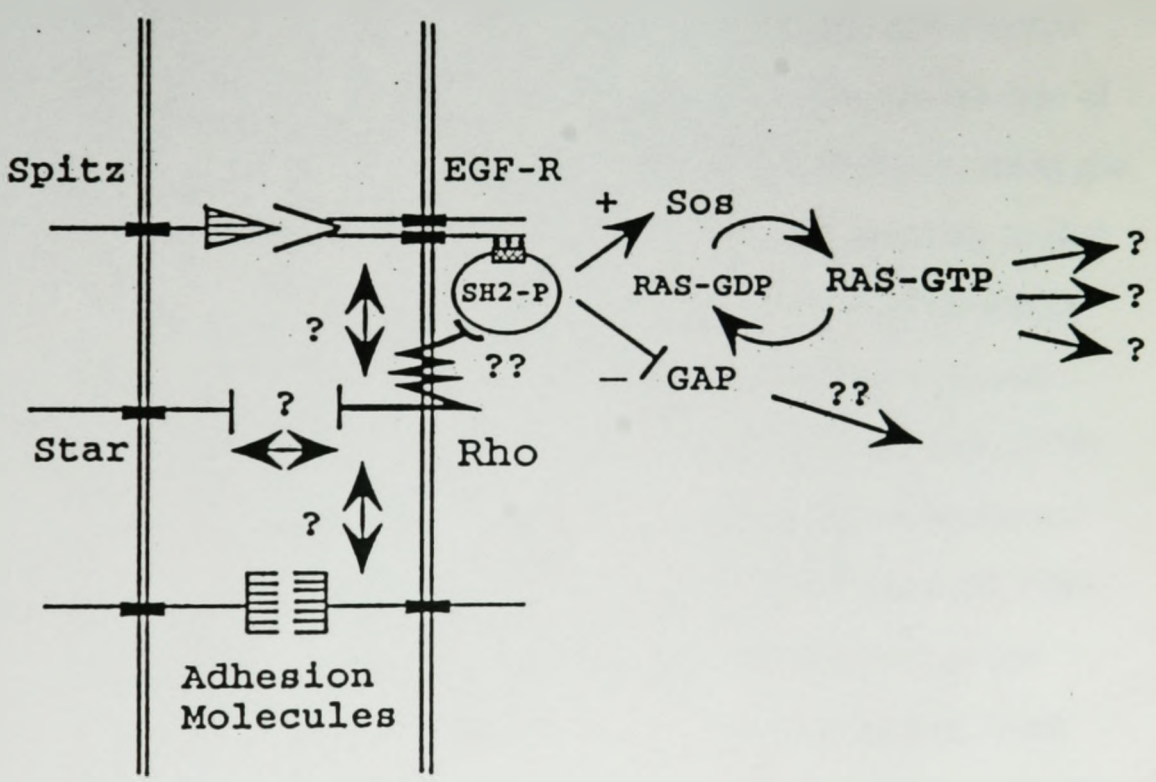
New dominant *rho* phenotypes have been recovered by the enhancer piracy method where a transposon containing the gene of interest and a heat shock element is allowed to randomly insert into the *Drosophila* genome. By this method resulting phenotypes include the conversion of interveins into veins, a serrated wing margin and the loss of halteres (Noll et al., 1994). The inserted gene will come under control of cell-specific enhancers and therefore be restricted in expression. To determine the basis for the dominant *rho* phenotypes, enhancer piracy lines were crossed with flies carrying mutations in various genes of the putative *Egfr* signalling pathway (*Star*, *Gapl*, *Rasl*, $Egfr^{elp}$ and *DfEgfr*). The results suggested that *rho* acts together with *S* in the *Egfr* pathway and that each of the genes assayed is essential for *Egfr* signalling.

The *S* and *rho* genes are co-expressed in the CNS midline precursor and their neuronal and glial progeny during *Drosophila* embryogenesis. The mutant phenotypes of the axon tracts are identical but are the mutant phenotypes of all midline cell lineages similar?

(4) Characteristics of glia in *Drosophila* embryos

Glia have been characterised in many invertebrate systems and it was originally thought that they function primarily to support and nourish neurons (Radojcic and Pentreath, 1979). However, more recent studies have revealed many additional potential functions including providing mechanical stability, permeability barriers as with the perineurium, ensheathment of axons, phagocytosis of neurons, guidance function during neurogenesis, regulating the ion composition surrounding neurons, transmitter uptake and metabolic exchange with neurons and guidance during axonal pathfinding (Bastiani and Goodman, 1986; Jacobs and Goodman, 1989a; Fredieu and Mahowald, 1989).

Figure 3. Models for the amplification of Spi-Egfr signalling by Rho. Rho may increase adhesion between cells in the signalling pathway resulting in a higher likelihood of contact between ligand and receptor. Alternatively, Rho may directly interact with components of the Egfr pathway. A combination of both models would include the binding of Rho to the Egfr and S resulting in a Rho-S; Spi-Egfr complex that is more stable that the Spi-Egfr (From Sturtevant et al., 1993).


Spitz
EGF-R
+
Sos
SH2-P
RAS-GDP
RAS-GTP
?
?
?
?
??
–
GAP
??
?
Star
Rho
?
Adhesion
Molecules

Invertebrate glia have been systematically categorized by light and electron microscopy in adult central ganglia (Wigglesworth, 1959; Radojcic and Pentreath, 1979). Four classes of glia included (1) glia composing the perineurium that control the passage of substances into and out of the ganglion (2) subperineurium glia that produce a myelin-type substance for motor axons (3) giant glial cells in the ganglion that have extensive invaginations into ganglion cells (4) glia that surround the neuropile also have cytoplasm that surrounds axons. Hoyle (1986) further subdivided glia of the adult locust, *Schistocerca americana* using serial electron microscopy. Based on location and the type of cytoplasm projections eight classes of glia were classified including transport glia, axon hillock glia, neuropilar glia and tracheal glia. Due to the variability in glial morphology and location it was proposed that glia may have roles other than neuronal support. Many invertebrate ganglia are avascular and the glia are located between the neurons and haemolymph (Radojcic and Pentreath, 1979).

Drosophila glia have been characterized by ultrastructure (Jacobs and Goodman, 1989a), with glial-specific antibodies (Fredieu and Mahowald, 1989; Ito et al., in press) and by enhancer trap analysis based on morphology and position (Klämbt et al., 1991; Klämbt and Goodman, 1991; Jacobs et al., 1989; Hartenstein and Jan, 1992; Ito et al., in press) and by molecular analysis (Klämbt et al., 1991; Xiong et al., 1994; Campbell et al., 1994).

Figure 4 shows the ensheathing-type glia recognized in *Drosophila* embryos (see Klämbt and Goodman, 1991 and Doe and Goodman, 1994). This group includes the midline glia (MG), the longitudinal glia (LG), the A and B glia, nerve root glia, exit glia and peripheral glia. There are other glia such as the

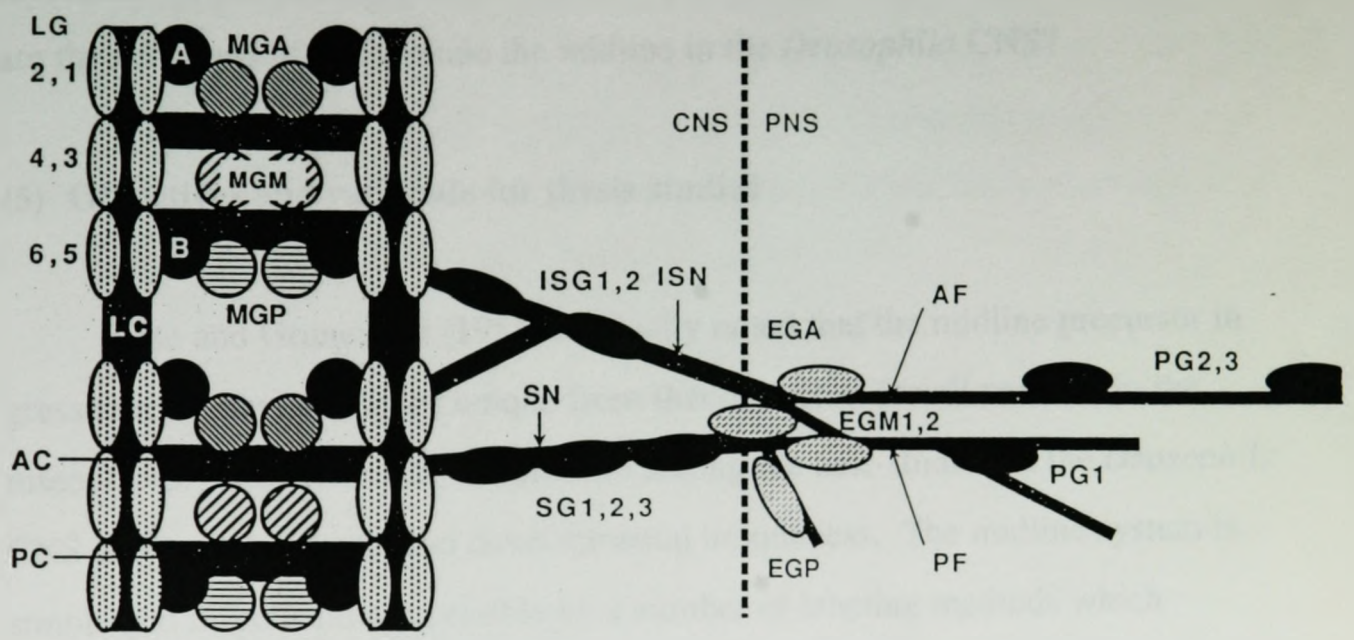
subperineurial glia described by Ito et al., (in press) and those that surround the cell bodies of the ventral unpaired median neuron neurons and the MNB.

Despite the morphological characterizations of *Drosophila* glia their functions have not been established. Disruptions in glial function during embryogenesis lead to aberrant commissural formation (Klämbt et al., 1991). In mutations of the *dropdead* gene, abnormal glia contribute to neural degeneration (Buchanan and Benzer, 1993). Genes that are expressed in midline glia (MG) as well as other cell types include *spitz* group genes (*sim*, S, *spi*, *rho* and *pnt*; Crews et al., 1988; Bier et al., 1990; Rutledge et al., 1992; Klämbt, 1993), *sli* (Rothberg et al., 1990), *argos* (Freeman et al., 1992), *breathless* (Klämbt et al., 1992) and *orthodenticle* (*otd*; Finkelstein et al., 1990) and *D-netrin* (Mitchell et al., 1994). Other genes including *propero* (*pros*; Doe et al., 1991), *otd* (Finkelstein et al., 1990), *pnt* (Klämbt, 1993) and *fushi tarazu* (*ftz*; Jacobs, 1993) are expressed in the longitudinal glia as well as other cell types. In mutants of these genes the longitudinal axon tracts are interrupted (Jacobs, 1993).

The gene product of *repo* contains a glial-specific homeodomain, and is expressed in all *Drosophila* embryonic glial cells excluding the midline glia and the glia of external sensory organs (Campbell et al., 1994; Xiong et al., 1994). Mutations in *repo* are embryonic lethal and affect the differentiation of glia (Halter et al., 1995).

Clearly, there is immense morphological diversity among insect glia. Attempts should be made to determine the developmental roles of glia in the embryonic CNS of *Drosophila*. The MG described in the embryonic CNS of *Drosophila* (Jacobs and Goodman, 1989a; Klämbt et al., 1991) occur in a discrete location and have a very specialized function in commissure morphogenesis

Figure 4. This schematically shows the distribution of glia along major axon tracts in *Drosophila* embryos. Three pairs of midline glia including anterior, middle and posterior (MGA, MGM and MGP, respectively) ensheath the anterior and posterior commissures. Three pairs of longitudinal glia (LG) ensheath the longitudinal axon tracts (LC). Two glia called A and B are located medial to the LC. Nerve root glia ensheath the intersegmental (ISG) and segmental (SG) nerves. Exit glia (EGA, EGM and EGP) are located on the nerve roots as they enter the peripheral nervous system (PNS). Peripheral glia ensheath the nerve roots in the PNS (From Klämbt and Goodman, 1991). Anterior fiber (AF) and posterior fiber (PF).


LG
2,1
A
MGA
4,3
MGM
6,5
B
MGP
LC
AC
PC
CNS
PNS
ISG1,2
ISN
SN
SG1,2,3
EGA
AF
PG2,3
EGM1,2
PG1
EGP
PF

(Klämbt et al., 1991). Molecular heterogeneity occurs between MG lineages as revealed by enhancer trap expression (Klämbt et al., 1991). Does this molecular heterogeneity create or reflect differences in development between MG lineages? Expression of the *repo* gene in all embryonic glia except the MG exemplifies the molecular distinction between the midline and the remainder of the CNS. What are the functions of glia outside the midline in the *Drosophila* CNS?

(5) Objectives and rationale for thesis studies

Bate and Grunewald (1981) originally noted that the midline precursor in grasshoppers have a lineage unique from that described for all neurons in the insect CNS. The cells in the midline are among the best-studied in the *Drosophila* CNS due to their genetic and developmental uniqueness. The midline system is simple and its cells now accessible by a number of labeling methods which facilitate analysis of their fate. However, given these attributes, the molecular and developmental details of midline cell determination, differentiation and death have not been established. This thesis attempts to address these issues by studying midline lineages during wildtype embryogenesis and in embyros mutant for genes expressed in multiple midline lineages.

Based on the proposed interactions between *spitz* group genes and the *Drosophila* epidermal growth factor receptor (*flb*), their co-expression in the midline cells and their similar mutant phenotypes it is prudent to analyse midline lineages in mutants that affect commissure morphogenesis in greater detail. It is important to characterize the fate of each lineage in detail in each mutant embryo

in order to study genetic interactions. Furthermore, it is not known how each midline lineage contributes to commissure morphogenesis.

How do segment-specific variations in cell lineages occur? Recent reports have shed light on the heterogeneity that exists during embryogenesis in both the midline precursor and their progeny in grasshoppers (Thompson and Siegler, 1993; Condron and Zinn, 1994; Condron et al., 1994) and in *Drosophila* (Udolph et al., 1993; Bossing and Technau, 1994). This issue of segment-specific variability in the MG lineage during *Drosophila* embryogenesis is addressed in this thesis (Sonnenfeld and Jacobs, 1995a).

The recent identification of the *reaper* gene, which is responsible for most programmed cell deaths in *Drosophila* embryos, paves the way for molecular analyses of the process (White et al., 1994). However, it is also important to understand the cell death process at the morphological level. For example, which cells in the CNS are involved in the cell death process in general? Given the morphological diversity among insect glia it is possible they have various roles during embryogenesis. Based on evidence that vertebrate glia perform immune system functions inside the CNS (Perry and Gordon, 1987; Streit and Graeber, 1993) we investigated whether glia in *Drosophila* embryos are involved in the cell death process.

Three major questions related to interactions between neurons and glia in the embryonic CNS will be addressed in this thesis. Which midline lineages are involved in commissural axon development? Is there a relationship between midline glia and survival and axon contact? Are glia involved in the cell death process of neurons in the embryonic CNS?

MATERIALS AND METHODS

1. *Drosophila* wild-type and mutant flies

Wild-type *Drosophila melanogaster* flies were from the Oregon-R stock from the Indiana Stock Centre. Unless otherwise stated, mutant alleles were obtained from the Indiana Stock Centre.

1.1. Second chromosome mutants.

slit^{IG107}: an EMS-induced allele located at 2-77 and originally identified in a screen for abnormal cuticle patterns (Nüsslein-Volhard et al., 1984; Rothberg et al., 1988).

Star^{1}: located at 2-1.3 and is a spontaneous allele originally identified by Bridges and Morgan (1919).

Star^{IIN}: an EMS-induced allele located at 2-1.3(Nüsslein-Volhard et al., 1984; Lindsley and Zimm, 1992).

Bic-$^{71.34}$*dpb/Cyo*: a maternal-effect semilethal, EMS-induced allele located at 2-52 and obtained from Beat Suter (Suter et al., 1989).

Bic-D^{IIIE48} *stau*^{HL} *b, cn bw/Cyo*: an EMS-induced allele located at 2-52 (Suter et al., 1989).

staufen (*stau*): a maternal-effect lethal EMS-induced allele located at 2-83.5. Embryos show deletions of the anterior-most head structures (Schüpbach and Wieschaus, 1986). One mutant copy of *stau* results in a double abdomen frequency of 100% (B. Suter, personal communication).

1.2. Second chromosome balancers:

These balancers were used to suppress crossing over in chromosome 2.

Cyo: *Curly of Oster* constitutes *In(2R)O, CY dp* ^{lvI} *pr cn*^{2} (Lindsley and Zimm, 1992). This balancer contains the dominant mutation Cyo producing curly wings.

1.3. Third chromosome mutants:

single-minded$^{B13.4}$: an EMS-induced protein null allele located at 3-52.2 originally identified by Hillicker et al., 1981; Mayer and Nüsslein-Volhard, 1988; Thomas et al., 1988).

rhomboid^{P38}: located at 3-0.2 and was generated from the excision of a p-element insertion into 62A (Bier et al., 1990; Freeman et al., 1992).

1.4. Deficiencies

Df(3L)WR4, ru, h, e, aa/TM6B= $W[^{+}R4]$ uncovers the *reaper* locus (White et al., 1994).

Df (3L)WR10, ru, h, sbd^{2}*/TM6B=* $W[^{+}R10]$ uncovers the *reaper* locus (White et al., 1994).

1.5. Third chromosome balancers:

TM6B: this is comprised of *In(3LR)TM6B, Hu e* (Lindsley and Zimm, 1992).

TM3: this is comprised of *In(3LR)TM3, y^+ ri p^p sep l(3)89Aa bx^{34e} e* (Lindsley and Zimm, 1992).

MKRS: this is comprised of *Tp(3;3)MKRS,M(3)76A, kar ry^2 Sb* and suppresses crossing over in the proximal regions of both arms of chromosome 3 (Lindsley and Zimm, 1992).

2. Enhancer traps

P-element vectors have been extensively used to identify genes required for development of the *Drosophila* embryonic CNS (Bier et al., 1990). They provide tissue-specific expression of a β-galactosidase (lacZ) fusion gene driven by a weak promoter of the P-element transposase gene (O'Kane and Gehring, 1987). These vectors contain bacterial plasmid sequences for rapid cloning and an eye colour gene (typically *white* or *rosy*) as a marker for P-element insertion.

AA142: P element insertion at cytological location 66D (Klämbt et al., 1991). This line expresses β-galactosidase strongly in the MGA, MGM and weakly in the MGP after stage 14 (Sonnenfeld and Jacobs, 1994).

T13: P element insertion at cytological location 60F (Klämbt et al., 1991). Until germ band retraction this line is expressed in six ventral unpaired median neuron neurons. After stage 13 this enhancer trap is expressed in the ventral unpaired median neuron neurons and in approximately 48 lateral neurons within each ventral nerve cord segment.

P223: P element insertion at cytological location 98D. This enhancer trap expresses β-galactosidase in the two MP1 neurons and the two MP2 neurons from stage 11 until the end of embryogenesis.

Q114: P element insertion at cytological location 64C (Klämbt et al., 1991). This enhancer trap is expressed in all three MG lineages and unidentified neurons at 12 hours of embryogenesis. It is expressed in the MGA and MGM after 8 hours of embryonic development and in the MGP after 9.5 hours.

X55: P element insertion at cytological location 56F (Klämbt et al., 1991). X55 is expressed in the ventral unpaired median neuron neurons, the MNB and its progeny and in the MGP. This enhancer trap was used to identify the MGP after late stage 13.

X81: This p-element was localized to cytological position 62A1,2 5' of the transcriptional start site of the *rhomboid* gene (Bier et al., 1990; Freeman et al., 1992). During gastrulation expression from this construct occurs on either side of the ventral furrow and then becomes restricted to a few cells in the midline of the CNS (Freeman et al., 1992).

3.0. Gene fusion reporter elements

p[*sim/lacZ*]: consists of a p-element transposon containing 7.8 kilobases of DNA from *sim* regulatory region fused to the β-gal gene (Nambu et al., 1990). Expression begins at stage 8 in midline precursors and becomes restricted to midline glia and a subset of ventral neurons during stage 15.

p[*sli/lacZ*]1.0: consists of a p-element transposon containing a 1.0 kilobase restriction fragment of *sli* DNA. After germband retraction p[*sli/lacZ*] is expressed in all six midline glia and becomes restricted to both MGM and one MGA in each segment (Wharton and Crews, 1993).

4.0 **Antibodies**

BP102: This is a mouse monoclonal Ab that recognizes an uncharacterized carbohydrate moiety present on the longitudinal and commissural axon tracts of the CNS (provided by C. S. Goodman, Berkeley). Used at a 1:10 dilution for wholemount embryo staining.

anti-β-galactosidase: This is a commercial mouse monoclonal antibody (Sigma) used to identify expression patterns of enhancer traps and gene fusion reporter constructs. It was used at a 1:100 dilution for embryo staining.

22C10: This antibody was used to identify the ventral unpaired median neurons at stage 14 and the MP1 and MP2 neurons at stage 12 of embryogenesis (Fujita et al., 1982). It was used at a 1:5 dilution for embryo staining (This was a gift from Corey Goodman).

anti-even-skipped: This rabbit monoclonal antibody recognizes the protein product of the pair rule gene even-skipped. This antibody was used to identify the RP neurons (Frasch et al., 1987) and was used at a 1:10 dilution for embryo staining.

anti-engrailed: This mouse monoclonal antibody recognizes the product of the nuclear engrailed protein during embryogenesis and was used to recognize the median neuroblast and its progeny during embryogenesis. (kindly provided by C. S. Goodman, Berkeley). It was used at a 1:5 dilution for embryo staining.

anti-peroxidasin: A mouse polyclonal antibody that recognizes peroxidasin which is produced and secreted by phagocytic haemocytes during embryogenesis (kindly provided by L. Fessler, UCLA; Abrams et al., 1993; Tepass et al., 1994). It was used at a 1:1 dilution for embryo staining.

anti-RK2: A rat polyclonal antibody that recognizes the amino-terminal region and excluding the homeodomain of the Repo protein expressed in most embryonic central nervous system glia excluding the midline glia (kindly provided by Andrew Tomlinson). It was used at a 1:100 dilution for wholemount embryo staining (Xiong et al., 1994; Campbell et al., 1994).

5.0 Embryo collections and staging

Embryos were collected on apple juice agar filled petri plates containing a small smear of live yeast paste (Fleischmann's dry yeast) for protein supply. Apple juice agar plates containing embryos were stored at $4^{o}C$ for a maximum of two days. Before use, these plates were thawed for two hours at room temperature to allow microtubule repolymerisation and restoration of axon morphology before further processing.

Embryos were staged according to Campos-Ortega and Hartenstein (1985) using a combination of morphological structures including protrusion of the procencephalon, subdivision of the yolk and length of the germband. The length of the germband during stage 12 was subdivided according to Klämbt et al., (1991).

6.0. Immunocytochemistry for *Drosophila* embryos.

Embryos from apple-juice agar collection plates were dechorionated with 50% sodium hypochlorite (commercial bleach) for ten minutes. Dechorionated embryos were rinsed with distilled water into a Nitex sieve which was then blotted dry on a kimwipe. The nitex sieve with the dechorionated embryos was immersed in a scintillation vial containing heptane: 3.7% formaldehyde (1:1 ratio) in phosphate buffered saline (PBS, pH, 7.4). Embryos were fixed for at least twenty minutes at the interface between the heptane and bottom aqueous layer containing the fixative. This interface ensures that the embryos were permeabilized to allow entry of fixative. The aqueous layer was removed with a pasteur pipette and methanol was added to the remaining heptane containing the fixed embryos. This solution was shaken for approximately thirty seconds to remove the vitelline layer surrounding the embryo. Devitellinated embryos fell to the bottom in the methanol layer and were transferred with a pasteur pipette to fresh methanol in 12mm disposable glass tube. Three subsequent methanol washes were performed to remove traces of heptane. Embryos were either stored in methanol at $4^{o}C$ until further use or were immediately processed for antibody staining.

To proceed with antibody staining, the methanol was replaced with PBT (PBS containing 0.1% Triton X-100). Embryos were washed for ten to thirty minutes in PBT on a rotator and were then incubated in 100 µl of PBT containing normal goat serum (NGS) (7.5%) to block nonspecific antibody binding for thirty to sixty minutes. A specific dilution of primary antibody was added (see section 3.0 Antibodies) to the embryos in block solution and they were incubated overnight at 4°C. The block and primary antibody solution were washed out with PBT for three hours to overnight with at least four changes in PBT. The washed embryos were blocked again in PBT containing NGS for thirty to sixty minutes. The secondary antibody (either goat anti-mouse IgG or goat anti-rat IgG conjugated to horse-radish peroxidase) was added to this solution at a dilution of 1:100. The embryos were incubated in secondary antibody for two hours at room temperature and were then washed with at least three changes in PBT for two hours to overnight on a rotator.

For visualization of the antibody reaction, embryos were incubated for ten minutes in PBT containing 0.3 mg/ml diaminobenzadine (DAB). To this solution 3 µl of 3% hydrogen peroxide was added as a substrate for the DAB and the reaction was allowed to proceed until the background staining in the embryos began to darken. This reaction was stopped by dilution in PBT. Waste DAB was neutralized in bleach. Embryos that were processed for single antibody labelling were dehydrated through an ethanol series (50%, 70%, 90%, 95% and 100% ethanol) and were then transferred to methyl salicylate in which the embryos and antibody stain can be preserved.

Single antibody labelled embryos to be processed with a second antibody were washed in PBT for at least two hours after the DAB-peroxide reaction. The

washed embryos were blocked in PBT containing NGS for thirty to sixty minutes and the second primary antibody was added and incubated overnight. Subsequent processing is described above.

An 8% cobalt chloride solution ($CoCl_2.6H_20$; Fisher, lot 884698) was added to the diluted DAB solution to a final volume of 4 µl/ml to produce a grey colour in the final reaction product. This solution was added in double labelling experiments when the same secondary antibody was used.

7.0. Immunocytochemistry with X-gal for light microscopy.

Embryos were dechorionated with 50% bleach, rinsed with distilled water and collected on a Nitex sieve. Embryos were fixed in a deep depression slide in 0.5 ml of heptane that was saturated with fixative (0.10M sodium cacodylate buffer, pH 7.4; 2.5% glutaraldehyde, EM grade, Fluca). The upper phase of this solution was used to fix the embryos for fifteen minutes at room temperature. To ensure proper fixation embryos were inspected under a dissection microscope for the presence of a yellow colour. Fixed embryos were transferred onto a glass slide with a pasteur pipette and excess heptane was removed with a filter paper. Embryos were transferred onto double sided tape and were placed in a well (silicone sealant well) on a glass slide, embryo side up. Embryos were covered with 0.1M sodium cacodylate buffer (pH 7.4).

The vitelline layer of the fixed embryos was removed with an electrolytically sharpened tungsten needle. Devitellinized embryos were transferred from the well with a pasteur pipette to 12mm disposable glass tubes. The 0.1M cacodylate buffer was exchanged for 300 µl of X-gal staining solution (10 mM phosphate buffer, pH 7.2; 150 mM sodium chloride; 1 mM magnesium

chloride; 3.1 mM potassium ferrocyanide; 3.1 mM potassium ferricyanide; and 0.3% Triton-X 100) and the embryos were incubated in this solution for at least five minutes. The X-gal reaction was initiated by the addition of 25 µl/ml X-gal (8% wt/vol. in dimethyl sulfoxide) to the staining solution containing the embryos. The embryos were reacted overnight at 18°C to reduce the size of X-gal crystals or for two hours at 37°C when screening transgenic fly lines. The staining solution was removed by several washes with of PBS. The embryos were either stored in 90% glycerol/PBS at four °C or were processed for light and electron microscopy as described in the following section.

8.0. Fixation and embedding *Drosophila* embryos for electron microscopy

Embryos were dechorionated with 50% bleach, rinsed with distilled water onto Nitex sieves and the sieves were blotted dry on a Kimwipe. Dechorionated embryos were immersed in heptane equilibrated with fixative containing 25% glutaraldehyde and 2% acrolein for ten minutes. The fixed embryos were transferred in a minimum volume in a pasteur pipette to a clean glass slide and the excess heptane was blotted dry with filter paper. Double sided tape was gently rolled over the embryos and this was placed embryo side up in a silicone sealant well. The well was filled with primary fixative containing 0.1M sodium cacodylate (pH 7.4), containing 2% paraformaldehyde and 2.5% glutaraldehyde. The vitelline membranes surrounding the embryos were removed with an electrolytically sharpened tungsten needle under a dissection microscope. The devitellinized embryos were collected in a pasteur pipette and transferred to 12mm glass disposable tubes containing 0.1M sodium cacodylate buffer (pH 7.4). Several changes of sodium cacodylate buffer (pH 7.4) were performed to

eliminate traces of primary fixative. Approximately 0.5 ml of 1% osmium tetroxide in sodium cacodylate buffer (pH 7.4) was added to the embryos for at least sixty minutes followed by two washes in sodium cacodylate buffer and then two washes in distilled water. Approximately 0.5 ml of 5% uranyl acetate was added to the embryos for thirty minutes.

Embryos were dehydrated by a series of washes in 50%, 70% and 90% ethanol for ten minutes each followed by two ten minute washes in 95% ethanol and three ten minute washes with 100% ethanol. The following plastic mixture for embryo embedding was made: 4.4 grams of araldite, 6.2 grams of Epon, 12.0 grams of DDSA and 0.3 ml of DMP.30. This mixture was stirred for at least one hour. For infiltration of embryos, the embryos were placed in a 1:1 mixture of ethanol: plastic mixture for twenty minutes, followed by a twenty minute incubation in 1:4 mixture of ethanol: plastic mixture and finally 100% plastic mixture. The plastic mixture was allowed to infiltrate into the embryos overnight in a dessicator. The plastic mixture was placed to half full in rubber block molds to which labels had been added and was allowed to polymerize at 70^{o}C for three hours to prevent the embryos from sinking to the bottom. The embryos were placed in the semi-polymerized plastic, the remainder of the wells were filled with plastic and the embryos were oriented to obtain cross-sections or sagittal sections. The tray containing the embedded embryos was placed in the oven at 70^{o}C for thirty minutes and the orientation of the embryos was inspected as softening of the plastic occurs. The tray was put back in the oven at 70^{o}C and the plastic was allowed to polymerize for two days. Sections from plastic embedded tissues were collected on slot grids and were stained with lead to increase contrast before inspection.

9.0. Embedding HRP-labelled wholemount embyros

Embryos labelled with HRP as described above can be embedded for light level histological analysis. The solution of methyl salicylate in which HRP-labelled embryos are stored is changed by several washes in 100% ethanol. Conventional methods for embedding for electron microscopy are then followed as described above.

10.0. Vital dye staining of *Drosophila* embryos.

This staining procedure was adapted from Abrams et al., (1993) to label dead cells in wholemount embryos. Embryos were dechorionated in 50% bleach, rinsed in distilled water and collected on nitex sieves. The embryos were placed into 5.0 ml of heptane to which an equal volume of 100 ug/ml nile blue A (Sigma, No. N 0766) in 0.1M phosphate buffer (pH 7.2) was added. After ten minutes, the embryos were removed from the heptane/dye interface with a pasteur pipette and were briefly rinsed in 0.1M phosphate buffer (pH 7.2). The rinsed embryos were placed on a slide containing a smear of series 700 Halocarbon oil to prevent dessication and were covered with a coverslip. These samples were immediately analysed with a Zeiss axiophot microscope as fading of the dye begins within thirty minutes of dye incorporation.

11.0. Terminal transferase labelling (Tunel) of *Drosophila* embryos.

This protocol was originally described by Gavrieli et al., (1992) and has been modified by Steve Robinow (University of Washington) and Kristin White (MIT). Embryos were dechorionated in 50% bleach, rinsed in distilled water and collected on nitex sieves. These embryos were fixed for thirty minutes at an interface of octane and 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2). After the fixative was removed, embryos were released from the vitelline membranes through vigorous shaking in 100% methanol and devitellinated embryos were recovered from the bottom layer. The embryos were rinsed in three changes of PBT and were then incubated in 1X terminal deoxynucleotidyl transferase (TdT) buffer (20 µl 5X TdT buffer and 40 µl of autoclaved water) for ten minutes. The 1X TdT buffer was replaced with reaction buffer (20 µl 5X TdT buffer, 20 µl of 100 µM dUTP mix, 3 µl TdT and 22 µlwater) and the embryos were incubated for three hours in a 37^{o}C water bath for incorporation of biotinylated dUTP. The embryos were washed for sixty minutes with at least four changes in PBT and the label was detected by peroxidase staining using the Vectastain Elite ABC mouse IgG kit (Vector Laboratories). The Vectastain elite ABC kit contains avidin and biotinylated horseradish peroxidase which form complexes for immunoperoxidase staining. 10 µl solution A and 10 µl of solution B were added to 1.0 ml of PBT and this mixture was preincubated at room temperature for 30 minutes to allow formation of the avidin-biotinylated horseradish peroxidase complex. The embryos were incubated in the avidin-biotin-HRP complex for 60 minutes at room temperature. After 60 minutes the complex was washed out with five minute washes in PBT three times. Embryos

were resuspended in 300 µl of 0.3 mg/ml of DAB in PBS to which 3% H_2O_2 was added and the reaction was allowed to develop under a dissection microscope until brown precipitate had formed. The reaction was stopped by dilution in PBT and embryos were dehydrated through an ethanol series and preserved in methyl salicylate as previously described.

12.0. Tunel solutions:

100 M dUTP: mix 13.4 µl of 100 µM dUTP (Cat# 1420 470) with 6.6 µl of Bio-16-dUTP (Cat# 1388 908) both from Boehringer, Mannheim.

Terminal deoxynucleotidyl transferase (TdT): This enzyme is a DNA polymerase isolated from calf thymus found in prelymphocytes. In the presence of a divalent cation it catalyses the addition of deoxynucleotides to the 3'-hydroxyl termini of DNA molecules. Co^{++} is the divalent cation used when the nucleotide to be added is pyrimidline. The enzyme prefers double-stranded DNA with a protruding 3'-hydroxyl terminus as is found in apoptotic cells with fragmented DNA. TdT (Cat# 18008-011) was ordered from Gibco BRL.

Vectastain kit solutions

5X TdT buffer: 0.5 M potassium cacodylate (pH 7.2), 10 mM $CoCl_2$ and 1 mM DTT. This buffer (Cat# 16314-015) was ordered from Gibco BRL.

Reagent A: avidin DH. Avidin is a glycoprotein with a high affinity for the vitamin biotin.

Reagent B: horseradish peroxidase enzyme

13.0. *Drosophila* culture media and solutions

Banana corn-meal food: Mix 1.5 litres of water and 20 grams of agar (Sigma, A-7002) and bring to a boil. Add 45 grams of corn-meal to the mixture and cook for 10 minutes while stirring constantly. While this cooks mix in a blender 2 bananas, 80 grams of torula yeast and 3 tablespoons of corn syrup and 0.5 litres of boiled water. After the corn/agar mixture has cooled for 10 minutes add the blended banana yeast mixture and cook for an additional 10 minutes. Once the food has cooled to 45^{o}C while constantly stirring, add 15 ml of acid mixture and 5ml/l of Tegosept and allow to stir for at least 5 minutes. Immediately fill vials with food.

Acid mixture: Add 83.6 ml of 100% o-propionic acid (BDH, lot 4745340N) and 8.3 ml of 85% phosphoric acid (Fisher, lot 902021) to 100 ml of double distilled water and bring final volume to 200 ml.

Tegosept: Make a 1 litre solution of 10% tegosept (p-hydroxybenzoic acid methyl ester; Sigma) in ethanol and add 1 ml of green food dye. Maintain stock at 4 ^{o}C.

Yeast paste: make a 1g per 2ml solution of active dry yeast paste in water and allow to sit overnight at 4^{o}C. Maintain stock at 4^{o}C.

Apple juice agar plates: In a 5 litre beaker add 100 grams of sucrose and 1 litre of apple juice. Autoclave both mixtures and combine in one beaker to let cool while

constantly stirring. Dissolve 6 grams of tegosept once the mixture has cooled to 45^{o}C. Aliquot the apple juice agar into 0.5 litre erlenmeyer flasks and cover with aluminum foil. This mixture is stored at 4^{o}C until further use. To make collection plates, dissolve solidified apple juice agar in the microwave and half fill small petri dishes (Fisher, 60 x 15 mm). Once solidified these plates are stored in the original plastic sleeves at 4^{o}C.

14.0. Histology

For light level analysis of embryonic tissue, 0.5-1.0 micron sections were obtained on a RMC-7 microtome. Sections were collected in a commercial plastic boat and the plastic was spread by waving a chloroform wick over top. In all cases serial sections were obtained and transferred to slides coated with a solution of 1% gelatin and 0.1% chromium potassium sulphate [$CrK(SO_4)_2.12H_20$]. Sections containing X-gal or bluo-gal stain were counter-stained with basic fuschin to allow visualization of the blue stain. Sections containing antibody staining were counter-stained with dilute methylene blue and those with no stain were counter-stained with methylene blue (0.1% in 0.1M sodium borate buffer). Counter-stained sections were preserved with permount (Fischer Scientific) solution underneath a cover slip. Analyses of sections were performed on a Zeiss Axiophot microscope and were photographed under a 63X oil immersion lens.

15.0. Light and electron microscopy solutions

Formaldehyde: This monoaldehyde (H-C=0H) is soluble in water and is available commercially as 37-40% formalin. Since formalin contains impurities formaldehyde is a more effective fixative when it is produced by dissociation of paraformaldehyde. Formaldehyde fixation causes swelling and distortion of cytoplasmic organelles and therefore was used only for light level fixation of embryos. Formaldehyde cross-links proteins by causing the formation of amino methylol groups which then condense with other groups including phenol and indole to form methylene bridges (-CH2-).

Glutaraldehyde: The structure of glutaraldehyde consists of a straight hydrocarbon chain that links two aldehyde groups. It is the most effective protein cross-linker of the aldehydes and causes the least protein conformational changes.

Acrolein: This fixative has a faster penetration rate and causes less shrinkage than either glutaraldehyde or formaldehyde. Acrolein (CH2=CH-CHO), is an olefinic aldehyde commonly made from dehydration of glycerol at high temperatures.

Paraformaldehyde: A form of formaldehyde that increases crosslinking of proteins and decreases loss of antigens through extraction.

Osmium tetroxide: This nonpolar substance is an effective fixative because it can penetrate both polar and nonpolar media including hydrophobic regions. It is useful as a postfixative because it preserves many lipids and also acts as an electron stain to produce high contrast of the tissue. It has a slow penetration

rate and is a poor cross-linker of proteins and therefore was not used as a primary fixative during embryo preparation for electron microscopy.

Uranyl acetate: This was used in en bloc staining of embryonic tissue prior to dehydration with ethanol to increase electron contrast and reduce extraction while the tissue is in alcohol. Staining of embedded tissue with uranyl acetate increases precipitate formation.

Plastics: The following plastics were used for routine embedding of embryos; araldite 502 resin (J.B. EM Services INC., #034), D.D.S.A-dodecenyl succinic anhydride (J.B.S. #031), EPON 812 resin (J.B.S., 030) and the catalyst DMP-30 tri(dimethylaminomethyl) phenol (J.B.S. #033).

Sodium cacodylate buffer (0.1M, pH 7.4): This buffer avoids phosphate groups which may interfere with cytochemical studies. It is incompatible with uranyl acetate and therefore washes in water were performed between the two steps. This buffer has the highest hydrogen bonding and therefore reduces extraction during tissue treatment.

Preparation of subbed slides: Dissolve 5.0 grams of gelatin and 0.5 grams of chromium potassium sulphate [$CrK(SO_4)_2.12H_2O$] (BDH; lot 95398/4319) in 500 ml of ddH_2O. Heat this mixture to 55^oC while stirring and fill a glass staining dish. Load slides into a stainless steel slide holder and immerse slides in subbing

mixture for approximately 3 minutes. Dry slides in a 60^{o}C oven. The subbing mixture is stored at 4^{o}C and at further use is thawed in a 60^{o}C oven.

16.0. Film Processing

Black and white pictures were taken on a Zeiss Axiophot microscope using technical pan 35 mm film (Kodak) at a speed of 25 ASA. The film was developed with Kodak technidol liquid developer.

RESULTS

The results section of this thesis is arranged as follows:

Chapter 1- Mesectodermal cell fate analysis in *Drosophila* midline mutants occurs as a reprint from the Mechanism of Development. (Sonnenfeld and Jacobs, 1994).

Chapter 2- Apoptosis of the midline glia during *Drosophila* embryogenesis: A correlation with axon contact. (Sonnenfeld and Jacobs, 1995a).

Chapter 3- Macrophages and glia mediate the removal of apoptotic cells from the *Drosophila* embryonic nervous system. (Sonnenfeld and Jacobs, 1995b)

Contributions to Sonnenfeld and Jacobs, 1994: all research and preparation of manuscript was performed by M. Sonnenfeld except for the electron microscopy which was performed by Dr. Jacobs.

Contributions to Sonnenfeld and Jacobs, 1995a. Electron microscopy was performed by Dr. Jacobs. Tunel labelling was performed by M. Sonnenfeld but figure 2 in the text was prepared by Dr. Jacobs. Final preparations of the manuscript were done by Dr. Jacobs.

Contributions to Sonnenfeld and Jacobs, 1995b. All electron microscopy was performed by Dr. Jacobs except for figure 4.

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Mesectodermal cell fate analysis in *Drosophila* midline mutants

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Abstract

We have used enhancer traps and antibodies as markers of cell identity to assess the relative contribution of individual mesectodermal cell (MEC) lineages to CNS midline morphogenesis in four mutations that disrupt commissure formation in *Drosophila*. The absence of commissures, leading to longitudinal tract collapse, was seen in embryos mutant for the genes *single-minded* and *slit*. MEC lineages did not survive in *single-minded* mutant embryos, in contrast to the survival of all MEC lineages in *slit* mutant embryos. The midline glial cells were displaced and appeared ultrastructurally normal in *slit* mutant embryos, yet the presence of the MG was not sufficient to generate commissures. Commissure formation requires correct MEC cytoarchitecture, dependent upon *slit* activity. In fused commissure mutants (*rhomboid* and *Star*) neuron number was reduced in the ventral unpaired median neuron (VUM) lineage and the median neuroblast lineage before commissure formation (stage 12). Subsequent to these neuronal defects, the midline glia died by apoptosis (stage 13). Commissure fusion and glial apoptosis may be triggered by the earlier perturbations in MEC neuronal lineages. These studies establish when the respective activities of each gene are required for the development of each MEC lineage.

Key words: Embryonic CNS; *Single-minded*; *Slit*; *Rhomboid*; *Star*; Lineage; Glia; P-Element

1. Introduction

The developing ventral nerve cord (VNC) of the *Drosophila* embryo is an attractive system for investigating the mechanisms of cell determination and differentiation. The neurons and glia of the midline of the embryonic VNC are the progeny of mesectoderm cells (MECs). The MECs demarcate the ventral ectoderm from the presumptive mesoderm during gastrulation and form a developmentally distinct compartment of the VNC (Crews et al., 1992). In each segment the MECs are composed of four neuronal lineages and three glial lineages which originate from eight midline progenitor cells (Klämbt et al., 1991). The pattern and identity of the MEC lineages have been characterised during embryonic VNC development and there now exist many enhancer trap lines which provide markers for each MEC lineage (Crews et al., 1992).

Among the first cells of the VNC to differentiate, the MECs are postulated to play a significant role in early CNS morphogenesis that is analagous to the role of the floor plate cells of the vertebrate nerve cord (Goodman and Shatz, 1993; Nambu et al., 1993). One proposed function of the MECs is the establishment and morphogenesis of the commissural tracts that carry contralateral axon projections. In *Drosophila*, growth cones of commissural axons interact with the MEC derived midline glia (MG). It has been proposed that the MG are necessary for commissural tract formation and morphogenesis (Jacobs and Goodman, 1989a; Klämbt et al., 1991). To address this question, we have examined the differentiation of a number of MEC lineages, including the MG, in mutations that perturb commissural tract genesis.

We have examined two groups of mutations that specifically affect the formation of the commissural tracts of the CNS axon scaffold. The first group of mutations, termed *collapsed longitudinal tract mutations* results in a collapse of midline structure, producing a

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single midline longitudinal tract from the bilateral pair of longitudinal tracts. This is identical to 'group 1' mutations of Klämbt et al. (1991) and the collapsed CNS phenotype described by Nambu et al. (1992). The second group of mutations, termed *fused commissure mutations*, have incomplete or no separation of the anterior and posterior commissures. This is identical to 'group 3' mutations of Klämbt et al. (1991). A lineage by lineage analysis of MEC differentiation within these groups should reveal the contribution of each cell type to midline cytoarchitecture and axon tract genesis.

The collapsed longitudinal tract phenotype is examined here in embryos mutant for the genes *single-minded* (*sim*) and *slit* (*sli*; Rothberg et al., 1988; Thomas et al., 1988). The *single-minded* gene encodes a nuclear protein containing a basic helix-loop-helix motif representative of certain transcription factors (Nambu et al., 1991). *sim* activity is necessary for the expression of numerous MEC specific genes, and may function as a master regulator of MEC cell differentiation (Nambu et al., 1991). The *slit* gene encodes an extracellular matrix protein produced by the MECs (Rothberg et al., 1990).

In the fused commissure phenotype, the anterior and posterior commissures form but they do not completely separate. This group contains the *spitz* group genes, (*spitz*, *pointed*, *sichel*, *rhomboid* [*rho*] and *Star* [*S*]). The gene products of some members of the *spitz* group are hypothesised to interact with the *Drosophila* EGF receptor in a signal transduction pathway in many different tissues in development (Rutledge et al., 1992; Sturtevant et al., 1993). It is not known which MEC lineages send or receive this putative signal, nor what role this signal may have in cell determination or differentiation.

Previous studies with some of these mutations support a role for the MEC cells in establishing the axon tracts during VNC development (Thomas et al., 1988; Klämbt et al., 1991). It is not known how or when the determination, differentiation, and survival of each MEC lineage is affected in these mutations, or how these events contribute to the axon tract phenotype. We have examined several MEC lineages in both collapsed and fused commissure mutants to determine how each might be affected by mutations in genes required for VNC midline morphogenesis. Our results support the role of *sim* as a master regulator of MEC cell differentiation. We also find that mutations in *sli* affect the position but not the differentiation or survival of the MECs, establishing that mispositioning of the MECs is sufficient to cause a complete collapse of longitudinal axon tracts. These data also suggest that midline glial cell differentiation is not sufficient for commissure formation. The genes *rho* and *S* are necessary for determining the correct number of cells in many neuronal MEC lineages. The correct number of midline glia are determined but later become apoptotic in embryos mutant for *rho* and *S*. Midline glial survival may depend upon the other MEC lineages disrupted in *spitz* group mutations. Multiple MEC lineages are likely required for commissure formation.

2. Results

2.1. Collapsed longitudinal and fused commissure mutant CNS phenotypes

A summary of the development of the collapsed longitudinal and fused commissure phenotypes is shown in Fig. 1. In wild-type embryos at stage 12/3, both the anterior and posterior commissures have been pioneered and have not separated from each other (Fig. 1A). During stage 12/0 the anterior and posterior commissures separate and result in a ladder-like arrangement of the two longitudinal connectives and commissures (Fig. 1F).

Early defects in the two collapsed longitudinal tract mutants (*sim* and *sli*) were apparent when the commissures were pioneered. In *sim* mutants all axons were at the midline (Fig. 1B). *sli* mutant embryos at stage 12/3 had a commissural phenotype intermediate between wild-type and *sim* mutant embryos (Fig. 1C). The phenotype by stage 14 in both *sim* and *sli* mutants was identical: longitudinal axon tracts were displaced towards the midline in the space that the anterior and posterior commissures would have occupied (Fig. 1G and H). In both *sim* and *sli* mutant embryos, contralaterally projecting axons can be seen within the midline axon bundle indicating that some contralateral projections persist despite commissure fusion (Jacobs, 1993).

In embryos mutant for *rho* or *S*, both commissures were pioneered, although early abnormalities were evident: both commissures were closer together and were broader than wild-type commissures (Fig. 1D and E). In these mutants, the anterior and posterior commissures remained incompletely separated. The longitudinal tract was thinner than wild-type in *rho* mutant embryos (Fig. 1I) and was sometimes absent in *S* mutant embryos (Fig. 1J, arrowhead).

2.2. Fate of the midline glial cells in collapsed longitudinal and fused commissure mutants

The fate of the midline glia (MG) in collapsed longitudinal and fused commissure mutants was analysed with the enhancer trap line AA142. This enhancer trap expresses β-galactosidase (β-gal) specifically in the two anterior midline glia (MGA) and the two middle midline glia (MGM) which are present at the dorsal midline in stage 12/5 wild-type embryos (Fig. 2A). Beginning at stage 12/0, the MGM migrate posteriorly over the MGA thereby separating the anterior and posterior commissures (Klämbt et al., 1991). By middle stage 13, the MGA sit in front of the anterior commissure while the MGM reside between the anterior and posterior commissures at the dorsal midline (Fig. 2B). In stage 15 ven-

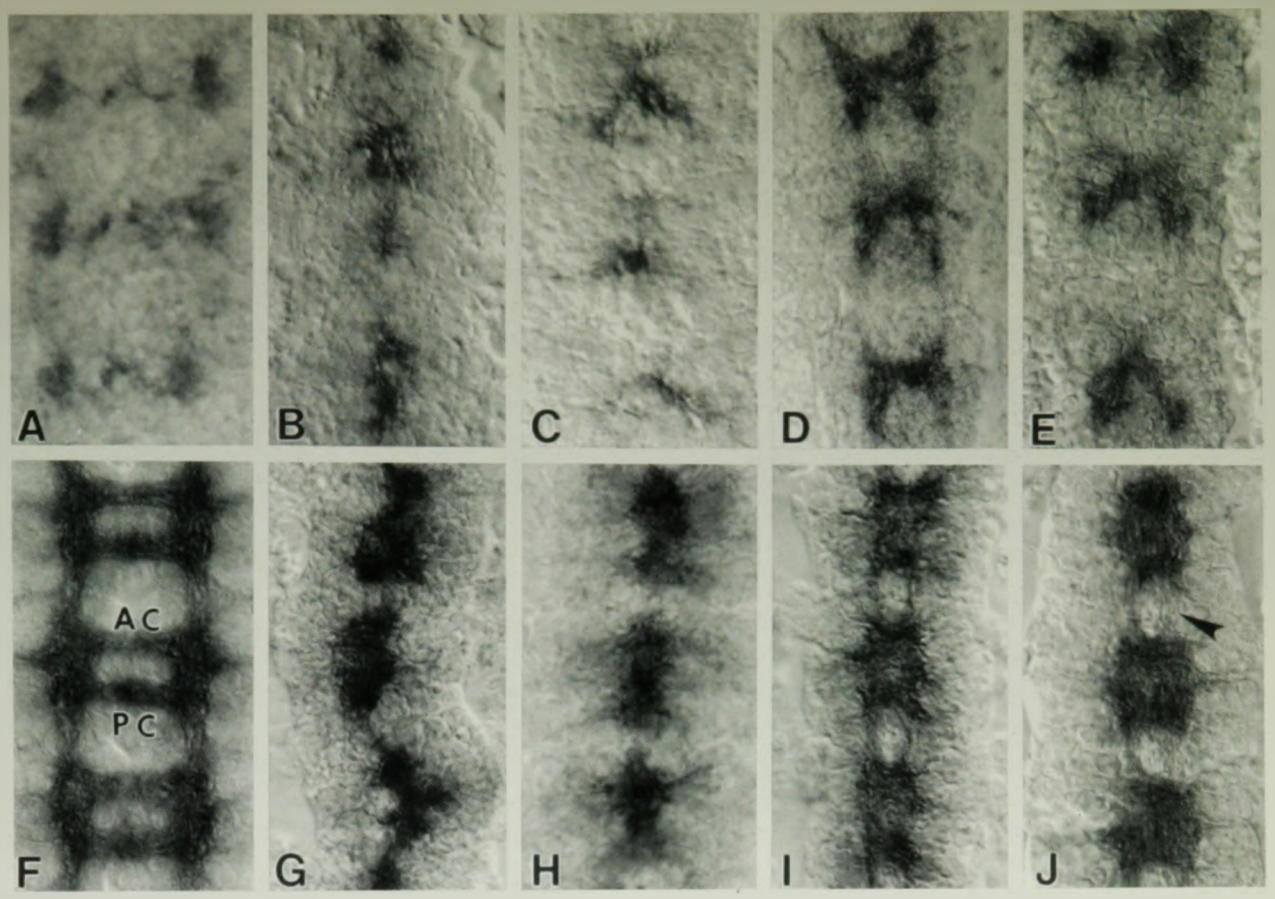

Fig. 1. Development of the commissures in collapsed longitudinal and fused commissure mutants. CNS axon tracts were visualised with the monoclonal antibody BP102 and HRP immunocytochemistry. Each column represents a single mutant. (A, F) Wildtype; (B, G) $sim^{B13.4}$; (C, H) sli^{IG107}; (D, I) rho^{38}; and (E, J) S^{1}. Each row presents a different stage of VNC development. Row 1 (A–E) axon and growth cone staining (BP102) at stage 12/3. Row 2 (F–J) axon and growth cone staining at stage 14. The pioneering of the anterior commissure occurs in stage 12/3 wild-type embryos (A), and by stage 14 both anterior (AC) and posterior (PC) commissures and longitudinal axon tracts form a ladder-like structure (F). In stage 12/3 $sim^{B13.4}$ mutant embryos the pioneering growth cones are collapsed at the midline (B) and by stage 14 the longitudinal axon tracts collapse at the midline (G). In stage 12/3 sli^{IG107} mutants the commissures are pioneered but are closer together (C) and by stage 14 the longitudinal tracts have collapsed at the midline (H). In stage 12/3 mutants of rho^{38} and S^{1} (D, E) the commissures are thicker and narrower and by stage 14 they remain incompletely separated (I, J). Anterior is at top.

tral nerve cords (VNCs) the MGA and MGM assume a final inverted L-shaped arrangement (Fig. 2C).

sim mutants did not express the enhancer trap AA142 (data not shown). In *sli* mutants, however, the MG were wild-type in both number and position during stage 12/5 (Fig. 2D). During formation of the anterior commissure (stage 12/3), some MG became displaced ventrally (Fig. 2E). However, not all MG were ventrally displaced and by stage 15 these cells were scattered from the dorsal to the ventral surface of the VNC (Fig. 2F).

In both fused commissure mutant embryos (*rho* and *S*), the MG were displaced dorsally in the VNC during stage 12 (Fig. 2G and J). In both mutants a reduction in midline glial cell number became apparent during stage 13 (Fig. 2H and K) and this reduction progressed until stage 14. At this stage, only three MG per embryo were generally present, they were smaller than normal and had been displaced dorsally (Fig. 2I) and rarely ventrally (Fig. 2L), out of the VNC. During stage 12/3 the MG were laterally dispersed from the midline in some segments in *rho* mutant embryos (data not shown). Midline glial dispersal was infrequent in *S* mutant embryos (data not shown).

We have performed a similar analysis of midline glial differentiation with an antibody to slit, a *sli* promoter reporter fusion construct (provided by S. Crews), and enhancer traps into the genes for *rhomboid* (provided by E. Bier) and *argos* (provided by C. Klämbt). All midline glial markers produced similar results (data not shown). Therefore, although the AA142 enhancer trap has not been characterised molecularly, it does provide an accurate reporter of midline glia specific gene activity.

Expression of the AA142 enhancer trap is a useful marker of the midline glial lineage but does not reveal whether the MG differentiate as glia in the collapsed and fused commissure mutants. We have used electron

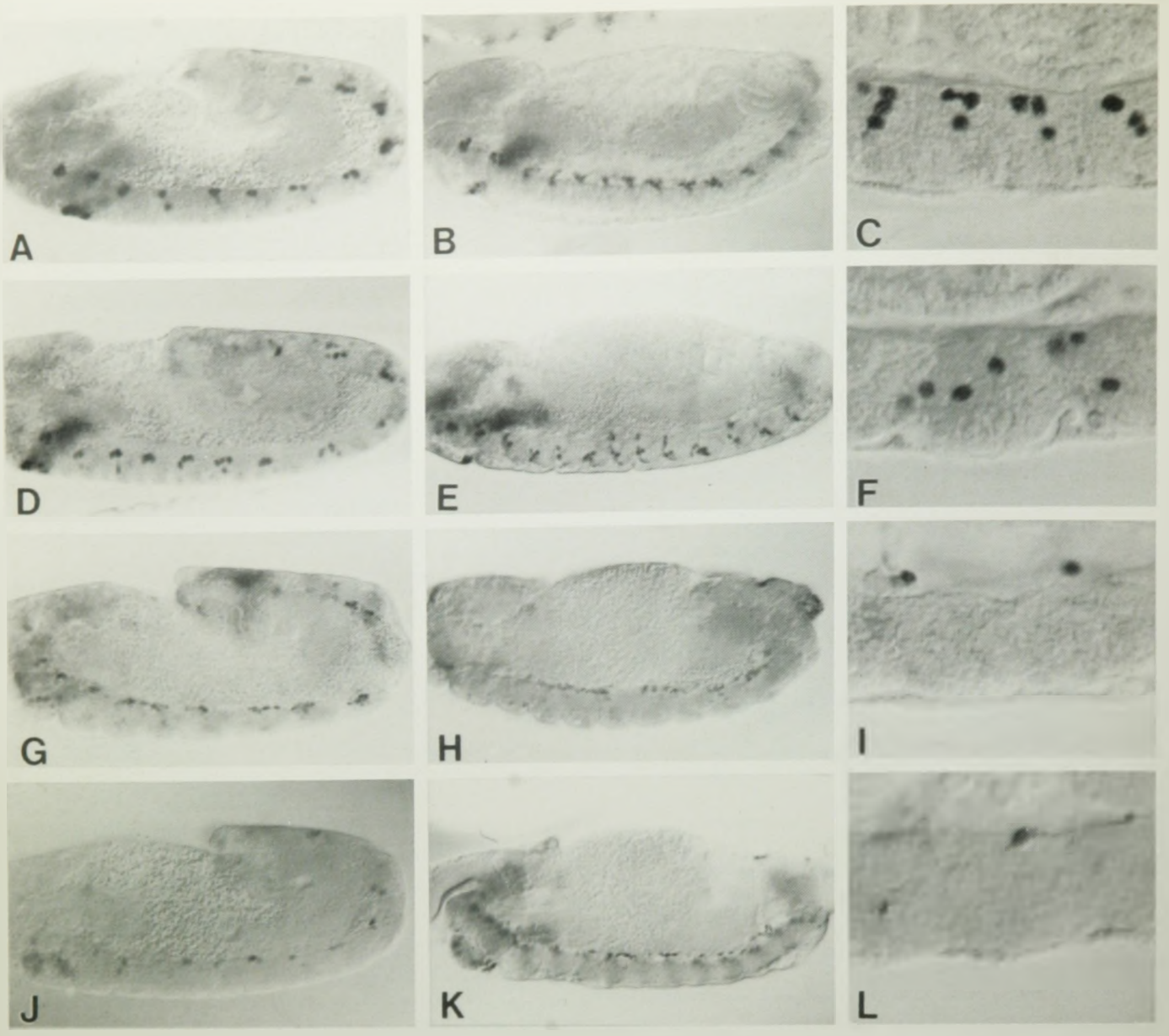

Fig. 2. Development of the midline glia in collapsed longitudinal and fused commissure mutants. All views are sagittal with anterior to the left. Each row represents a single mutant. (A–C) wildtype; (D–F) *sli*^{IG107}; (G–I) *rho*^{38}; and (J–L) S^1. Each column presents a different developmental stage: in column 1 (A, D, G, J), position of the midline glia during stage 12/5. In column 2 (B, E, H, K), MG distribution at stage 13. In column 3 (C, F, I, L) MG differentiation during stage 14 at higher magnification. All embryos carry the AA142 enhancer trap expressing β-galactosidase in the midline glia which was visualised with an antibody to β-gal. In wild-type stage 12/5 embryos the MG are at the dorsal midline (A) and at stage 13 the MGM migrate over the MGA to separate (the commissures (B). By stage 14 the MGA and MGM's appear in an inverted L-shape at the dorsal midline (C). In *sli*^{IG107} mutants the MGs reach the dorsal midline during stage 12/5 (D), become ventrally displaced during stage 13 (E) and by stage 14 are spread from the dorsal to ventral surface (F). In stage 12/5 mutants of both *rho*^{38} and S^1 the wild-type number of MG are present but they appear tightly clustered and displayed dorsally (G, J). Note the reduction in cell number from stage 13 (H, K) until stage 14 (I, L).

microscopy to assess midline glial differentiation in the two mutant groups. The MG were identified in wild-type and mutant embryos carrying the AA142 enhancer trap with a substrate for β-galactosidase that produces an electron opaque product. In stage 14 wild-type embryos the MG enwrap the commissures at the VNC midline (Fig. 3A, arrow). In *sli* mutants of the same age, differentiated MG were also found in contact with both longitudinal and contralaterally projecting axon bundles (Fig. 3B). Although ventrally displaced, these MG showed many aspects of glial differentiation, including an electron lucent cytoplasm, extensive endoplasmic reticulum and lamelipodial contact with axon surfaces (Jacobs and Goodman, 1989a). Differentiated MG were present in stage 16 *sli* mutants, but were absent in similarly aged *rho* mutants. Cell death of MG in *rho*

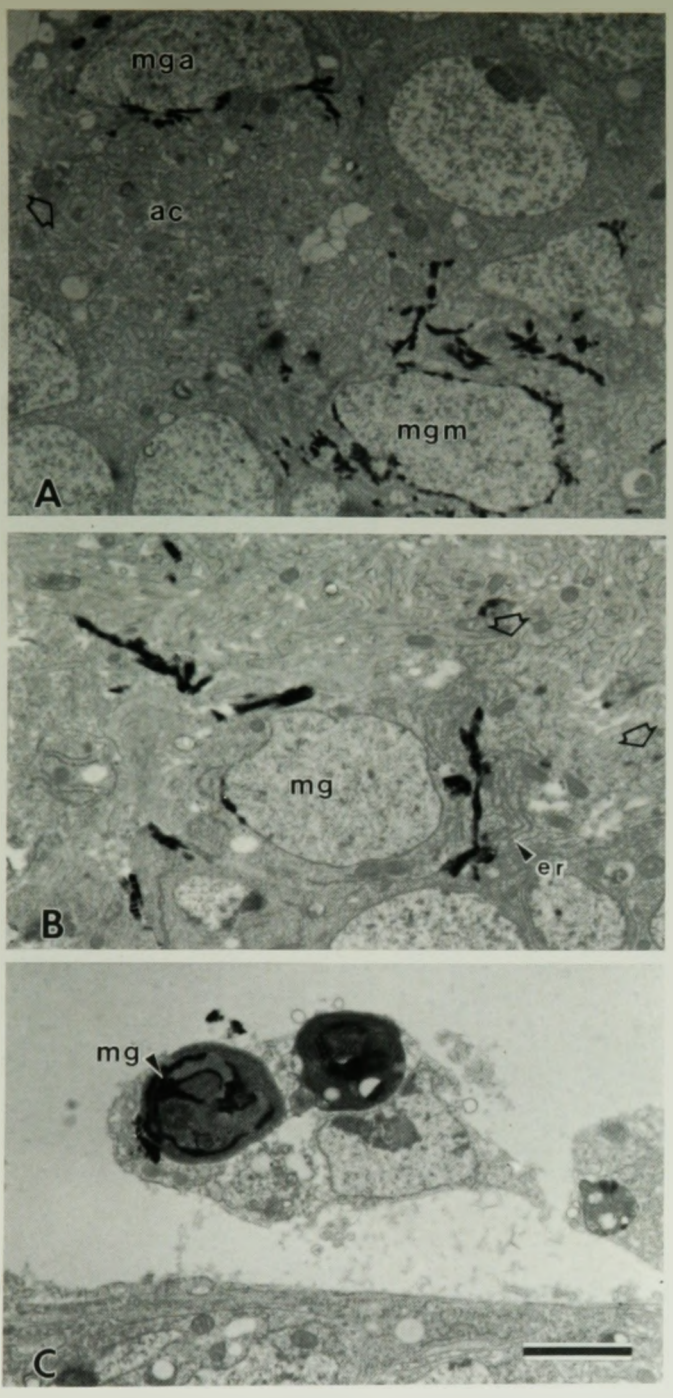

Fig. 3. Differentiation of the midline glia in wildtype, *rho*^{38} and *sli*^{IG107} mutants. β-Galactosidase activity is detected as electron dense precipitate in the MG cell bodies in wild-type (A), *sli*^{IG107} mutant (B) and *rho*^{38} mutant (C) VNCs. In (A), both the MGM and MGA are seen. Lamellipodial processes are identified with an open arrow. MG in *sli*^{IG107} mutant embryos (B) also show lamellipodia (open arrow) and extensive endoplasmic reticulum (er). In a *rho*^{38} mutant (C), the nucleus of the apoptotic MG cell is engulfed by a macrophage on the dorsal surface of the VNC. All panels show sagittal views of stage 14 VNCs. Scale bar: 2 μm.

mutant embryos was observed as early as stage 12/3 and possessed the morphological characteristics of apoptosis (Abrams et al., 1993; Wyllie, 1980). By stage 14 most apoptotic bodies had been expulsed from the VNC and were located within macrophage-like cells (Fig. 3C). This is consistent with the observed decline in the number of MG in *rho* mutants, assayed with AA142 and with slit antibody. Identical changes in midline glial cell apoptosis and slit production were also found in *S* mutant embryos (data not shown).

In contrast to *slit*, which has no commissures, midline glial differentiation and survival were more severely affected in the fused commissure mutants, despite their less dramatic commissural phenotype. If midline glial differentiation is not sufficient for commissure formation, and midline glial survival not necessary for commissure survival, it is likely that other MEC cell types contribute significantly to the development of VNC midline structures. The possible involvement of other MEC lineages in perturbed midline morphogenesis was therefore investigated using MEC lineage-specific molecular markers.

2.3. Fate of the VUM neurons in collapsed longitudinal and fused commissure mutants

There are six ventral unpaired median neurons (VUMs) located behind the posterior commissure at the ventral midline of the mature embryonic VNC. This neuronal lineage arises from the mesectoderm and maintains close contact with the MGM during commissure separation (Klämbt et al., 1991). We have followed the fate of the VUMs in collapsed and fused commissure mutants using the enhancer trap T13.

The enhancer trap T13 is expressed in six neurons which are arranged dorsally to ventrally in wild-type stage 12/5 VNCs (Fig. 4A). During stage 13, T13 is expressed in two well organised rows of neurons which begin to migrate ventrally (Fig. 4B). By stage 14, β-gal continues to be expressed in the VUM neurons at the ventral midline as well as in 48 lateral VNC neurons in each segment (Fig. 4C).

In *sim* mutant embryos, T13 was expressed in three ventrally displaced cells within each segment of the VNC (stages 12 and 13, Fig. 4D and E). The absence of these ventrally displaced cells from the midline of stage 14 *sim* mutants suggests that these cells had died (Fig. 4F, arrow). The cells of the lateral VNC that expressed T13 in *sim* mutants were reduced in number relative to wild-type and had been displaced further laterally. In contrast, we did not observe abnormalities in VUM neuron number and position in stage 12 embryos of *sli* mutants (Fig. 4G). However, during stage 12/3, the dorsal-most VUM neurons lost contact with the dorsal midline and subsequently were more ventrally positioned at stage 13 relative to wildtype embryos (Fig. 4H). Despite this ventral displacement, the VUM neurons were wildtype in size and number at the ventral midline of stage 14 *sli* VNCs (Fig. 4I, arrow). As in *sim* mutants, T13-expressing cells of the lateral VNC were displaced further laterally in *sli* mutant embryos.

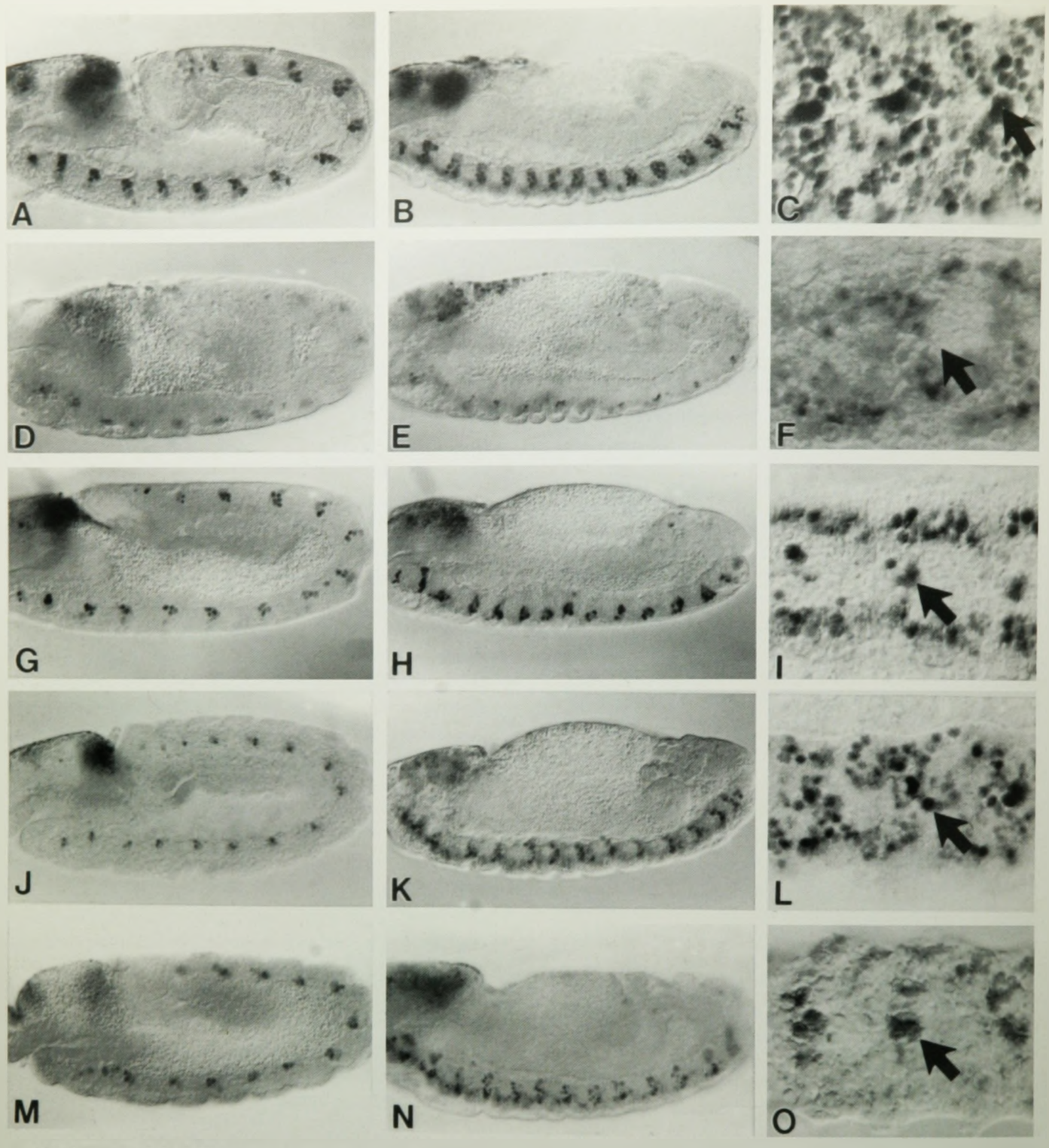

Fig. 4. Development of the VUM neurons in collapsed longitudinal and fused commissure mutants. Each row represents a single mutant. (A–C) wildtype; (D–F) *sim*$^{B13.4}$; (G-I) *sli*^{1G107}; (J–L) *rho*^{38}; and (M–O) *S*^{1}. Each column presents a different developmental stage. (A, D, G, J, M) stage 12 in sagittal view; (B, E, H, K, N) stage 13 in sagittal view; and (C, F, I, L, O) stage 14 in frontal view. All embryos contain the T13 enhancer trap which was visualised with an antibody to β-gal. During stage 11 T13 is expressed in 6 VUM neurons (A) and by stage 13 these neurons span from the dorsal to ventral surface of the VNC (B). In stage 14 embryos a cluster of VUM neurons is present at the ventral midline (arrow in C). From stage 11 until stage 13 in *sim*$^{B13.4}$ mutants, only a very small cluster of neurons express T13 which never migrate in the VNC (D, E). By stage 14 no T13-positive neurons are present at the ventral midline(arrow in F). In *sli*^{1G107} mutants the VUM neurons are present at the proper position during stage 11 (G) but are misplaced ventrally by stage 13 (H). These neurons are still present at the ventral midline at stage 14 (arrow, I). In both *rho* and *S*, note the reduction in cell number at stage 11 (J, M). During stage 13 half the wild-type number of neurons migrates ventrally (K and N) but by stage 14 are still present (arrow, L, O).

In stage 12 embryos of the fused commissure mutants (*rho* and *S*) half the wild-type number of T13-expressing cells remained at the dorsal midline (Fig. 4J and M). During stage 13, T13-expressing cells were arranged in a disorganized column but these cells did begin to migrate ventrally (Fig. 4K and N). By stage 14, the remaining VUM neurons were present at the ventral midline but were smaller than those of wild-type or *sli* mutant embryos (Fig. 4L and O). Studies with 22C10 confirmed that the remaining VUM neurons in fused commissure mutants were reduced in number compared with wildtype embryos (data not shown). These 22C10-expressing VUM neurons still established a medial axon fascicle, although pathfinding beyond the segment boundary was affected (data not shown). We have also noted a reduction in the number of lateral neurons that express T13 in both *rho* and *S* mutant embryos.

2.4. Fate of the MP1 neurons in collapsed longitudinal tract and fused commissure mutants

The MP1 neurons, which are MEC progeny, participate in the establishment of the longitudinal tracts (Jacobs and Goodman, 1989b; Grenningloh et al., 1991). The fate of the MP1 neuron was followed with the enhancer trap line P223. In addition to the MP1 neuron, P223 also labels the two MP2 neurons which arise from the MP2 neuroblast (see Doe, 1992). At mid-stage 13, three P223 expressing neurons flank the dorsal VNC midline (Fig. 5A). During late stage 13 of wild-type development the neurons begin to migrate ventrally and by stage 14 the soma of each MP1 neuron contacts its contralateral homologue at the midline (Fig. 5B). Their axons extend posteriorly within the longitudinal connectives of each segment (Fig. 5B, arrow).

The number of P223-expressing neurons in *sim* mutant embryos was under-represented (Fig. 5C and D). The remaining neurons were closer to their contralateral homologues than those of wildtype embryos but these neurons retained their wildtype dorsal position within the VNC. We interpreted this result to represent the absence of the MP1 neurons and the medial displacement of the two MP2 neurons. In contrast, in *sli* mutants the wildtype number of P223-expressing neurons was present during all embryonic stages of development. Yet during stage 12 all P223-expressing neurons were ventrally displaced in comparison to wild-type or *sim* mutant embryos (Fig. 5E). As in *sim* mutants, the P223-expressing neurons in *sli* mutants were closer together at the midline (Fig. 5F). These results for both *sim* and *sli* have been confirmed with the monoclonal antibody 22C10, which labels the MP1 and MP2 neurons (Grenningloh et al., 1991).

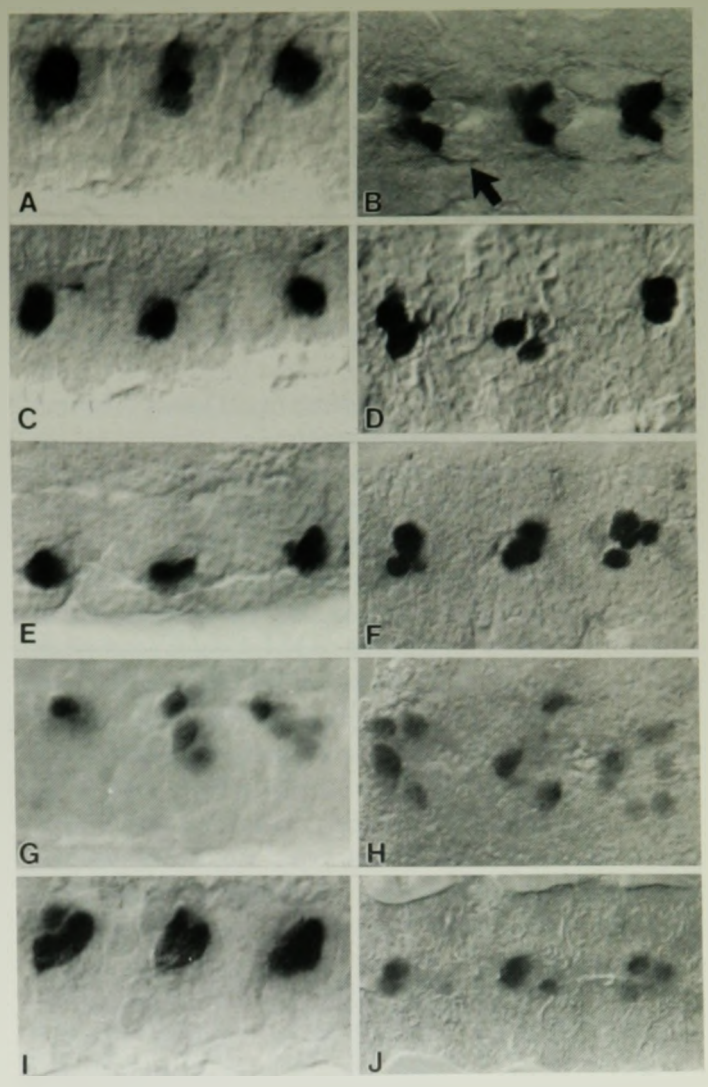

Fig. 5. Development of the MP1 neurons in collapsed longitudinal tract and fused commissure mutants. All embryos contain the enhancer trap P223 which is expressed in MP1, dMP2 and vMP2 neurons and was detected using an antibody to β-gal and HRP cytochemistry. Each row represents a single mutant. (A, B) wildtype; (C, D) $sim^{B13.4}$; (E, F) sli^{IG107}; (G, H) rho^{38}; and (I,J) S^{1}. Each column presents a different view of a different developmental stage. Column 1 (A, C, E, G, I) sagittal views of stage 13 VNCs. Column 2 (B, D, F, H, J) frontal views of dissected stage 14 VNCs. In wild-type embryos the MP1, dMP2 and vMP2 neurons are located at the dorsal midline at stage 13 (A) and by stage 14 the posteriorly projecting axons of MP1 and dMP2 can be visualised (B, arrow). In $sim^{B13.4}$ mutants only 1/3 of the wild-type neurons are present at the dorsal midline (C) and by stage 14 are fused at the midline (D). In stage 13 sli^{IG107} mutants the wild-type number of neurons is present but ventrally displaced (E). At stage 14 all neurons are still present but are fused as in $sim^{B13.4}$ mutants (F). In rho^{38} mutants the neurons are all present but are disconnected from each other (G) and by stage 14 this separation has grown (H). In S^{1} mutants, the P223 neurons appear wild-type at stage 13 (I) but by stage 14 there is a reduction in their number (J).

In stage 13 VNCs of the fused commissure mutant, *rho*, some P223-expressing neurons lost contact with one another and were occasionally reduced in number (Fig. 5G). By stage 14 all P223 neurons in *rho* mutants had lost contact with each other and became laterally dispersed from the midline (Fig. 5H). In contrast, in *S* mutant embryos P223-expressing neurons were wildtype in number and position at stage 13 (Fig. 5I). In this mutant, a reduction in cell number and size, as compared to wildtype, was observed by stage 14 (Fig. 5J).

2.5. Fate of the median neuroblast in collapsed longitudinal and fused commissure mutants

The *engrailed* (*en*) segment polarity gene product is expressed in a number of neurons in the posterior region of each segment in the embryonic CNS (Patel et al., 1989). In the mesectoderm, this includes the MNB and its neuronal and glial progeny. We have used a monoclonal antibody to the en protein to follow the fate of the MNB and its progeny in collapsed longitudinal and fused commissure mutants. In wild-type embryos, six large engrailed labeling (*en*+) neurons were detected at the ventral midline of stage 14 VNCs (Fig. 6A). There was no midline *en* expression in *sim* mutant embryos (Fig. 6B). In *sli* mutant embryos, the MNB progeny were wildtype in number and randomly displaced about the midline (Fig. 6C).

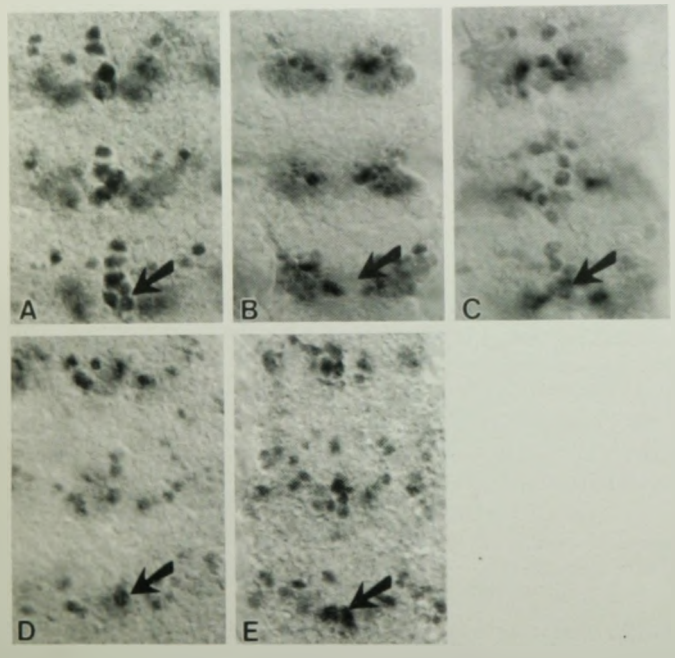

Fig. 6. Fate of the MNB in collapsed longitudinal and fused commissure mutants. The MNB and its progeny were identified in wildtype (A) and in homozygous mutant embryos of $sim^{B13.4}$ (B), sli^{1G107} (C), rho^{38} (D) and S^{1} (E) by using a monoclonal antibody to the engrailed protein and HRP cytochemistry. All panels show dissections of stage 14 VNCs (ventral view) with anterior at the top. In wild-type embryos 6 en^{+} neurons are present at the midline (A, arrow). Mutants of $sim^{B13.4}$ (B) at stage 12 have no en^{+} neurons present at the midline (arrow). In mutants of *sli*, *en*+ cells are present, but displaced (arrow, C). In both mutants of rho^{38} (D) and S^{1} (E) there is a reduction of en^{+} neurons at the midline (arrow).

In both fused commissure mutants, the *en*+ neurons were under-represented at the midline (Fig. 6D and E), raising the possibility that some of the MNB progeny require *rho* and *S* function for normal differentiation.

3. Discussion

We have traced the differentiation of individual MEC lineages in mutations that perturb morphogenesis of the commissures of the *Drosophila* VNC. Although previous studies associate defects in MEC cell differentiation with collapsed longitudinal and fused commissure CNS phenotypes (Nambu et al., 1990; Klämbt et al., 1991) the fate of individual MEC lineages through development in these mutations had not been determined. This study shows that multiple lineages are affected by the four mutations examined. In addition, it is clear that commissure development is influenced by many MEC cell types, and the mutant commissure phenotypes seen here represent the summation of morphogenetic influences of different MEC lineages through development.

3.1. Collapsed longitudinal tract mutants

Mutations in the genes *sim* and *sli* result in a collapse of the longitudinal connectives at the midline. Previous studies have shown that the *sim* gene is required for the expression of many midline genes and is considered a master regulator of MEC specific transcription (Nambu et al., 1990). The *sli* gene encodes a multi-domain secreted protein which has homology to molecules involved in protein-protein interactions (Rothberg et al., 1990). The *sim* and *sli* genes are co-expressed in the MECs from gastrulation onwards. After stage 13, expression of both become restricted to the MG cells (Crews et al., 1988; Rothberg et al., 1988).

It has been suggested that the collapsed longitudinal tract phenotype of *sim* mutants may be due to a lack of *sli* protein (Nambu et al., 1990). Although both *sim* and *sli* mutants result in identical CNS axon tract phenotypes, the ontogeny is very different. Collapse of midline structures, including commissures, lags in *sli* mutant embryos relative to *sim* mutant embryos. Previous studies show that the MEC lineages in *sim* mutants fail to complete their final division and do not migrate into the VNC layer (Nambu et al., 1991). We have confirmed this specifically for the VUM neuron precursors since expression from the T13 enhancer trap persisted in *sim* mutants. The VUM precursors exist at stage 11, but they do not survive in *sim* mutants. In contrast, all MEC lineages in *sli* mutants were represented in normal numbers and in normal position at least until stage 12/3, which is the beginning of commissure formation.

Lineage detection using the enhancer trap P223 revealed an additional difference in the cellular defects in *sim* and *sli* mutant embryos. This line labels the MP1 neurons as well as the non-mesectodermally derived

MP2 neurons. The axons from these neurons fasciculate and pioneer the first intersegmental axon fascicle (Jacobs and Goodman, 1989b). The MP1 neurons were absent in *sim* mutants, and the P223-expressing cells were likely to be the MP2 neurons. In the absence of the MP1 neurons, the MP2 neurons maintained their dorsal-ventral position in *sim* mutants. In contrast, in *sli* mutants all P223-expressing neurons were ventrally displaced. It is therefore possible that adhesion between the MP1 and MP2 neurons result in the ventral displacement of the MP2 neurons along with the MP1 neurons. Other neurons adjacent to the midline, such as RP2 and aCC (assessed with antibodies 22C10 and to protein even-skipped) are not similarly displaced in *sli* mutant embryos, suggesting that the ventral displacement of the MP2 neurons is specific (M.J.S., unpublished observations).

We observed a significant decrease in the number of neurons that express T13 in the lateral CNS of *sim* mutant embryos. These results indicate that some lateral neurons may indirectly depend on the presence of MEC lineages for proper development. Ventral ectodermal formation is also aberrant in *sim* mutants, suggesting that epidermal development depends on signals originating from the MEC progenitors (Kim and Crews, 1993).

What is the function of *sli* in MEC cell development? In contrast to previous reports, our lineage analyses in *sli* mutants suggest that *sli* is not necessary for the survival or differentiation of any of the MEC lineages assessed (Klämbt et al., 1991; Rothberg et al., 1990). Combining enhancer trap lineage detection and ultrastructural analysis enabled us to establish that differentiated MG ensheath axons in stage 14 *sli* mutant VNCs. Rothberg et al. (1990) propose an early and late function for *sli* in VNC development. In early development, *sli* is detected in many MEC cells. Slit protein is also found on cardioblasts during heart formation and at points of muscle attachment to the ectoderm (Rothberg et al., 1990). This pattern of expression is suggestive of a role in cell organisation or adhesion. In the nervous system, *sli* expression is required to maintain the cytoarchitecture of MEC lineages. During later embryogenesis, *sli* expression is restricted to the MG cells and the axons that bind secreted *sli* protein to their surface. We note that axon fasciculation and adhesion of axons to the MG cells is not severely disrupted in *sli* mutant embryos (Fig. 3B). Slit function at this stage remains to be elucidated.

3.2. *Fused commissure mutants*

Previous studies of MEC cell lineages in fused commissure mutants revealed arrested migration of the midline glial cells (Klämbt et al., 1991). On this basis a correlation between MG differentiation and commissure morphogenesis was suggested. We report that all MEC lineages we have examined are perturbed in *rho* and *S* mutant embryos. Two neuronal lineages, such as the VUM and MNB progeny are under-represented before a midline glial cell defect is detectable. It is likely that abnormalities in the development of neuronal MEC lineages contribute to the fused commissure phenotype.

The survival of MP1 beyond stage 13 was reduced in both commissure fusion mutants. The phenotype of *rho* differs from *S* in that the MP neurons lose contact with one another by stage 12/3. Other MEC cells also show lateral dispersion, but to a lesser extent. Ultrastructural analysis shows that *rho* mutant MECs adhere normally to other VNC cells (unpublished observations). This defect is not a general loss of adhesiveness in MECs, but represents a loss of neighbour to neighbour contiguity involving MECs, suggesting that *rho* is necessary to maintain adhesion between MEC neighbours.

We find that the midline glial lineage marker, AA142, shows normal expression in *rho* and *S* mutant embryos prior to stage 12/3. This suggests that these genes are not required for MG determination. However, we have found that apoptotic profiles of MG are present during stage 12/3 and by middle stage 13 the MG no longer express the midline glial protein *slit* (unpublished observations). We have confirmed that the MG die by light and electron microscopic analysis. These findings support the hypothesis that lack of MG function in *rho* and *S* mutants can result in a failure of commissure separation. These data also reveal that a lack of sli protein after stage 13 does not result in the collapsed longitudinal tract phenotype characteristic of the *sli* mutant.

Recent studies provide evidence for a strong interaction between *rho* and *S* during wing vein development (Sturtevant et al., 1993). Strong suppression of heat-shock-*rho* phenotypes by *S* mutations suggests it is possible that *rho* might mediate adhesion by binding the *S* product. Since both proteins contain putative transmembrane domains and are co-localised during development, they may interact physically during a putative signal transduction event. Transcripts from both the *rho* and *S* genes are co-expressed in the MEC cells and adjacent ventral ectoderm during and after gastrulation (Bier et al., 1990; Kolodkin et al., in press). Our results show that the fate of the MEC lineages in *rho* and *S* mutants are very similar, supporting the hypothesis that these two genes may participate in a common pathway governing MEC differentiation.

Mutations that disrupt the morphogenesis of the VNC commissural tracts affect the proliferation, survival and morphogenesis of a number of MEC lineages. The *sli* mutation is distinct from the others studied here because MEC differentiation and survival are not perturbed. The collapsed longitudinal tract phenotype in *sli* mutant embryos results from a displacement of all MEC lineages from their normal positions within the VNC. Even though the MECs differentiate, their absence from

normal position is equivalent to a loss of their function in a morphogenetic event like commissure formation. In dramatic contrast, position of the MECs may be near normal as in the mutation *commissureless* (*comm*), and yet no commissures form (Seeger et al., 1993). The presence of the MG in *slit* and *comm* mutants is not sufficient to establish commissures. Other aspects of midline glial phenotype such as their position, or cell surface proteins like slit may be necessary. Observations reported here suggest a contribution by other MEC lineages to commissure establishment and morphogenesis. In both *S* and *rho*, defects in all MEC neuronal lineages precede the midline glial apoptosis. The possible role of other MEC lineages in directing commissure morphogenesis requires further examination. This issue may be addressed by cell ablation experiments.

4. Experimental Procedures

4.1. Drosophila strains

Wildtype Drosophila were Oregon-R. The following mutant alleles were used: $sim^{B13.4}$ (a protein null allele provided by S. Crews, Nambu et al., 1991), sli^{IG107} was originally isolated in a screen for abnormal cuticle patterns and is a protein null allele (Nusslein-Volhard et al., 1984; Rothberg et al., 1988), rho^{P38} (generated from excision of a p-element insertion into 62A, Freeman et al., 1992), and S^{1} (Bridges and Morgan, 1919). The *slit* and *Star* alleles were obtained from the Indiana Stock Centre.

4.2. Egg collection and embryo staging

Embryos were collected at 25°C on apple juice agar plates. Embryos were staged according to Campos-Ortega and Hartenstein (1985) incorporating the subdivisions of stage 12 introduced by Klämbt et al., (1991).

4.3. Cell fate analysis in mutant embryos

The following enhancer trap lines were used to follow the fate of individual MEC cells in various mutant backgrounds; AA142, a P element insertion at 66D, labels the midline glia (MG) (Klämbt et al., 1991), T13, a P element insertion at 60F labels the VUM neurons (Klämbt et al., 1991), P223, a P-element insertion in 98D, marks the MP1 and MP2 progeny and was generated by Y. Hiromi. Each enhancer trap was incorporated into various mutant backgrounds using standard genetics and stable mutant lines were identified by X-gal immunocytochemistry (Jacobs et al., 1989). Homozygous mutant embryos were identified by lack of expression from the second (*CyO* P[ry^{+}, *elav/lacZ*]) and third (TM3 P[ry^{+}, *ftz/lacZ*] balancer chromosomes. Stable mutant lines were checked for proper mutant phenotypes using the monoclonal antibody BP102 (below).

4.4. Antibodies and immunocytochemistry

Embryos containing a P[lacZ] element were stained with a monoclonal antibody against β-galactosidase (Sigma) and detected using HRP cytochemistry according to Patel et al., (1987) and Grenningloh et al. (1991). The monoclonal antibodies BP102, engrailed (4D9) and BP104 were kindly provided by C. Goodman (Patel et al., 1989). Antibody to the even-skipped protein was provided by M. Frasch (Frasch et al., 1987). The antibody 22C10 was kindly provided by S. Benzer (Fujita et al., 1982). Slit antibody was provided by S. Artavanis-Tsakonas (Rothberg et al., 1988).

Electron microscopy was performed as described by Jacobs (1993), employing 5-bromo 3-indolyl-β-D-galactopyranoside as a β-galactosidase substrate.

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CHAPTER 2

Apoptosis of the midline glia during *Drosophila* embryogenesis: A correlation with axon contact.

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SUMMARY

We have examined cell death within lineages in the midline of *Drosophila* embryos. Approximately 50% of cells within the anterior, middle and posterior midline glial (MGA, MGM and MGP) lineages died by apoptosis after separation of the commissural axon tracts. Glial apoptosis is blocked in embryos deficient for *reaper*, where greater than wildtype numbers of midline glia (MG) are present after stage 12. Quantitative studies revealed that MG death followed a consistent temporal pattern during embryogenesis. Apoptotic MG were expelled from the central nervous system and were subsequently engulfed by phagocytic haemocytes. MGA and MGM survival was apparently dependent upon proper axonal contact. In embryos mutant for the *commissureless* gene a decrease in axon-glia contact correlated with a decrease in MGA and MGM survival and accelerated the time course of MG death. In embryos mutant for the *slit* gene, MGA and MGM maintained contact with longitudinally and contralaterally projecting axons and MG survival was comparable to that in wildtype embryos. The initial number of MG within individual ventral nerve cord segments was increased by ectopic expression of the *rhomboid* gene, without changing axon number. Extra MGA and MGM were eliminated from the ventral nerve cord by apoptosis to restore wildtype numbers of midline glia. Ectopic *rhomboid* expression also shifted MGA and MGM cell death to an earlier stage of embryogenesis. One possible explanation is that axon-glia contact or communication promotes survival of the MG and that MG death may result from a competition for available axon contact.

Introduction

Cell death is a major feature during development of the central nervous system (CNS) in both vertebrates and invertebrates (Oppenheim, 1991; Bate et al., 1981; Truman et al., 1992). Although death has been noted in both neuronal and glial cell populations, neuronal cell death has received closer attention (Oppenheim, 1991). There is growing evidence, however, that glial cell number is also regulated by differential cell proliferation and cell death, leading to speculation that glial cell number may be regulated by mechanisms similar to those described for neuronal cell number (Raff et al., 1993).

In some neuronal populations, cell death results from the matching of cells to their efferent targets and is believed to operate by a trophic mechanism (Hamburger and Oppenheim, 1982). The deletion of targets result in reductions in the survival of the innervating population of neurons (Oppenheim, 1985; Hamburger and Oppenheim, 1982). Considerable evidence indicates that trophic support may also originate from afferent inputs, glia, and the extracellular matrix (Okado and Oppenheim, 1984; Sohal, 1986; Furber et al., 1987; Walicke, 1989; Johnson and Deckwerth, 1993), and is modulated by both neurotrophins and afferent activity (Cunningham et al., 1979; Sendtner et al., 1990; Yan et al., 1992; Neff et al., 1993).

Death of ensheathing-type glia such as oligodendroctyes and Schwann cells has also received attention in the developing nervous systems of vertebrates (Knapp et al., 1986; Barres et al., 1992a; Raff et al., 1993; Louis et al., 1993; Doyle and Colman, 1993). Survival of newly formed oligodendrocytes in culture can be promoted by growth factors produced by their neighbour astrocytes or by growth factors present in the optic nerve (Louis et al., 1993; Barres et al., 1992b). In addition, there is evidence that glial-axonal contact plays a role in the survival of oligodendroglia. Oligodendroglia degenerate in neonatally transected rat optic nerves (David et al., 1984; Raff et al., 1993). Raff et al., (1993) proposed a model describing an early dependence of oligodendroglia on growth factors for differentiation followed by a dependence on axon contact for survival. The limited availability of axon-derived survival signals would serve to match the number of oligodendroglia to the length of axons requiring myelination. Glial cell death in insects contributes to the remodeling of the central nervous system (CNS) during metamorphosis. For example, glial cell death occurs in the outer layer of the perineurium and the glial cover of the larval and pupal neuropil in the thoracic ganglia of *Manduca sexta* (Cantera, 1993). Natural glial cell death has been observed in metamorphic adults of the insect *Manduca sexta* where afferent input from antennal axons is required for the survival of glial cells in the glomeruli (Tolbert and Oland, 1989; Oland and Tolbert, 1987). The midline glia (MG) of the midline of the *Drosophila* nerve cord participate in the morphogenesis of the commissural tracts during early embryogenesis (Jacobs and Goodman, 1989; Klämbt et al., 1991). Early MG survival and commissure morphogenesis requires the function of the *rhomboid* and *Star* genes (Sonnenfeld and Jacobs, 1994). Here we report that subsequent to their function in commissure morphogenesis,

50% of the *Drosophila* embryonic MG in normal embryos undergo apoptosis. The extent and time-course of MG apoptosis is altered in mutant and transgenic embryos which modify the degree of commissural axon contact with the MG. One possible explanation is that CNS axons may play a role in regulating the number of MG that survive to hatching. MG number may therefore be regulated during later embryogenesis by trophic mechanisms.

MATERIALS AND METHODS

Stocks and enhancer traps

AA142, a p-element insertion at 66D, labels the midline glia (MG) (Klämbt et al., 1991). The promoter fusion construct, *slit-lacZ* 1.0, is described in Wharton and Crews (1993) and was kindly provided by S. Crews. The *rhomboid*^{P38} (*rho*^{P38}) allele was generated from excision of a p-element insertion into 62A (Freeman et al., 1992). The *slit*^{IG107} (*sli*^{IG107}) allele (Nüsslein-Volhard et al., 1984; Rothberg et al., 1988) and two deficiencies that uncover *reaper* (*Df(3L)WR4* and *Df(3L)WR10;* White et al., 1994) were obtained from the Indiana stock centre. The *commissureless*^{1} (*comm*^{1}) allele was kindly provided by C. S. Goodman (Seeger et al., 1993). Flies carrying a HS-*rho* p-element on the X chromosome (HS-*rho*-1B) were generously provided by E. Bier (Sturtevant et al., 1993).

Embryo staging

Embryos were staged according to Campos-Ortega and Hartenstein (1985), incorporating three subdivisions of stage 12 (Klämbt et al., 1991).

Immunocytochemistry

Embryos containing a P[*lacZ*] element were incubated with a monoclonal antibody against β-galactosidase (Sigma) which was detected using HRP cytochemistry according to Patel et al. (1987) and Grenningloh et al. (1991). A mouse polyclonal antibody to *Drosophila* peroxidasin (a-X) was used to identify phagocytic haemocytes (kindly provided by L. Fessler; Abrams et al., 1993;

Tepass et al., 1994) which were visualized by HRP cytochemistry. The monoclonal antibody BP102 was used to visualize commissural and longitudinal CNS axon tracts (kindly provided by C. S. Goodman).

β-galactosidase histochemistry using X-gal (5-bromo-4-chloro-3-indolyl-B-C-galactopyranoside) and bluo-gal (halogenated indolyl-β-D-galactoside) was performed according to Jacobs et al., (1989) except that embryos were reacted for 12-16 hours at 18°C. For combined immunolabeling with X-gal and anti-peroxidasin, embryos were fixed in 4% formaldehyde.

Light and electron microscopy

Embryos containing AA142 and *sli-lacZ* 1.0 and reacted with X-gal were embedded and processed for light level microscopy according to Jacobs et al., (1989). Embryos reacted with bluo-gal were processed for electron microscopy as previously described (Jacobs, 1993).

Heat shock protocols

Wildtype and HS-*rhomboid* (*rho*) embryos carrying the AA142 enhancer trap were collected on apple juice agar plates for three hours at 25°C. These embryos were then aged for two hours at 25°C and the plates were transferred to a 37°C water bath for one hour. The embryos were then allowed to develop for an additional five hours at 25°C or twelve hours at 18°C and were then processed for anti-β-galactosidase and BP102 staining. The ectopic expression of *rho* was verified by examining the wing phenotype (Sturtevant et al., 1993). To determine the effects of late ectopic *rho* expression, wildtype and HS-*rho* embryos expressing the AA142 enhancer trap were collected for three hours, aged for six

to eight hours at 25°C and the plates were transferred to a 37°C water bath for one hour. These embryos were allowed to develop at 25°C for one hour and were then processed for anti-β-galactosidase and BP102 staining.

RESULTS

Variations in midline glia numbers and position after axon tract establishment

The midline glial lineage is derived from the mesectoderm of the *Drosophila* embryo. Three precursors divide once during stage 9 to produce three pairs of midline glia (MG), identified by position as anterior, middle and posterior (MGA, MGM and MGP, respectively) (Jacobs and Goodman, 1989; Klämbt et al., 1991). To determine glial function after commissure separation we have used enhancer traps and a reporter construct to identify cells within the midline glial lineage during later embryogenesis. The enhancer trap AA142 is expressed in the MGA, MGM and weakly in the MGP in the embryonic CNS (Klämbt et al., 1991). The reporter fusion construct for the *slit* gene (*sli-lacZ*) is expressed in many MEC lineages prior to stage 13. Expression of *sli-lacZ* is restricted to the MGA and MGM during stage 15 and therefore is a useful marker of mature MG (Wharton and Crews, 1993).

We have observed variations in the position and number of MGA and MGM between ventral nerve cord (VNC) segments and embryonic stages, assessed with both AA142 and *sli-lacZ* 1.0 as shown in figure 1. Decreases in number and variations in position of the MGA and MGM were first observed during stage 13 and were present until the end of embryogenesis (Table 1; Figure

1). Quantitative analyses revealed a 21% decrease in the number of MGA and MGM from stage 13 until stage 17 of embryogenesis. There are no changes in MG number from stage 17 (3.0 +/- .52 cells/segment, n=190) through first and second instar larva (L1 3.2+/-.23 cells/segment, n=90). In 76.9% of segments more MGM than MGA survived while in 7.8% of segments more MGA than MGM survived. These results suggested that the MGM had a greater probability of survival than the MGA (Table 1). The MGM differs from the MGA because they contact axons of both the anterior and posterior commissure while MGA contacts anterior commissure axons only. Certain combinations of surviving MGA and MGM were not observed within ventral nerve cord segments. For example, no segments were found to contain only one or no MG. Of the scored segments containing two MG, 3% of all segments scored contained one MGA and one MGM, 1.9% of all segments scored contained no MGA and two MGM, while no segments contained two MGA and no MGM (Table 1).

β-galactosidase expression of AA142 in the two MGP was weak and transient and was excluded from cell counts in this analysis. The β-galactosidase staining in the MGP was diffuse during stage 13 (Fig. 1A) and was no longer detected after stage 14. The number of MGP was instead analysed using an enhancer trap (X55) that is expressed in the MGP, the ventral unpaired median neurons (VUMs) and the median neuroblast (MNB) (Klämbt et al., 1991). Two MGP arise from a midline glial precursor cell (Klämbt et al., 1991) and by stage 14 of embryogenesis there was an average of 1.6 MGP within ventral nerve cord segments (n=67) assessed with the X55 enhancer trap (Fig 2A). During stages 15 and 16 there was an average of 0.6 and 0.3 MGP within ventral nerve cord segments, respectively (n=100 and n=123; Fig. 2B). During stage 17 there was

an average of 0.04 cells within ventral nerve cord segments (n=178) while ventral nerve cord segments (n=169) in early first instar larvae contained no MGP. Ultrastructural studies confirm that there is a reduction in the number of MGP after stage 14 (Jacobs, unpublished observation).

These quantitative analyses revealed that there was a combined 50% decrease in the number of MG from stage 13 to stage 17 of embryogenesis. We therefore investigated the possibility that the MG undergo natural apoptosis in the midline during embryogenesis.

Midline glia die by apoptosis

Initiation of apoptosis is blocked in embryos deficient for *reaper* (White et al., 1994) We examined X55 and *sli-lacZ* 1.0 expression in embryos homozygous for *Df(3l)W4*, which uncovers *reaper*, to determine whether the reduction in MG number could occur when apoptosis was blocked. In stage 16 embryos, 3 to 5 X55 expressing MGP (Fig. 2C), and 9-10 *sli-lacZ* 1.0 (Fig. 2D) expressing cells were observed per segment. Small labeled profiles outside of the nervous system were not seen with either marker in *Df(3l)W4* embryos. These results indicate that the decline in MG number during development is blocked in *reaper* deficient embryos, and the deficiencies also result in a 60% increase in the number of cells expressing MG specific markers.

We used electron microscopy to gain ultrastructural confirmation of midline glia cell death. Midline glia were identified with a substrate for β-galactosidase (bluo-gal) in sagittal sections from wildtype embryos carrying the AA142 enhancer trap. The bluo-gal substrate forms rod-shaped crystals around the nuclei of surviving and apoptotic midline glia (Fig. 3; Sonnenfeld and Jacobs,

1994). Surviving MG in wildtype embryos ensheath commissural axon tracts and have characteristic ultrastructural features such as extensive *rough* endoplasmic reticulum and electron lucent cytoplasm (Fig. 3A). In wildtype embryos, midline glia labeled with the bluo-gal substrate were found in macrophages outside the dorsal surface of the ventral nerve cord(Fig. 3B, 3A). These expulsed MG displayed features of apoptosis including condensed nuclei, reduced cytoplasmic volume and increased electron density (Fig. 3B, 4A) (Abrams et al., 1993). The nuclei of apoptotic glia in embryos mutant for the *rhomboid* (*rho*) gene are also found in macrophages dorsal to the ventral nerve cord and possess similar apoptotic characteristics as the MG in wildtype embryos (Fig. 3C; Sonnenfeld and Jacobs, 1994).

To determine the time course of MG death during wildtype embryogenesis, we scored the number of whole-mount embryos containing apoptotic MGA and MGM from stage 12 until stage 17 using the AA142 enhancer trap as a cellular marker. Apoptotic MGA and MGM were first detected during stage 13 in 76.7% of scored embryos (n=34). During stage 14, 100% of embryos (n=57) contained apoptotic MG and by stage 17, 21.7% of embryos (n=23) contained apoptotic MG. AA142 embryos were double labeled with terminal transferase to identify when DNA fragmentation began (TUNEL, White et al., 1994). TUNEL labeled apoptotic glia first appear at stage 13, in rounded cells already outside of the CNS. We conclude that DNA fragmentation was detectable at the same time that apoptotic cells can be identified by histological means. The temporal pattern of apoptosis within the MGP lineage was determined using the X55 enhancer trap. By stage 14, 83.3% of embryos (n=6) contained X55 labeled apoptotic cells and by stage 15 100% of embryos (n=9) contained apoptotic cells. By stage 17, 50%

of scored embryos (n=16) contained X55 labeled apoptotic cells while no first instar larvae contained apoptotic cells (n=14). Although the X55 enhancer trap is also expressed in some midline neurons, other markers unique to those lineages (such as T13 and P223; Sonnenfeld and Jacobs, 1994) did not label apoptotic profiles.

Apoptotic midline glia are found in macrophages

In addition to the variation in MG position and number within ventral nerve cord segments of embryos, small AA142 and *sli-lacZ* expressing cells were present in the haemolymph space dorsal to the ventral nerve cord (Fig. 1A-D, arrowheads). These small displaced cells appeared to be contained within larger macrophage-like structures (Fig. 1A-D, 4A). Identical results were observed using the X55 enhancer trap for MGP identification (data not shown). Two lines of evidence suggested that the small displaced AA142, *sli-lacZ* and X55 expressing cells were MG that had died and were expelled from the VNC: (1) the proximity of most profiles to the midline (Fig. 1G,H, arrowheads; Fig 4A) and (2) the fact that MG that undergo apoptosis in embryos which are mutant for the genes *rho* and *Star* (*S*) appear in similar positions (Sonnenfeld and Jacobs, 1994).

To confirm that the b-galactosidase expressing cells in whole mount embryos were in macrophages we used an antibody to peroxidasin, which is an extracellular matrix protein produced and secreted by macrophages (Abrams et al., 1993; Tepass et al., 1994). Expulsed MG were found in macrophages that were recognised by anti-peroxidasin, (Fig. 4B-D). These expulsed MG were found in macrophages in various locations in the embryo including dorsal (Fig. 4B), lateral (Fig. 4D) and posterior to the ventral nerve cord (Fig. 4C). All

expulsed MG were located in macrophages outside the ventral nerve cord as determined by histology (Fig. 4A; n=15) and whole mount embryo analysis with the polyclonal antibody to peroxidasin (Fig. 4B-D; n=43).

Macrophages containing apoptotic MG were occasionally observed at considerable distances from the CNS, revealing mobility of the macrophages. Although we could not detect MG in the early stages of cell death by light microscopy, the fact that we rarely observed apoptotic MG within the ventral nerve cord suggests that they were expelled before nuclear condensation was complete. Apoptotic MG are also rarely found within the ventral nerve cord of embryos mutant for the *rhomboid* gene, suggesting that the expulsion of apoptotic MG before nuclear condensation may be a common occurrence (Sonnenfeld and Jacobs, 1994).

Possible non-autonomous regulation of MG survival

The identity of the surviving MG varied between embryonic ventral nerve cord segments and stages suggesting that a non-autonomous signal may be involved in MG survival. Axon contact has been proposed as a determining factor in the regulation of glial numbers in the rat optic nerve (Raff et al., 1993), and may explain some of the variation observed in the MG lineages. We therefore investigated the potential role of axon contact in regulating MG survival by analyzing MG fate in three contexts that altered MG-axon contact.

In *commissureless* (*comm*) mutant embryos, commissural axons rarely cross the midline of the ventral nerve cord so that MG have minimal contact with axons (Seeger et al., 1993; Fig. 5A, B). The highest frequency of apoptotic MG (assessed with AA142) was observed in stage 13 *comm* mutant embryos (n=16)

rather than stage 14 as observed in wildtype embryos. Apoptotic cellular profiles were no longer detected in stage 17 embryos (n=6). The temporal pattern of MG death in *comm* mutant embryos correlated with a 39% decrease in the number of MGA and MGM from stage 13 until stage 17 of embryogenesis, relative to a 21% decrease over the same period for wildtype (Fig. 6). Surviving MG became laterally displaced towards the longitudinal axon tracts (compare Figures 5A,and 5B; Seeger et al., 1993).

The relationship between the MG and the CNS axons is altered in embryos mutant for the *slit* (*sli*) gene (Rothberg et al., 1988). In *sli* mutants, commissural and longitudinal axon tracts are collapsed at the midline (Fig. 5C,D; Rothberg et al., 1988). We have previously demonstrated that the MG in *sli* mutant embryos differentiate and ensheath axons at the ventral midline (Sonnenfeld and Jacobs, 1994). The temporal pattern of MG cell death in *sli* mutant embryos (assessed with AA142) was similar to that in wildtype embryos. In *sli* mutant embryos there was a net 23% decrease in the number of MGA and MGM within ventral nerve cord segments from stage 13 to 17 compared to a 21% decrease in wildtype embryos over the same period (Figure 6).

It was possible to increase the number of MGA and MGM within ventral nerve cord segments by ectopic expression of the *rhomboid* (*rho*) gene. Transgenic flies carrying a p-element containing the heat shock (HS) promoter-70 fused to the *rho* gene (kindly provided by E. Bier; Sturtevant et al.,1993) were used to induce an increase in the number of MGA and MGM during embryonic development of the CNS. MG were identified using the AA142 enhancer trap (Figure 5E,F) and the *sli-lacZ* reporter (data not shown). Increases in the number of MGA and MGM were observed in stage 12 and stage 13 embryos that had

been subjected to a one hour heat shock during stages 9 and 10 of embryogenesis (Figure 5E; Figure 6). The increases in MG numbers were detected as early as the reporters are expressed. At stage 13 there was a 50% increase in the average number of MGA and MGM within ventral nerve cord segments (Figure 6). In embryos subjected to an identical heat shock but examined at later stages of development, the number of MGA and MGM declined towards the wildtype numbers of MG (Figure 5F; Figure 6). The onset of observed MG death in HS-*rho* embryos was shifted earlier to stage 12 compared to stage 13 in wildtype embryos. It was therefore possible that the extra MG died by apoptosis and was reflected by a 40% decrease in the number of MGA and MGM from stage 13 until stage 17 of embryogenesis. The extra MGA and MGM did not perturb formation or separation of the anterior and posterior commissural axon tracts (data not shown).

DISCUSSION

The midline glia are responsible for the morphogenesis of the anterior and posterior commissures during embryonic development. A subset of the midline glia migrate between and separate the anterior and posterior commissures during stage 12, and subsequently ensheath axons of the commissural tracts (Klämbt et al, 1991). In the current study, we have shown that 50% of the MG died by apoptosis after the commissural axon tracts were separated. The use of enhancer traps allowed us to detect MG during late stages of apoptosis due to perdurance of b-galactosidase. Apoptosis within the MGA, MGM and MGP lineages resulted in decreases in the number and variations in position of surviving MG within

embryonic ventral nerve cord segments after stage 13. The reduction of MG number by variable patterns of apoptosis represents one of the earliest examples of stochastic variation in cell fate in *Drosophila* development. Our analysis revealed that the MGM have a greater probability of survival within ventral nerve cord segments than either the MGA or MGP. These observations suggest that the MG are not equivalent in their developmental potential. Whether more MGM survive because they are necessary to stabilize commissure separation remains to be determined.

Apoptosis of the MG was blocked in embryos deficient for the gene *reaper*, previously established to be required for apoptosis (White et al, 1994). In the absence of apoptosis, there is an increase in MG number relative to the wildtype MG number. It is possible that these extra MG are derived from MECs that undergo apoptosis before glial specific reporters can detect them. Alternatively, the absence of apoptosis may alter cell interactions during determination, thereby diverting extra cells to the MG fate. We did not observe apoptotic profiles in embryos carrying enhancer traps expressing b-galactosidase in the ventral unpaired median neurons (VUM's) and the MP1 and MP2 neurons (enhancer traps T13 and P223, respectively) (Sonnenfeld and Jacobs, 1994). These observations suggest that cell death within the mesectoderm may be limited to the three midline glial lineages.

Death of identified neurons in the embryonic grasshopper ventral nerve cord can establish segmental specialisations and remove cells that have become obsolete (Bate et al., 1981). We did not find regional differences in MG survival between ventral nerve cord segments. However, the correlation between the

beginning of MG death and the separation of the anterior and posterior commissure suggests that fewer MG are required after this morphogenic event.

The MG are involved in commissure separation and also ensheath these axons during embryogenesis (Klämbt et al., 1991; Jacobs and Goodman, 1989). Perhaps MG survival after commissure morphogenesis is modulated by contact with the commissural axons they ensheath. We tested the hypothesis that axonal contact may be involved in MG survival by investigating three contexts where axon-glia contact had been altered. The decrease in contact between the MG and the commissural axons in *commissureless* (*comm*) mutant embryos was correlated with an 85% greater loss of cells from the MGA and MGM lineages. The decrease in axonal contact in *comm* mutant embryos was also correlated with an earlier peak in detectable apoptosis (stage 13) revealing that the MG may begin to respond to axon contact by late stage 12.

Although CNS axon tracts are abnormal in embryos mutant for the gene *sli*, the MG maintain axonal contact throughout embryogenesis (Sonnenfeld and Jacobs, 1994). Our analysis showed that in *sli* mutant embryos there was a decrease in the number of MGA and MGM (23%) comparable to wildtype MG decreases (21%) by the end of embryogenesis. It is possible that the collapse of CNS axon tracts toward the midline in *sli* mutants did not prohibit the axon-glial contact that may be involved in MG survival. Ectopic expression of the *rhomboid* gene during stage 9 and 10 increased the number of mesectodermal cells expressing MG specific genes. This increase was corrected between stages 12 and 17 by increased levels of apoptosis. Commissure morphogenesis was unaffected.

For all three situations, an alteration in the ratio of MGs to commissural axons is corrected by changes in the number of MGs that undergo apoptosis. The genes *sli*, *rho* and *comm* are all expressed in the mesectoderm however, so mechanisms autonomous to the MG could also account for these observations (Rothberg, et al., 1988; Bier et al., 1990; Goodman, personal communication). It is possible that *comm* is expressed by the MG themselves, and is necessary to maintain MG differentiation and survival, as previously established in *rho* and *Star* mutant embryos (Sonnenfeld and Jacobs, 1994). Alternatively, the absence of *comm* activity, or excess *rho* activity could interfere with the ability of the MG to ensheath axons, resulting in their expulsion from the CNS. Analysis of MG survival in embryos genetically mosaic for *comm* and *HS-rho* could resolve these alternates.

Recent studies show that a rise in the level of ecdysteriods at pupariation controls the cessation of midline glial proliferation during the third larval instar, as well as triggering the eventual programmed cell death of the MG after 50% of metamorphosis (Awad and Truman, personal communication). Similarly we have found that the maximum number of glia are required during embryonic commissure separation and after this morphogenetic event some of the MG die. In both situations, a subset of the glia are transient, acting to facilitate morphogenesis of the nervous system, after which they are disposed. This is analogous to the transient mouse subplate neurons, which provide the template for the lateral geniculate body projection to the visual cortex. After this projection is established, and cortical dominance columns are formed, the subplate neurons die (Allendoerfer and Shatz, 1994).

Studies on trophic modulators of neuronal survival have noted a non-linear relationship between the amount of afferent or efferent input and the number of surviving neurons (Oppenheim, 1991). Similar effects are emerging in studies of glial survival. Glial-axonal contact appears to modulate oligodendrocyte survival in neonatally transected rat optic nerves (David et al., 1984; Raff et al., 1993). However, not all oligodendroglia die after optic nerve transection (David et al., 1984). Similar results have been found by studying the effects of a lack of afferent sensory input on glial cell differentiation in the antennal lobes of *Manduca sexta* during metamorphosis (Oland and Tolbert, 1987). Interestingly, we have found that not all MG die in *comm* mutant embryos. We cannot exclude the possibility that minimal axon contact could influence MG survival in *comm* mutant embryos. Nevertheless, the relationship between numbers of MG and numbers of axons is not linear. There are likely other factors which also modulate MG number in later embryogenesis.

A model by Raff et al., (1993) proposes that as oligodendroglia precursors differentiate into oligodendrocytes they lose sensitivity to certain growth factors. They then become dependent on axon-derived signals for survival. During a critical period, 50% of the oligodendroglia contact a myelin-free region of axon, and the remainder die. The competition for axon-derived signals may match the number of oligodendrocytes to the length of axons requiring myelination. We propose that a similar mechanism may function in *Drosophila* to regulate MG number.

Genes of the *spitz* group, including *rho*, are hypothesized to function in a signal transduction pathway (Rutledge et al., 1992; Sturtevant et al., 1993) and are required for MG survival after determination (Sonnenfeld and Jacobs, 1994).

The increase in MG numbers generated by ectopic *rho* expression supports a role for the *rho* gene in MG determination. The midline cells are not responsive to ectopic *rho* expression after stage 13 of embryonic development, or once MG migration is complete. It is possible that once the MG complete their migration they become dependent on axon contact for survival. The correlation between decreased axon contact and decreased MG survival in *comm* mutant embryos supports this model.

These studies reveal that interactions between neurons and the MG may regulate glial cell number during embryonic insect development in a manner similar to that described in some vertebrate systems. The embryonic MG may serve as a useful *in vivo* model system to identify and characterise the role of trophic factors involved in glia survival.

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Table 1. Variations in the position an number of midline glia within ventral nerve cord segments.

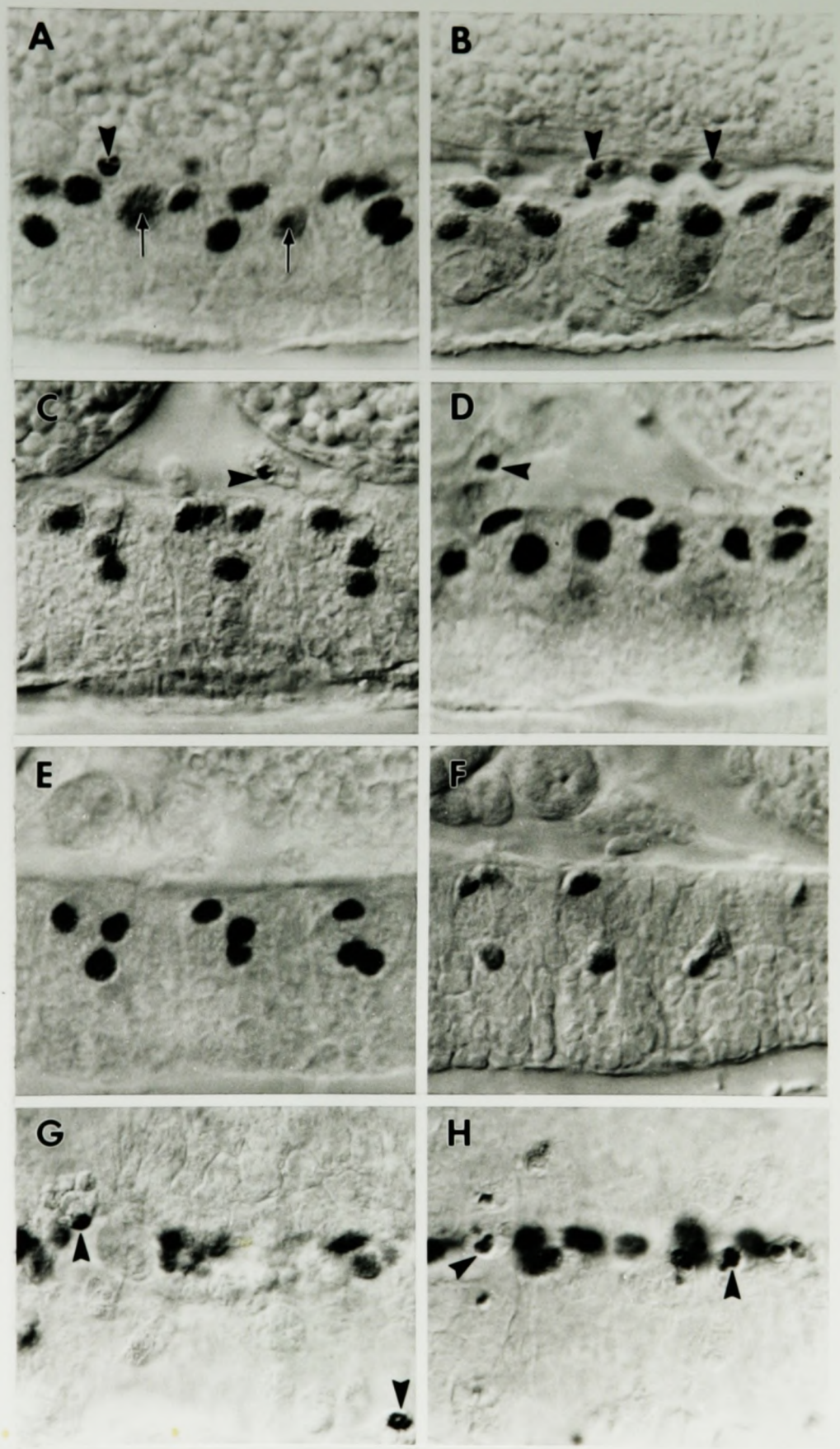
Table 1. Variations in the position and number of midline glia within VNC segments

MGA:MGM		0:0	1:1	1:2	2:1	2:2	0:2	2:0
Stage								
13	n=70	0	0	28.6*	*	71.4	0	0
14	n=141	0	1.7	51.4	0.7	46.2	0	0
15	n=98	0	9.0	55.3	0	35.7	0	0
16	n=132	0	8.8	66.5	0	24.7	0	0
17	n=96	0	3.0	76.9	7.8	10.4	1.9	0

All data are presented as percentage of embryonic ventral nerve cord segments containing the above combinations of MG based on position. All embryos carried the AA142 enhancer trap for identification of surviving MG and the number of segments scored is shown in column 2.

* 28.6% represents the percentage of segments containing three midline glia indistinguishable by position at this stage.

Fig. 1. Variations in the position and number of MG in embryonic ventral nerve cord (VNC) segments. Embryos containing the AA142 enhancer trap (A, C, E, G) and *sli-lacZ* 1.0 reporter construct (B, D, F, H) were stained with antibodies against anti-β-galactosidase. In each case, the anterior part of the embryo is on the left of the photomicrograph. All embryos are in sagittal view except for G and H which are shown in frontal (ventral) view. Stage 14 embryos containing AA142 (A) and *sli-lacZ* 1.0 (B) have variations in the number and position of MG in each ventral nerve cord segment. Small displaced b-gal stained cells are present in the haemolymph space dorsal to the ventral nerve cord (A, B arrowheads). Positions of the anterior (a) and posterior (p) commissures are identified in (A) and (E). MGP is denoted by the arrows in (A). (C, D) Variations in MG number and position continue during stage 15 while the displaced anti-β-galactosidase stained cells (arrowheads) are evident within macrophage-like structures. (E, F) Stage 17 of embryogenesis. A decrease in MG numbers within individual segments is apparent at stage 17 and the variation in MG position persists. (G, H) Distribution of the β-galactosidase stained displaced cells relative to the midline in embryos containing AA142 and *sli-lacZ* 1.0. These small cells are present within macrophage-like structures (arrowheads) in positions directly ventral to the midline as well as lateral to the VNC.


A
B
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G
H

Fig. 2. Variation in the number of MGP in wildtype and *reaper* deficient embryos. Embryos containing the X55 enhancer trap (A,B,C) and *sli-lacZ* 1.0 reporter construct were labeled with antibodies to b-galactosidase. At stage 14 (A) a pair of prominent MGP are seen at the posterior of each segment (arrowhead). Smaller X55 labeling neurons extend from the posterior boundary of the neuromere and ventrally. A few small X55 positive profiles are also present in the haemolymph dorsal to the nerve cord (arrow). At stage 16 (B) most MGP are no longer present (arrowhead) but a few small cells remain in the haemolymph (arrow). In embryos homozygous for the deficiency *Df(3L)W4*, which uncovers *reaper*, 3 to 5 MGP are seen per segment at stage 16 (arrowhead in C). At stage 16, 9-10 *sli-lacZ* 1.0 expressing cells surround the commissures (arrow) in each segment in *Df(3L)W4* homozygous embryos (D). Some cells are on the surface of the nerve cord (arrowhead).

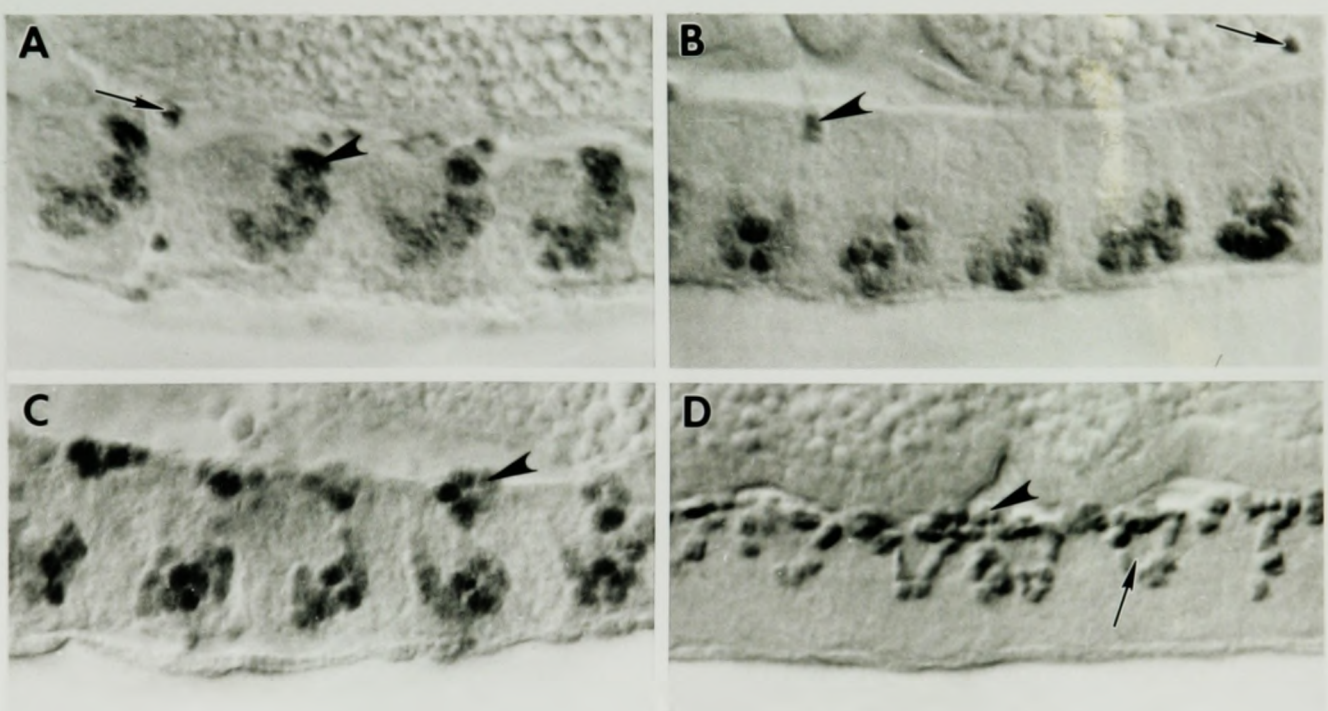
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Fig. 3. Electron microscopic analysis of MG in wildtype and *rhomboid* (*rho*) mutant embryos. (A, B) Sagittal sections of stage 14 wildtype embryos containing the AA142 enhancer trap processed with a substrate for β-galactosidase (bluo-gal). (C) Sagittal sections of stage 14 embryos mutant for the *rho* gene. The bluo-gal crystals surround the nuclei of MG expressing AA142. Surviving MG within the ventral nerve cord (A) possess distinctive glial characteristics such as extensive *rough* endoplasmic reticulum (RER), electron lucent cytoplasm and lamellipodial protrusions ensheathing the axons (arrowhead). Displaced MG in wildtype embryos (B) identified with the bluo-gal product (arrowhead) are present within macrophages dorsal to the ventral nerve cord and possess apoptotic features including reductions in cytoplasmic volume, increased electron density, condensed chromatin and intact cytoplasmic organelles. (C) Identical apoptotic characteristics and positions are observed in MG expelled from the ventral nerve cord of a stage 14 *rho* mutant embryo. Scale bars are 2.0 μm.

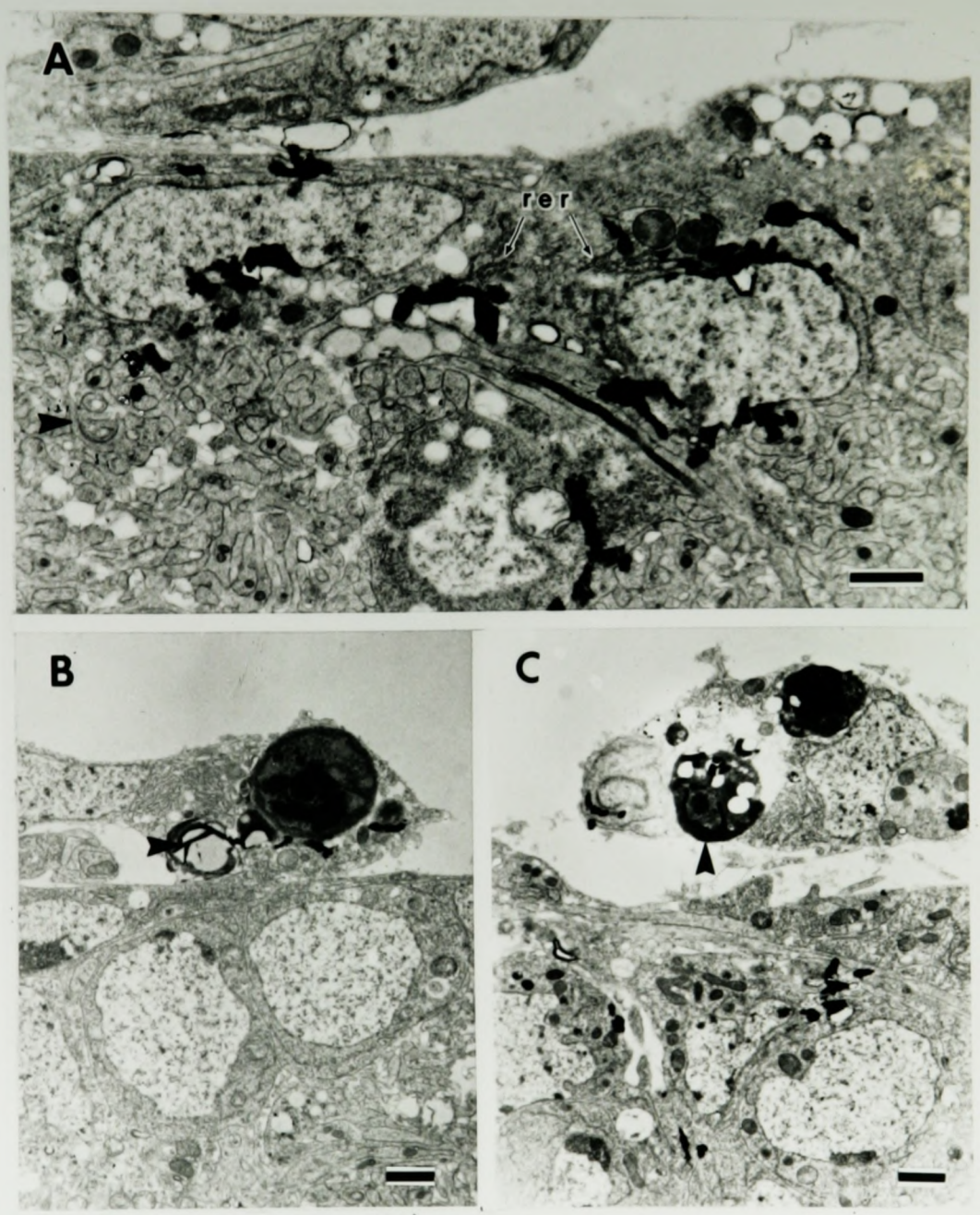
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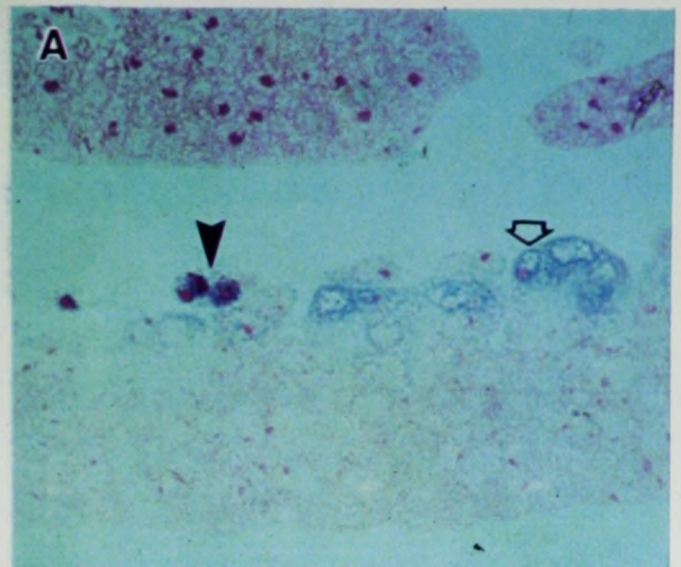
Fig. 4. Apoptotic MG are located within macrophages. (A) Plastic sagittal section (1 μm) of a stage 14 embryo containing AA142 and stained with X-gal (blue). Anterior of the section is to the left. Displaced MG recognized by X-gal (arrowheads) are condensed and intensely counter-stained with basic fuscin indicating apoptosis (A). The dead MG are contained within macrophage-like structures dorsal to the ventral nerve cord(arrowhead). Surviving MG (open arrow) identified by X-gal appear larger than their dead counter-parts and are located within the VNC. (B, C, D) Whole-mount views of stage 15 embryos containing AA142 and double-labeled with anti-peroxidasin antibody (HRP histochemistry) and X-gal (blue). Anterior of the embryos is to the left of the photomicrograph. In B displaced MG (arrowheads) are co-localized to anti-peroxidasin-positive macrophages (sagittal view). (D) Lateral to the ventral nerve cord of the same embryo as in (B), additional displaced MG (arrowheads) are present in macrophages recognized by anti-peroxidasin. (C) The MG and anti-peroxidasin-positive macrophages are also co-localized in positions posterior to the ventral nerve cord(arrowhead) (horizontal view).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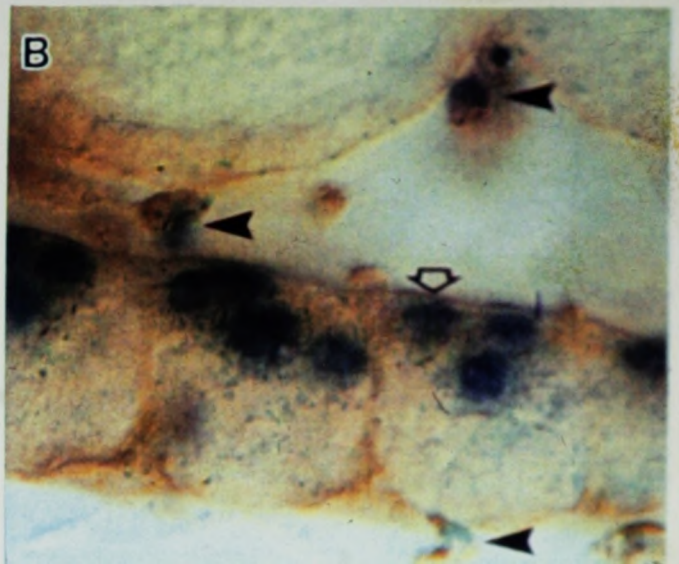
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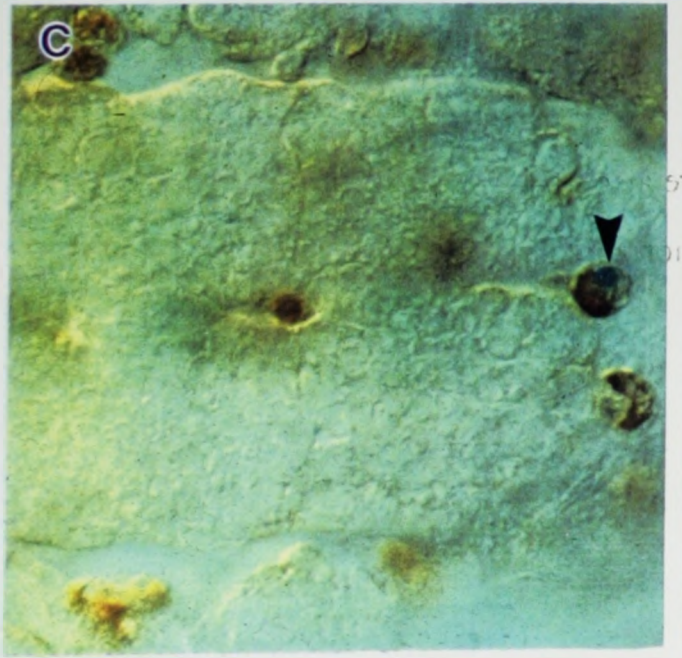
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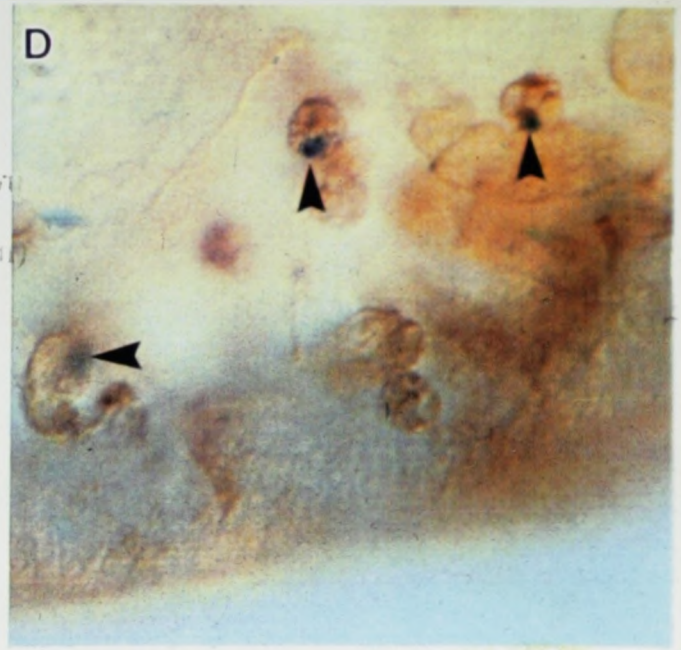
D

Fig. 5. Midline glia survival may be influenced by axonal contact. In *commissureless* (*comm*) mutant embryos axon contact by glia at the midline is minimal during stage 13 (A) although it can be seen (long arrow). By stage 15, the MG in *comm* mutants are reduced in number relative to wildtype and have migrated towards the longitudinal axon tracts (B) (arrow). In *slit* mutant embryos, the MG (open arrow) maintain contact with commissural and longitudinal axons (closed arrow) at the midline during stage 13 (C) and stage 15 (D). Ectopic expression of the *rhomboid* (*rho*) gene results in increased numbers of MG by stage 13 (E) some of which begin to die (arrowheads). By stage 14 (F) MG numbers are reduced while apoptotic cellular profiles remain visible (arrowhead). Embryos are oriented with anterior to the left in all panels. Embryos in A-D are shown in horizontal (ventral) view while those in E and F are shown in sagittal view.

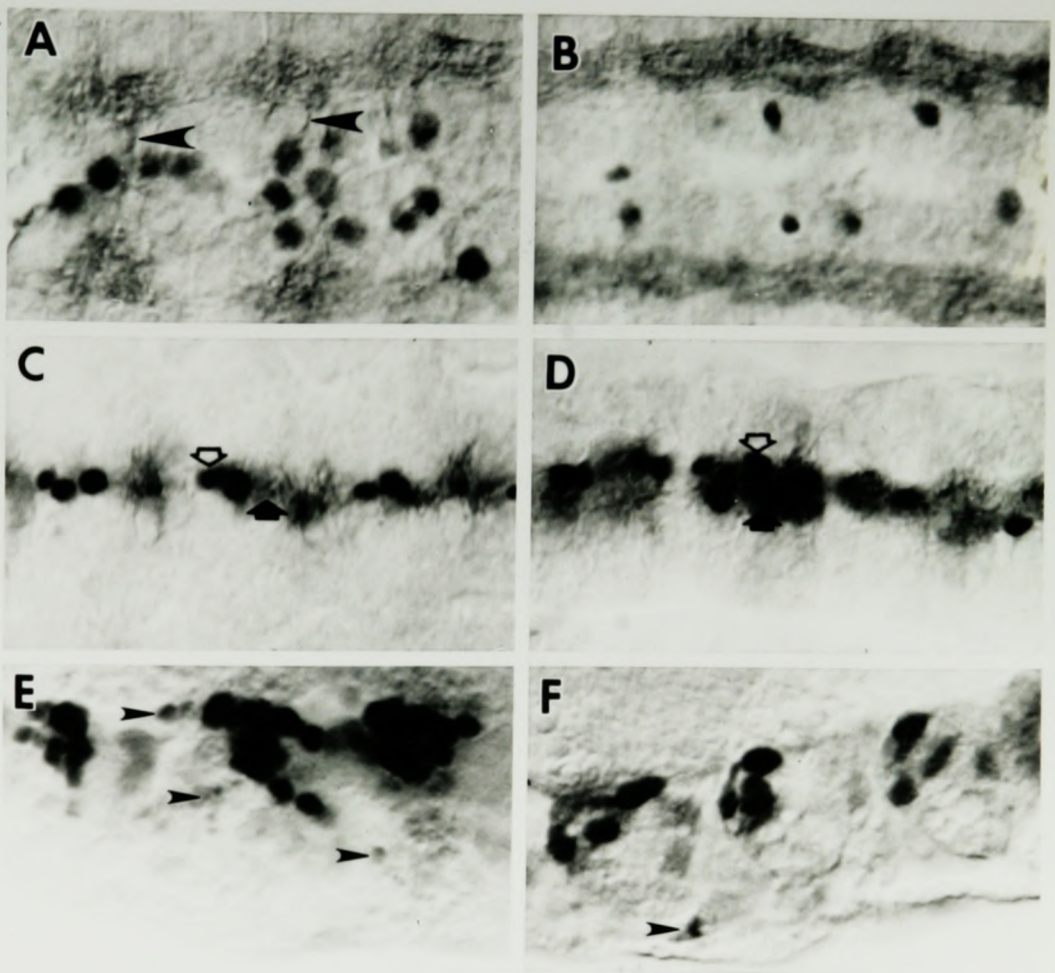
A
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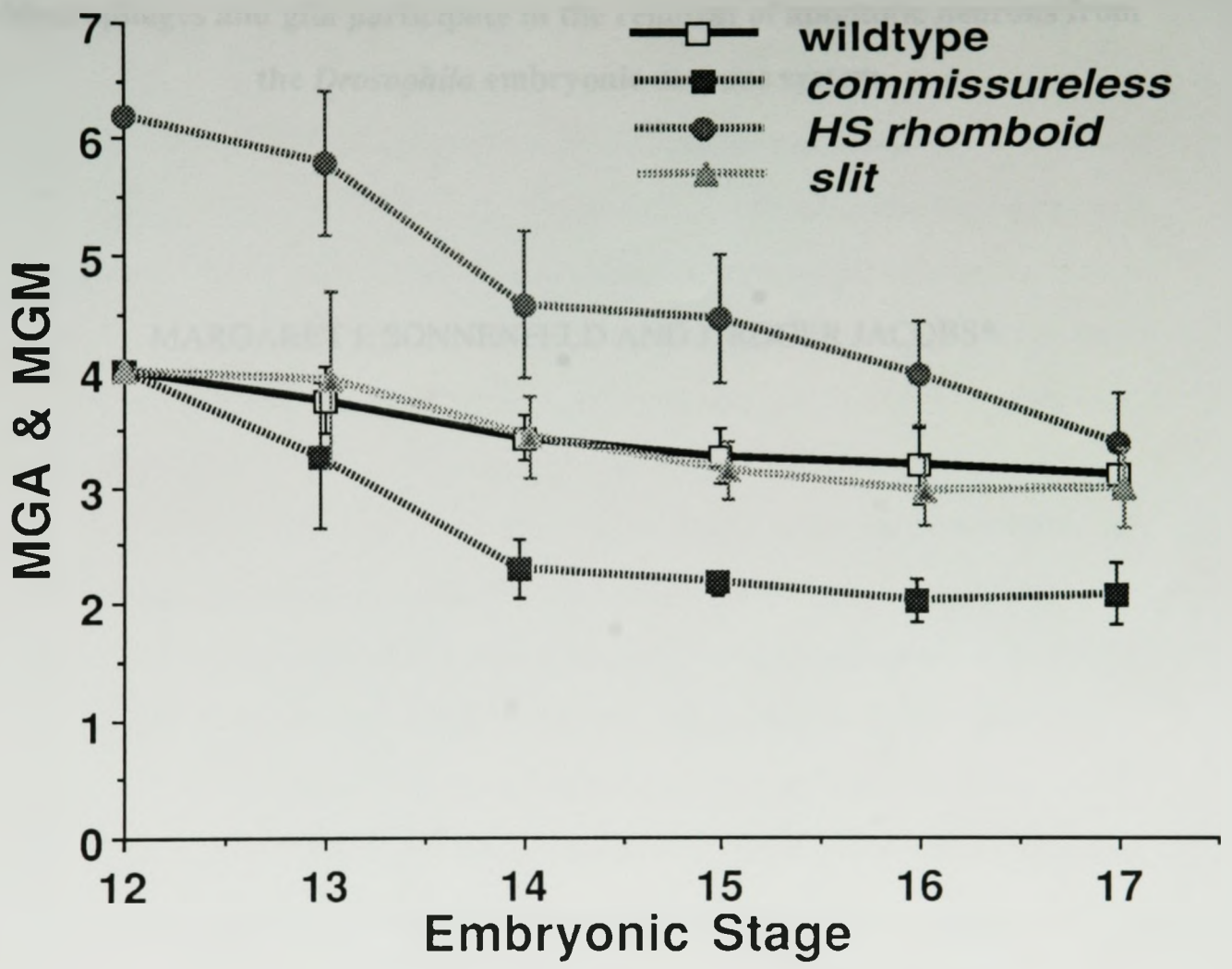
Fig. 6. Comparisons of MGA and MGM numbers during embryogenesis in embryos with alterations in MG-axon contact. The MG were assessed by position and number within ventral nerve cord segments using the AA142 enhancer trap as a marker. Decreases in MG numbers in embryos mutant for the *slit* gene are similar to those in wildtype embryos. Decreases in axon contact with the MG in *comm* mutant embryos correlates with a rapid decrease in MG numbers between stages 12 and 14 of embryogenesis. After stage 14, MG numbers in *comm* mutant embryos remain reduced from wildtype MG numbers by 20%. Ectopic expression of the *rho* gene correlates with a 50% increase in the number of MGA and MGM by stage 12 of embryogenesis. The number of MGA and MGM in HS-*rho* embryos rapidly decline by stage 14 of embryogenesis and after stage 14, MG numbers decline less rapidly towards wildtype MG numbers. Error bars identify the standard deviation from the mean of the embryo averages in each sample.


wildtype
commissureless
HS rhomboid
slit
MGA & MGM
0
1
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7
12
13
14
15
16
17
Embryonic Stage

CHAPTER 3

Macrophages and glia participate in the removal of apoptotic neurons from the *Drosophila* embryonic nervous system.

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ABSTRACT

Cell death in the *Drosophila* embryonic central nervous system (CNS) proceeds by apoptosis as revealed ultrastructurally by nuclear condensation, shrinkage of cytoplasmic volume and preservation of intracellular organelles. Apoptotic cells do not accumulate in the CNS, but are continuously removed and engulfed by phagocytic hæmocytes. To determine whether embryonic glia can function as phagocytes we studied serial electron microscopic sections of the *Drosophila* CNS. Apoptotic cells in the nervous system are engulfed by a variety of glia including midline glia, interface (or longitudinal tract) glia, and nerve root glia. However, the majority of apoptotic cells in the CNS are engulfed by subperineurial glia in a fashion similar to the microglia of the vertebrate CNS. A close proximity between macrophages and subperineurial glia suggests that glia may transfer apoptotic profiles to the macrophages. Embryos affected by the maternal-effect mutation *Bicaudal-D* have no macrophages. In the absence of macrophages most apoptotic cells are retained at the outer surfaces of the CNS and subperineurial glia contain an abundance of apoptotic cells. Some apoptotic cells are expelled from the CNS suggesting that the removal of apoptotic cells can occur in the absence of macrophages. The number of subperineurial glia is unaffected by changes in the rate of neuronal apoptosis.

INTRODUCTION

In the developing vertebrate central nervous system (CNS) glia play an important role in tissue modeling by the phagocytosis of degenerating cells and their processes (for review see Nakajima and Kohsaka, 1993). Phagocytosis of cells by microglia (Hughes, 1961), astrocytes (O'Connor and Wyttenbach, 1974), radial ependymal cells and mononuclear leukocytes (Chu-Wang and Oppenheim, 1978) has been observed during embryonic development of the brain and spinal cord. This coincides with a time of developmental neuronal apoptosis (Oppenheim,1991; Barres et al., 1992). In the developing mouse cerebellum (Ashwell, 1990; Perry et al., 1985) and developing mouse retina (Hume et al., 1983) microglia have been observed at both the light and electron microscopic levels to phagocytose pyknotic cells. Microglia in the vertebrate CNS are considered the primary resident macrophages and also represent the main immune effector cells in the brain (Perry and Gordon, 1988; Streit et al., 1988; Graeber and Streit, 1990; Gehrmann et al, 1992; Streit and Graeber, 1993). The expression of genes in the normal adult CNS glia that function in the immune system may reflect glial competence for immune function. For example, major histocompatibility complex (MHC) class II antigens are expressed by microglia in the white matter of the human CNS (Hayes et al., 1987). The CD4 molecule is expressed by microglia in white matter of normal rat brains (Perry and Gordon., 1987).

How do glia and hæmocytes perform these functions in simpler organisms with less specialized immune systems? In the *Drosophila* retinal degeneration mutant rdg^{BKS22}, phagocytosis and gliosis occur in the lamina (Stark and Carlson, 1982), and this is the basis of the capacity for phagocytosis in this

embryo collection. Embryos were inspected morphologically for duplication of posterior features and were either checked for the absence of macrophages with an antibody to peroxidasin (protein X; Abrams et al., 1993; Tepass et al., 1994) or were processed for electron microscopy as described below. The embryonic pattern of cell death was determined by vital dye staining with nile blue (Abrams et al., 1993). Embryos were staged according to Campos-Ortega and Hartenstein (1985).

Antibodies and Immunocytochemistry

A polyclonal antibody to anti-peroxidasin (protein X; Abrams et al., 1993; Tepass et al., 1994), used to identify macrophages, was provided by L. Fessler. anti-*repo* antibody (RK2-5', a gift of Andrew Tomlinson) was used to identify CNS glial cells . *Repo* is a homeobox protein expressed in all embryonic glia except the midline glia (Campbell et al., 1994; Xiong et al., 1994). Glial nomencalture used here has been adopted from J. Ito, J. Urban and G. Technau, (in press). For antibody detection, embryos were processed according to Patel et al. (1987) and Grenningloh et al. (1991). In all cases the antibody reaction was visualized with HRP immunocytochemistry. Neurons were identified with the P[*elav/lacZ*] reporter construct #1, characterised by Yao and White (1994). *elav* expression is CNS specific (Campos et al., 1987; Robinow and White, 1991; Yao and White, 1994) and is not expressed by *repo* positive cells (Campbell et al., 1994).

Sectioning and electron microscopy

Electron microscopy and *lacZ* histochemistry was performed as described by Jacobs (1993), employing 5-bromoindolyl-ß-D-galactopyranoside as a ß-galactosidase substrate. Serial electron micrographs of the embryonic CNS were examined.

RESULTS

Cell death in the embryonic CNS

The ultrastructural features of apoptotic cells in the CNS include nuclear condensation, increased electron density, preservation of intracellular organelles and reduced cytoplasmic volume (Fig. 1A; Abrams et al., 1993). While single, un-engulfed apoptotic cells were found in the CNS, most apoptotic cells were found in subperineurial glia at the ventral surface of the CNS (Fig. 1A). The subperineurial glia were identified by their long cytoplasmic extensions into the neural layer, their electron lucent cytoplasm, labeling with *repo* (RK2-5') antibody and by their location on the surface of the CNS (Fig. 1A, Fredieu and Mahowald, 1989; Hoyle, 1986; Cantera, 1993; Nelson and Laughon, 1993; K. Ito, J. Urban and G. Technau, in press). Subperineurial glia can be identified by these criteria from late stage 12. They are distinguished from the mesodermally derived perineurial glia which do not express *repo* and appear at stage 16 (Edwards et al., 1993; personal observations). There is little neuronal apoptosis after the establishment of the perineurial sheath, and the possible involvement of these glia in engulfment has not been assessed.

Macrophages enclosing apoptotic cell profiles were present outside the ventral region of the early (stage 12/0) embryonic CNS (Fig. 1B). At stage 12/0, subperineurial glia were too immature to be identified by their characteristic morphological features but perineurial cells expressing the glial antigen RK2-5' were present in the CNS of these embryos (data not shown). No cells inside the CNS contain apoptotic profiles before stage 13. Macrophages were identified at the electron microscopic level by an abundance of *rough* endoplasmic reticulum, characteristic lamellipodia and the presence of multiple apoptotic cellular profiles (Fig 1B; Campos-Ortega and Hartenstein, 1985; Abrams et al., 1993; Tepass et al, 1994). Macrophages were never observed inside the embryonic CNS but accumulated between the ventral surface of the CNS and the epidermis as determined by ultrastructural analysis (Fig. 1B). Macrophages were also consistently present immediately dorsal to the CNS (Fig. 1A).

Glia engulf apoptotic cells in the embryonic CNS

Embryonic development of the CNS in *Drosophila* proceeds at a relatively rapid rate in a confined space (Jacobs and Goodman, 1989b). The embryonic CNS of *Drosophila* develops in 14 hours to a neuronal density of 10^7 cells/mm^3, approximately 100 times faster and at 10 to 100 times higher cell density than mammalian cortical tissue (Jacobs, personal observation; Hartenstein et al., 1987). Rapid generation of this compact nervous system requires efficient removal of apoptotic cells.

The presence of apoptotic cells in subperineurial glia prompted us to investigate whether glia participate in phagocytosis in the CNS. Glia that have

been identified in the *Drosophila* embryonic nervous system include longitudinal glia (also known as interface glia), midline glia, nerve root glia, and the A and B glial cells (Fredieu and Mahowald, 1989; Jacobs and Goodman, 1989a, b; Klämbt and Goodman, 1991; Nelson and Laughon, 1993) and have been recently classified (K. Ito, J, Urban and G. Technau, in press). Ultrastructural analysis of the embryonic CNS revealed that various glia contained apoptotic cellular profiles including the longitudinal tract glia which ensheath axons of the longitudinal tract (Fig. 2A) and the midline glia which ensheath axons of the anterior and posterior commissures (Fig. 2B). However, the subperineurial glia most commonly contained apoptotic debris (Fig. 2C). There was a close proximity between macrophages and the phagocytic subperineurial glia at the ventral surface of the CNS (Fig. 2C). We also found macrophages in contact with phagocytic glia surrounding axons of the peripheral nerve root in the peripheral nervous system (Fig. 2D). These observations suggested the possibility of transfer of apoptotic neurons from the phagocytic glia in the nervous system to the macrophages present in the surrounding hæmolymph.

To confirm that apoptotic neurons were expelled from the CNS and were engulfed by the surrounding macrophages, we used a ß-galactosidase reporter with expression driven by the nervous system-specific enhancer elements of the *elav* gene (Yao and White, 1994). The *elav* gene is expressed in the majority of embryonic CNS neurons and not in glia (Campos et al., 1987; Robinow and White, 1991; Campbell et al., 1994). Macrophages surrounding the CNS carried apoptotic cells labeled for ß-galactosidase activity, confirming that apoptotic neurons were expelled from the CNS and were engulfed by the surrounding macrophages (Fig. 2E). Previous analysis has also shown that apoptotic glia from

the midline of *Drosophila* are expelled from the CNS and are engulfed by macrophages present in the hæmolymph during both normal and aberrant embryonic development (Sonnenfeld and Jacobs, 1994; Sonnenfeld and Jacobs, in press).

To determine whether macrophages can enter the CNS as in vertebrates, we used a polyclonal antibody to peroxidasin which is produced and secreted by macrophages (Fig. 3A; Abrams et al., 1993; Tepass et al., 1994). Macrophages labeled with anti-peroxidasin were found in the hæmolymph around the CNS and other embryonic tissues but were not found inside the CNS (Fig. 3A).

These observations suggested that the removal of apoptotic cells from the embryonic CNS may include multiple routes such as: (1) single, un-engulfed apoptotic cells are expelled from the CNS and are engulfed and degraded by macrophages, (2) apoptotic cells are engulfed and degraded within the CNS by glia cells or (3) apoptotic cells engulfed by subperineurial glia are expelled from the CNS, and are engulfed and degraded by macrophages.

Fate of apoptotic CNS cells in the absence of macrophages

To determine whether subperineurial glia were able to substitute for the phagocytic activity of macrophages we determined the fate of apoptotic CNS cells in the absence of macrophages. This was achieved by ultrastructural examination of the CNS in embryos mutant for the *Bicaudal-D* (*Bic-D*) gene (Tepass et al., 1994). *Bic-D* is a maternal-effect gene that is required for anterior-posterior polarity during oogenesis and has sequence similarity to myosin heavy chain tail domains, intermediate filament proteins and kinesin (Suter et al., 1989;

Ran et al., 1994). Transheterozygote embryos of two *Bic-D* alleles (*Bic-D*$^{71.34}$ and *Bic-D*^{IIIE48}) show a disruption in the localization of anterior and posterior factors resulting in embryos with a duplication of posterior structures and a lack of anterior structures (Fig. 3B; Suter et al., 1989). Embryos from *Bic-D* mothers lack macrophages because they are produced in a structure anterior to the headfold (Fig. 3B; Tepass et al., 1994). In wildtype embryos macrophages labeled with anti-peroxidasin were found at various locations throughout the embryo (Fig. 3A).

In the absence of macrophages in *Bic-D* mutant embryos, numerous apoptotic cellular profiles accumulated at the ventral surface of the CNS (Fig. 4B) relative to the distribution of apoptotic cells in the wildtype CNS (Fig. 4A). Inside the CNS of *Bic-D* mutant embryos, subperineurial glia were found to contain an abundance of apoptotic cells (Fig. 4C and 4D). There were also single un-engulfed apoptotic cells inside the CNS in *Bic-D* mutant embryos, rarely encountered in wildtype embryos. This suggested that there was either an increased rate of cell death in *Bic-D* mutant embryos and/or a decreased rate of removal of apoptotic cells from the CNS. Despite the absence of macrophages, apoptotic cells were expelled from the CNS, including apoptotic bodies in late stages of apoptosis (Fig. 4B, C and D).

The accumulation of apoptotic cells in subperineurial glia may have resulted from the lack of transfer of apoptotic material to macrophages. We determined whether the subperineurial glia or other CNS glia compensated for the absence of macrophages by an increase in the number of glia. CNS glia were identified in *Bic-D* mutant embryos with an antibody to *repo* (RK2-5') the product of a *Drosophila* homeobox-containing gene that is specific to glial nuclei

(Campell et al., 1994; Xiong et al., 1994; Fig. 3C). In *Bic-D* mutant embryos the distribution of *repo* expressing glia was slightly altered from wildtype (Fig. 3D), however, the number of subperineurial glia per abdominal segment at stage 16 in *Bic-D* mutant embryos (15.5 ± 1.9, n=34) was not significantly altered from wildtype (17.8 ± 1.7, n=30).

We also determined whether subperineurial glia responded to a reduction in the rate of apoptosis, by examining embryos deficient for a gene required to initiate programmed cell death, *reaper*. We examined embryos homozygous for the deletions *Df(3L)WR4* and *Df(3lWR10)* which are deficient for *reaper* (White et al., 1994). The number of subperineurial glia labeled with antibody RK2-5 in these embryos (16.0 ± 1.2, n=33) was close to wildtype, however their nuclei were smaller. These results suggested that embryonic CNS glia did not proliferate to accommodate changes in the number of apoptotic cells in and around the CNS.

DISCUSSION

Role of CNS glia in the removal of apoptotic cells

The *Drosophila* embryonic CNS is non-vascular and communication with hæmocytes is more limited than that in vertebrate systems. A variety of embryonic glia in the *Drosophila* CNS were capable of phagocytic activity. Some of these glia were ensheathing glia (longitudinal glia and midline glia), analogous to vertebrate oligodendroglia. However, most of the apoptotic cells

were engulfed by subperineurial glia. The subperineurial glia appeared to play a dominant role in the phagocytosis of neurons (neuronophagia) and may therefore be functionally analogous to the vertebrate microglia. Using the neural cell marker *elav*, we have demonstrated that apoptotic neurons are subsequently degraded by macrophages. Most apoptotic cells are first engulfed by glia, suggesting that glia transfer apoptotic material to macrophages, however direct physical proof of this transfer has not been generated.

Neuronophagia by glia has been documented in invertebrate post-embryonic nervous systems. It has been described in the CNS of molluscs (Reinecke, 1976), during motoneuron degeneration, and in the prothoracic ganglion of *Manduca sexta* during metamorphosis (Stocker et al., 1978; Truman, 1985; Cantera, 1993).

Neuronophagia by glia also occurs after experimentally induced damage to the CNS in invertebrates. In adult cockroaches, hæmocytes are recruited to the site of injury, endogenous glia proliferate and glia from adjacent sites migrate into sites of injury in the CNS (Smith and Howes, 1987; Treherne et al., 1988; Smith et al., 1991). After nerve injury in the segmental ganglia of the leech, microglia, analogous to the vertebrate microglia, cluster at the sites of injury and become phagocytic (Elliott and Muller, 1981). After amputation of the cercal sensory pathway in the terminal abdominal ganglion of the house cricket, *Acheta domesticus*, glia engulf debris (Edwards and Meyer, 1985).

Similarly, during vertebrate development, various populations of glia are capable of phagocytic activity including resident ameboid microglia in the developing mouse cerebellum (Ashwell, 1990; Hughes, 1961) and astrocytes (O'Connor and Wyttenbach, 1974). In vertebrates, astrocytes, oligodendrocytes,

ependymal cells and microglia in the post-embryonic CNS may become phagocytic (Hao et al., 1991). Ferrer et al. (1990), showed that naturally occurring cell death occurs in the cortical subplate and plate of the rat during the first two weeks of postnatal life. Large numbers of macrophages with the characteristics of ameboid microglia appear in these regions during the same time. Dead cells decrease after day 10 in the cerebral cortex and underlying white matter and are absent from the adult brain. The phagocytic cells disappear in the cerebral cortex and underlying white matter when dead cells disappear.

It therefore appears that phagocytic activity by glia coincides with periods during which tissue remodeling and cell death are predominant in both vertebrates and invertebrates (Ashwell, 1990; Oppenheim, 1991; Abrams et al., 1993). Glia number changes in post-embryonic nervous systems, to accommodate changes in the number of apoptotic cells, have not been observed in embryonic nervous systems.

Relationship between macrophages and the CNS in vertebrates and invertebrates

Macrophages were not present inside the avascular *Drosophila* CNS, and therefore the subperineurial glia may normally substitute for their function. To test this hypothesis we examined the response of glia and apoptotic cells to an absence of macrophages. The accumulation of apoptotic cells inside subperineurial glia and at the ventral surface of the CNS in the absence of macrophages suggested that macrophages may normally participate in the removal of apoptotic cells from the periphery of the CNS. There was no evidence

that the subperineurial glia proliferated to compensate for the overabundance of apoptotic cells in the CNS. However, apoptotic cells accumulated around the CNS suggesting that macrophages were not essential for the removal of apoptotic cells from within the CNS. These results agree with recent reports that cell death still occurs in the absence of macrophages (Tepass et al., 1994). In contrast, in the developing mouse eye, macrophages are required for cell death and tissue remodeling (Lang and Bishop, 1993). The disruption of macrophages in transgenic mice results in the persistence of two transient ocular tissues, the hyaloid vasculature and the pupillary membrane.

It is notable that the mesodermally derived perineurial sheath, which provides a barrier between subperineurial glia and hæmocytes (Edwards et al., 1993), is established after most embryonic neuronal apoptosis is complete. Apoptotic profiles have not been observed in the perineurial cells (Jacobs, unpublished) and an organised perineurium is not present in hæmocyte depleted mutant *Drosophila* (Edwards et al., 1993; Jacobs, unpublished). Studies of glial regeneration in cockroaches have revealed a contribution of hæmocytes to the perineurial cell layer, as well as possible trophic interactions between the perineurial cells and the subperineurial glia (Smith et al., 1986; Treherne et al., 1988).

Programmed cell death does not occur in embryos mutant for the gene *reaper*, and although these embryos have no apoptotic cells, macrophages are present, although smaller than those circulating in wildtype embryos (White et al., 1994). The number of hæmocytes and subperineurial glia in mutant embryos showing increased or decreased cell death is the same as wildtype, suggesting

that embryonic cell death does not trigger hæmocyte or glial proliferation (Tepass et al., 1994; this work).

How do macrophages recognize degenerating cells? In vertebrates there is evidence of multiple recognition mechanisms as macrophages can recognize and remove apoptotic cells by the vitronectin receptor (Savill et al., 1990; Fadok et al., 1992), by the phosphatidylserine receptor (Fadok et al., 1992) and by macrophage scavenger receptors (Kodama et al., 1990). These authors suggest that the macrophage population determines which mechanism is used to remove apoptotic cells. Macrophages in *Drosophila* embryos express macrophage receptor activity suggesting that this mechanism for the recognition and removal of apoptotic cells by macrophages may be conserved between vertebrates and invertebrates (Abrams et al., 1992).

A role for CNS glia and for macrophages has been demonstrated in the removal and degradation of apoptotic neurons during *Drosophila* embryogenesis. Some molecular and cellular aspects of this function mirror the analogous events in vertebrate development, suggesting that *Drosophila* can provide a useful model for the genetic dissection of basic neuro-immune interaction, in particular, the response of hæmocytes and glia to injury and during regeneration.

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Fig. 1. Apoptosis in the embryonic CNS. (A) A sagittal view of a stage 14 wildtype CNS demonstrates the distribution of apoptotic cells. Single, un-engulfed cells (open arrow) and engulfed apoptotic cells (solid arrow) can be found inside the CNS. Most commonly, apoptotic cells are engulfed by subperineurial glia at the ventral surface of the CNS (arrowheads). Macrophages containing apoptotic cells were consistently found outside the ventral and dorsal surface of the CNS (m). (B) A sagittal view of a younger (stage 12/0) CNS reveals that phagocytic macrophages are observed outside the ventral surface (m). At this stage subperineurial glia could not be morphologically identified. Anterior is to the left. Dorsal is at top in all figures. Scale bars are 7 μm.

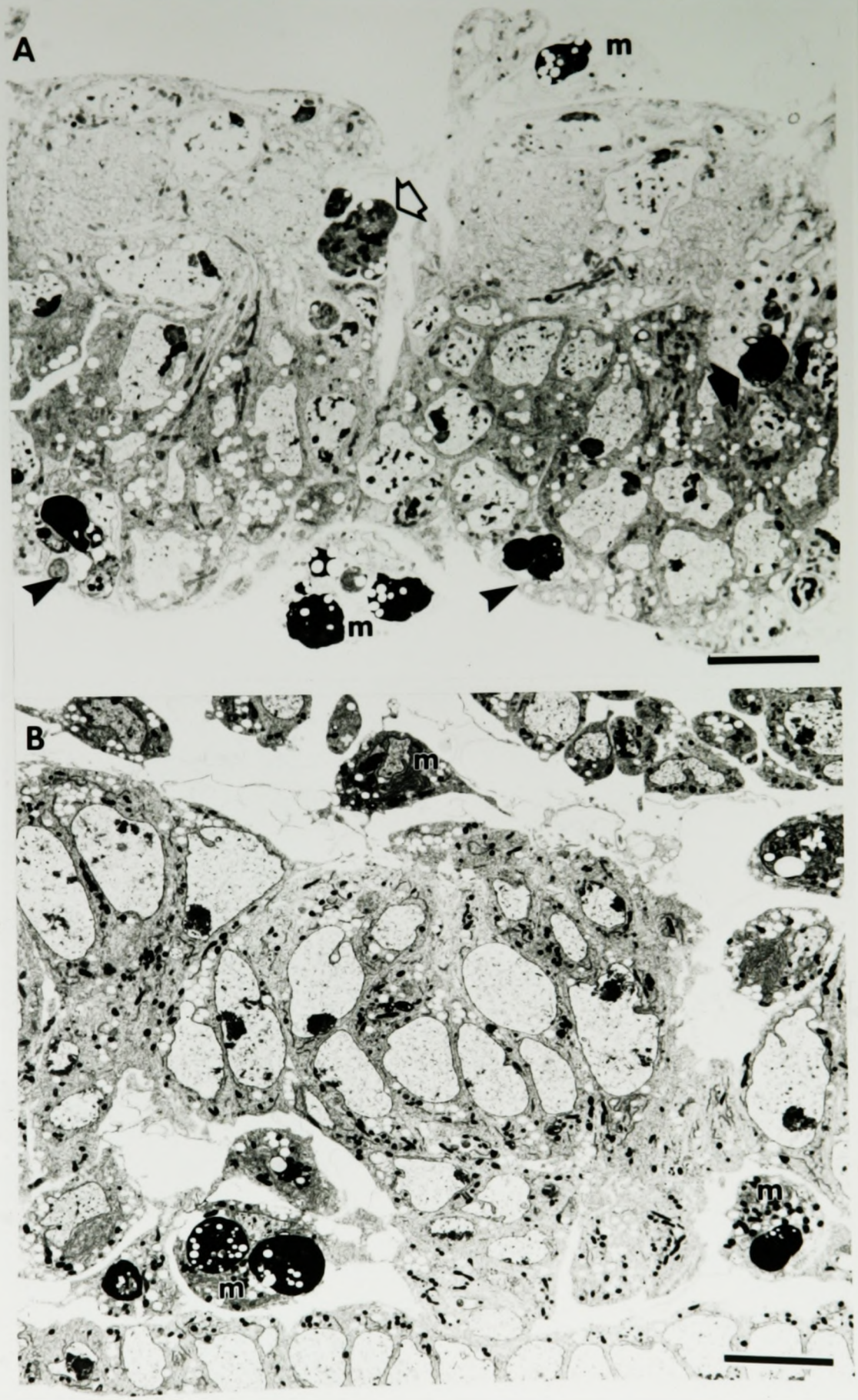
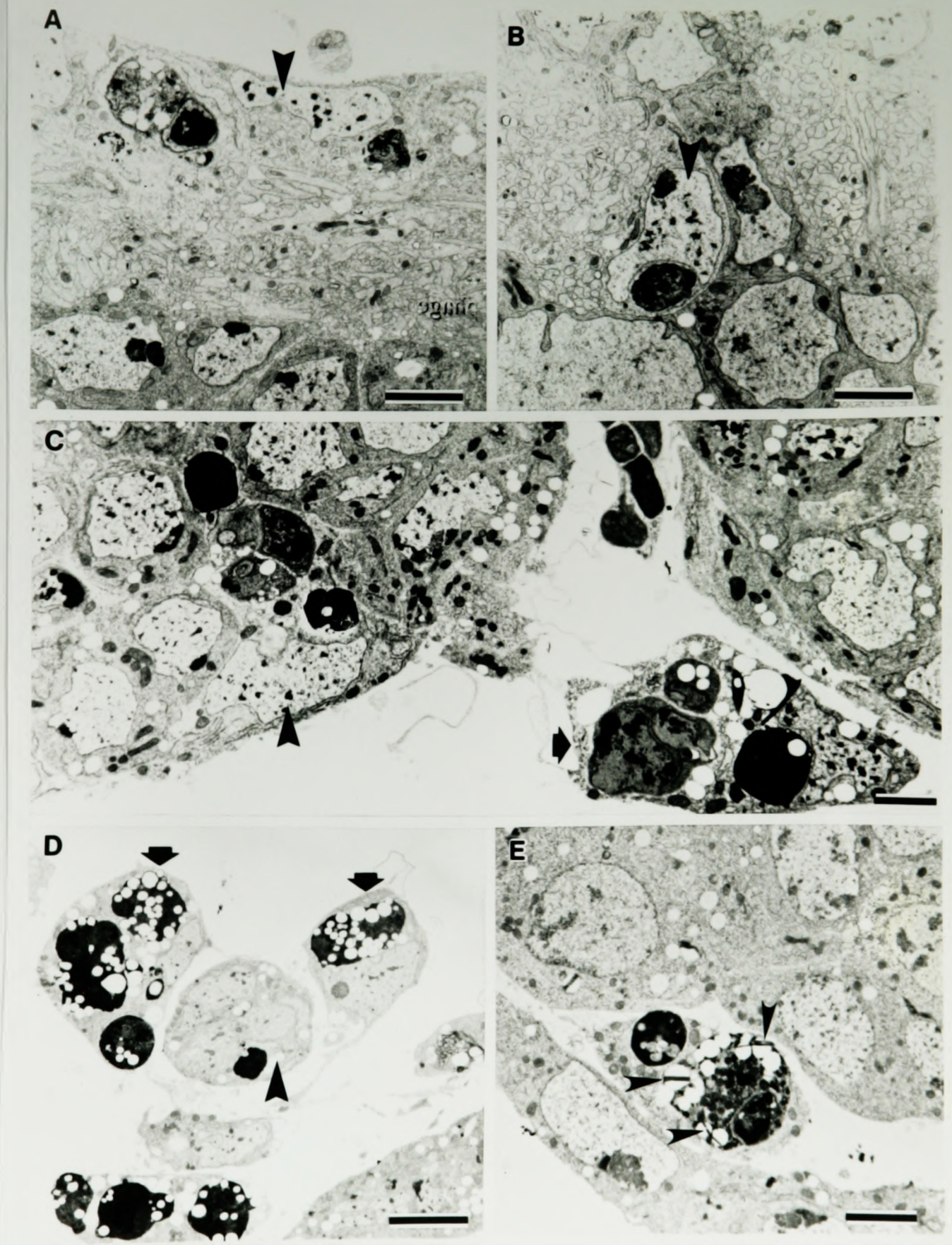

A
m
m
B
m
m
m

Fig. 2. Glia in the CNS and PNS contain apoptotic cells. Electron micrographs show parasaggital views of late stage 13 embryonic central nervous systems. Longitudinal tract glia (A; arrowhead) and middle midline glia (B; arrowhead) have both engulfed apoptotic debris in the CNS. A subperineurial glia (arrowhead) contains multiple apoptotic cells at the ventral surface of the CNS and is in close proximity to a macrophage (arrow) (C). In the PNS, a glia cell (arrowhead) that ensheaths the nerve root has engulfed apoptotic material (D). This portion of the nerve root is surrounded by macrophages (arrows) containing numerous apoptotic cells. (E) A macrophage outside the CNS encloses an apoptotic cell that labels hisotchemically (arrowheads) for the neuron specific reporter *elav-lacZ*. Note that the profile to the left does not label. Anterior is to the left in parasaggital views in A, B, and C. Panels D and E are cross-sections. Scale bars are 2 μm.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E

Fig. 3. Distribution of macrophages and glia in the CNS of wildtype (A and C) and *Bic-D* mutant embryos (B and D). The distribution of macrophages in stage 15 wildtype and *Bic-D* mutant embryos was determined using an anti-peroxidasin antibody and is shown in sagittal views of embryos with anterior to the left (A and B). In wildtype embryos, macrophages (arrowheads) are restricted to the hæmolymph space and do not enter the CNS (A) while there are no macrophages in *Bic-D* mutant embryos (B). The distribution of CNS glia was determined with an antibody to RK2-5' in wildtype (C) and *Bic-D* mutant embryos (D). Although the pattern of CNS glia was slightly altered in stage 15 *Bic-D* embryos compared to the wildtype pattern, there was no significant increase in glia numbers. Subperineurial glia were identified by position in the CNS (arrowheads). C and D show frontal views of RK2-5' labeled CNS with anterior oriented to the left of the photomicrograph.

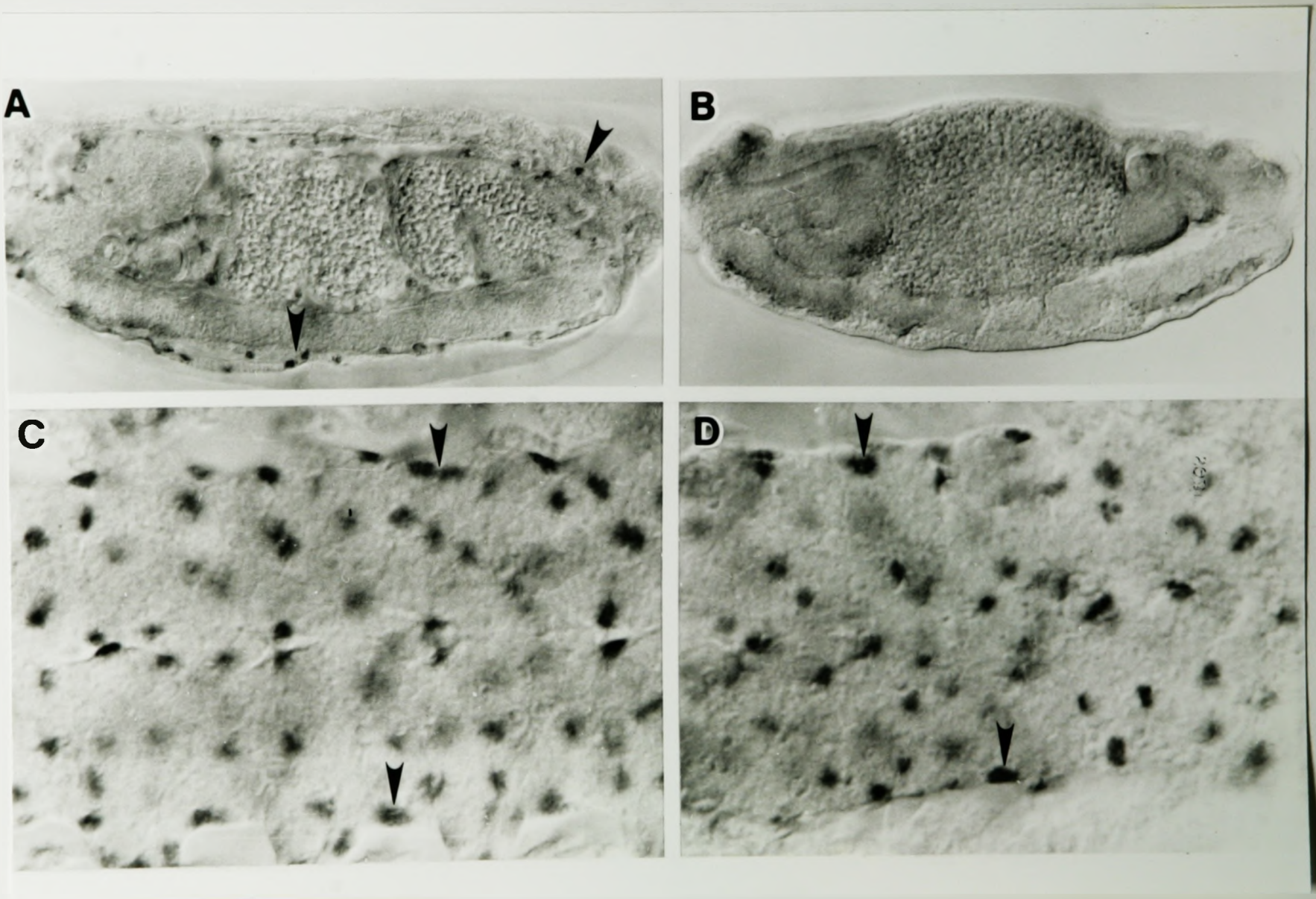
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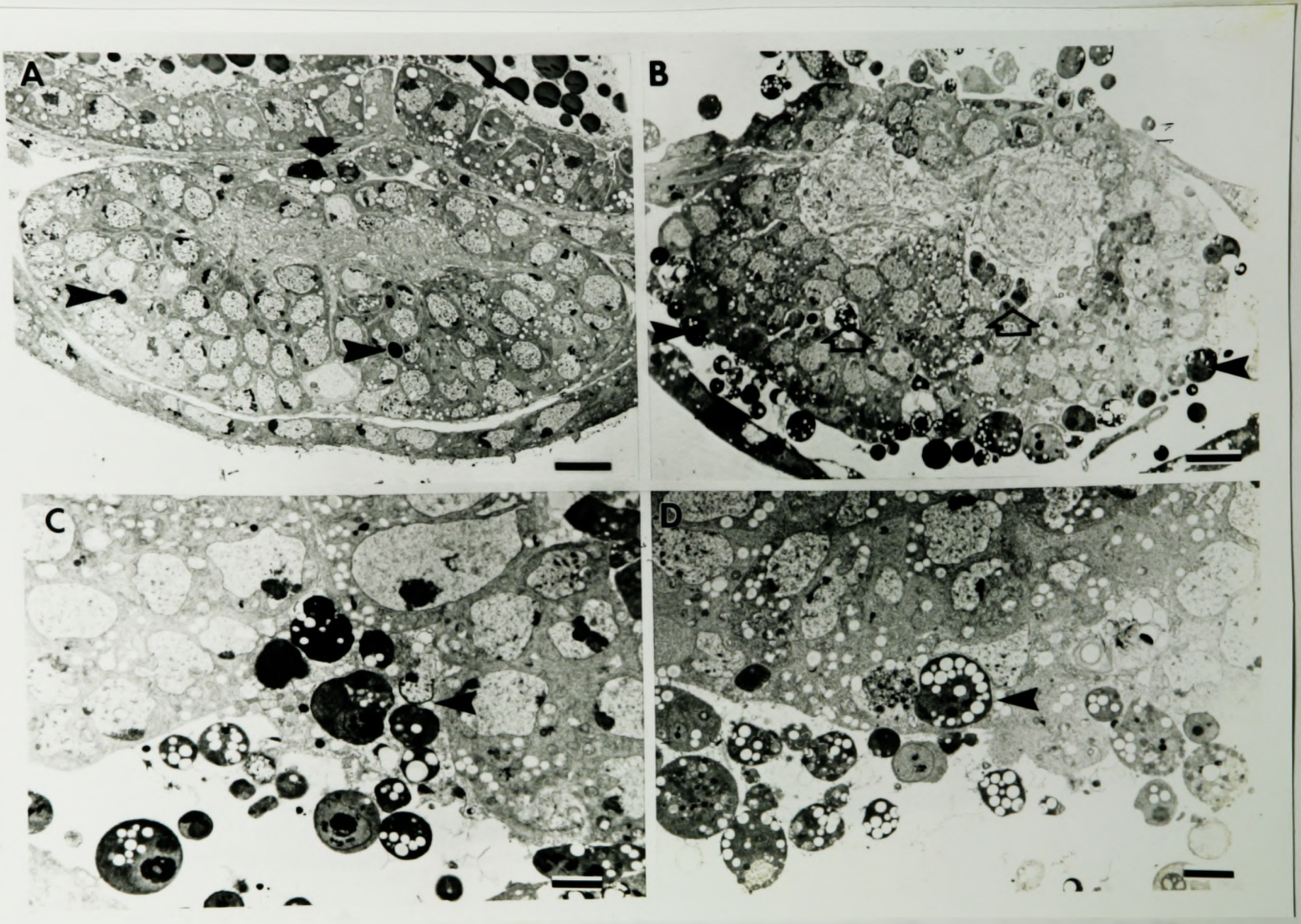
Fig. 4. Fate of apoptotic CNS neurons in the absence of macrophages in *Bic-D* mutant embryos. Electron micrographs from the CNS of stage 14 wildtype (A) and *Bic-D* mutant embryos (B-D) are shown. In a cross-section of the wildtype CNS, two apoptotic cells are present inside the CNS (A, arrowheads). A macrophage containing apoptotic cells is present dorsal to the CNS (arrow). In a cross-section of the CNS of a stage 14 *Bic-D* mutant embryo, apoptotic cells accumulate inside the ventral surface (arrowheads) while other cells appear to be expelled (B). Some un-engulfed apoptotic cells remain inside the CNS (open arrows). Panels C and D show higher magnification views of the *Bic-D* CNS. Multiple apoptotic cells accumulate inside subperineurial glia at the ventral surface of the CNS (C and D; arrowheads). The scale bars in A and B are 10 μm. The scale bars in C and D are 4 μm.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D

SUMMARY AND DISCUSSION

1) Genetic control of midline cell development during *Drosophila* embryogenesis.

1a) Which midline lineages are involved in commissural axon development?

Previous studies attributed a collapse of the axon tracts and lack of commissures at the midline to an absence of differentiated midline lineages (Rothberg et al., 1990; Klämbt et al., 1991; Nambu et al., 1991). In *sim* mutant embryos all midline cells fail to divide and extend into the nerve cell layer and ultimately die (Nambu et al., 1991). These observations were based on light level histology and the use of a promoter construct for the *sim* gene (p[*sim/lacZ*]) which is simultaneously expressed in neuronal and glial midline lineages until stage 14 of embryogenesis. Our results extend the above observations as they show specifically that the ventral unpaired median neuron neurons do not migrate into the dorsal VNC, do not divide and die in *sim* mutant embryos (Sonnenfeld and Jacobs, 1994).

It was determined that all midline lineages in embryos mutant for the *sli* gene were displaced to the ventral midline and failed to differentiate (Klämbt et al., 1991; Rothberg et al., 1990). In contrast, our midline lineage analysis in *sli* mutant embryos revealed that while midline neuronal and glial lineages were ventrally displaced, the survival and differentiation of these cells was not altered from wildtype. Midline neurons in *sli* mutant embryos were not reduced in number and expressed markers of differentiation such as the 22C10 antigen

(VUM neurons) and the engrailed protein (MNB and its progeny). In addition, a combination of enhancer trap analysis and electron microscopy showed that differentiated MG ensheath commissural axons in *sli* mutant embryos (Sonnenfeld and Jacobs, 1994). Given the structural similarities of Sli motifs to adhesive properties in other proteins and the midline cell phenotype, it has been proposed that Sli functions as an adhesion protein. Sli is a protein secreted by the MG and is found on the commissural and longitudinal axons (Rothberg et al., 1990). The leucine-rich-repeats (LRR's) and epidermal growth factor (EGF) repeats in the predicted protein may participate in protein-protein interactions in other proteins. In addition, there is a motif at the carboxy-terminal of Sli that is conserved in proteins such as laminin, agrin and perlican, that are exported from cells (Rothberg and Artavanis-Tsakonas, 1992). Therefore, an analysis of the functional domains of Sli by site-directed mutagenesis can be performed to determine the purpose of each repeat to Sli function.

Given the initial expression of *rho* and *S* in multiple midline lineages and their later restriction to the midline glia (Bier et al., 1990; Kolodkin et al., 1994) we hypothesised that multiple lineages could be affected in embryos with mutations in these genes. Four midline lineages were analysed using enhancer traps and antibodies as cellular markers in *rho* and *S* mutant embryos (Sonnenfeld and Jacobs, 1994). Our results showed that both neuronal and glial midline lineages are affected by mutations in the *S* and *rho* genes. This invalidates the conclusion that the fused commissure phenotype results only from failure of midline glia migration (Klämbt et al., 1991). The contributions of the neuronal and glial lineages to axon tract development can be more accurately assessed by cell ablation methods.

The abnormalities in T13 expressing neurons in the lateral CNS in both collapsed and fused commissure mutants can be explained by the results of Kim and Crews (1993). The cell fate of lateral neuroblasts is thought to be influenced by the midline since they are derived from the same precursor cells as the VE.

1b) Common phenotypes of mutations in *spitz* group genes in the midline.

Two lines of evidence suggest that *rho* and *S* function in a common pathway during midline cell development (1) both genes are expressed in the mesectoderm and the midline cells (Bier et al., 1990; Kolodkin et al., 1994) and (2) the cellular and axon phenotypes of embryos mutant for *S* and *rho* are similar (Klämbt et al., 1991; Sonnenfeld and Jacobs, 1994).

The number of neurons in the ventral unpaired median neuron and MNB lineages are under-represented in both *S* and *rho* mutant embryos before defects in the MG are apparent. The reduction in neuronal numbers from wildtype in *rho* and *S* may result from lack of proliferation as there was no evidence for death of these neurons by enhancer trap analysis. Alternatively, the fate of the absent neurons may not have been specified. The remaining neurons express markers for neuronal differentiation such as 22C10 (VUM's) and the engrailed protein (MNB and its progeny).

Genetic evidence suggests that Rho is involved in the Egfr signaling pathway during wing vein development (Sturtevant et al., 1993). It is proposed that Rho increases cell adhesion and therefore the likelihood of a receptor-ligand interaction. By this mechanism the local concentration of interacting molecules is increased indirectly by the adhesive interaction. The lateral dispersion of midline

neurons in *rho* mutant embryos suggests there may be defective adhesion between the cells and supports this proposal (Sonnenfeld and Jacobs, 1994). In *rho* mutant embryos the MP1 and MP2 neurons are laterally dispersed from the midline but do not all appear to die. This may reflect a decrease in the amount of interacting molecules while the signaling mechanism remains intact.

There is no genetic evidence that the *spitz* group genes function in a common pathway during embryonic midline cell development. In order to assay for genetic interactions between genes expressed in the midline it is necessary to characterise the phenotypes of mutations in each gene alone and the result of ectopic expression of the genes. Descriptions of the phenotypes of midline cells in *rho* and *S* mutant embryos lays the groundwork for investigating whether they function in a similar pathway during development of midline neuronal and glial lineages. Genetic interactions during midline cell development can be investigated by studying epistatic relationships between *spitz* group genes. In addition, the phenotype resulting from ubiquitious expression of the *rho* gene can be used to assay changes in glial phenotypes in combination with weak alleles of *spi* and *S* and temperature sensitive alleles of *flb*.

Given the hypothesized function of *rho* in the spatial restriction of Egfr activity during wing vein development it is possible that *rho* acts in a similar fashion during midline glial cell development. The extra MG phenotype we observed after ectopic *rho* expression (Sonnenfeld and Jacobs, 1995a) suggests that *rho* may function in MG determination. Since *rho* is expressed initially in cells lateral to the midline (Bier et al., 1990) it is possible that it activates *flb* expression after ectopic expression in these cells, and induces a glial fate. In this case there would be a depletion of cells expressing ventral ectodermal cell

markers. Alternatively, a depletion of midline neurons would reveal whether extra MG resulting from ectopic *rho* expression are derived from midline cells. There are variations in the initial number of extra glia between segments during stage 12 and this may result from variations in the amount of Rho available for the Egfr after heat shock. It is also possible that the variations result from variable penetrance of the administered heat shock.

1c) Multiple pathways for midline glial differentiation?

It has recently been shown that the *pnt* gene encodes two putative transcription factors of the Ets family (P1 and P2) which direct glial cell differentiation in the embryonic CNS (Klaes et al., 1994). P1 RNA is expressed in the longitudinal glia, the A and B glia and the ventral unpaired median neuron support cells (Klämbt, 1993; Klaes et al., 1994). P2 RNA is expressed exclusively in the anterior and middle midline glia.

A phenotype resulting from ectopic expression of P2 was not observed using the GAL4 system (Klaes et al., 1994). Ectopic expression of P1 in all midline cells and adjacent neuroectoderm (using a *rho*GAL promoter) resulted in extra glia that expressed AA142. It was concluded that all midline cells differentiated as MG, however this was not justified by determining whether midline neuronal lineages were absent. The presence of dead MG was noted and supports our hypothesis that cell death serves to rid the CNS of unwanted cells (Sonnenfeld and Jacobs, 1995a). The extra MG phenotype resulting from ectopic P1 expression correlated with the absence of commissural axons and was proposed to reflect the important function of midline cells for commissure

formation (Klaes et al., 1994). This is in contrast to the lack of commissural phenotype we have observed in the presence of extra MG in *reaper* mutant embryos and resulting from ectopic *rho* expression (Sonnenfeld and Jacobs, 1995a). This discrepancy may be reconciled by noting that the glial phenotype described by Klaes et al., (1994) consists of disorganized glia that are laterally dispersed from the midline. However, the extra MG resulting from ectopic *rho* expression and in *reaper* mutant embryos are found only in the midline (Sonnenfeld and Jacobs, 1995a).

The relevence of induction of an MG fate by P1 to wildtype midline development remains to be determined since P1 is normally expressed in the LG and ventral unpaired median neuron glia. The phenotypes resulting from lack of *pnt* function (Klämbt, 1993) and resulting from ectopic P2 expression (Klaes et al., 1994) suggest that *pnt* is required for MG differentiation and not MG fate specification. However, during eye development the *pnt* gene is required for the determination of all photoreceptor cells and genetic evidence suggests that *pnt*P2 acts in a signal transduction cascade downstream of Ras1 (O'Neill et al., 1994). It is therefore possible that *pnt* has different functions during eye and CNS development.

In addition, the mutant midline glial phenotype of *pnt* embryos is different from that of the *spitz* group (Klämbt, 1993). In *pnt* mutant embryos the MG are arranged along the width of the commissural axons and eventually die (Klämbt, 1993). Double mutant combinations of *pnt* and *rho* result in a reduction in the number of commissural axons that cross the midline. In double mutant combinations of *pnt* and *S* no commissures are formed (Klämbt, 1993). The additive and severe phenotypes of double mutants suggests that both pathways

may be necessary for proper commissural formation. Therefore, experiments designed to identify genes interacting in the same pathway as *pnt* may reveal an alternate route for MG determination.

Future studies in this field will focus on the following questions: (1) what genes regulate expression of *spitz* group genes and the *DER* gene? (2) how do midline lineages become distinct from each other?

2) Cell death in the nervous system of *Drosophila* embryos

2a) Cell death in the midline glia lineage

Studies of midline cell fates in *spitz* group mutant embryos revealed that apoptotic cells could be identified in wholemount embryos by β-gal expression from enhancer traps (Sonnenfeld and Jacobs, 1994). I therefore investigated whether cell death occurs during development in the CNS midline of *Drosophila* embryos using enhancer trap analysis of neuronal and glial lineages. Apoptotic cells were identified in wholemount embryos expressing β-gal in the MGA and MGM (AA142 and P[*sli/lacZ*]) and those expressed in the MGP (X55). Apoptotic cells were not observed in embryos expressing β-gal from enhancer traps specific to midline neuronal lineages including the VUM's (T13) and the MP1 (P223). However, it is possible that cell death occurs in the MNB lineage in *Drosophila* embryos as it does in grasshopper embryos (Thompson and Siegler, 1993).

Quantitative studies of MG by enhancer trap analysis during embryogenesis revealed the following; (1) variations in the number and position of the surviving MG nuclei, (2) some surviving combinations of MG were more

common than others and (3) there was a temporal consistency of apoptotic MG in wholemount embryos. Approximately 50% of all MG (identified by AA142 and X55) die by apoptosis following a consistent temporal pattern during wildtype embryonic CNS development. We showed that the displaced MG in wholemount embryos were in macrophages recognized by anti-peroxidasin (Sonnenfeld and Jacobs, 1995a; Tepass et al., 1994). The ultrastructural characteristics of apoptotic MG were confirmed by electron microscopy. These results ruled out the possibility that decreases in MG numbers during embryogenesis were due to variable expression of β-gal from enhancer traps.

Variations in the number of midline progenitors and their progeny have recently been shown by *in vivo* labeling methods of *Drosophila* embryos (Bossing and Technau, 1994). A clone of cells arising from a progenitor labeled with the fluorescent dye DiI can be followed *in vivo*. By this method variations were found in the number of MG in a labeled clone in a ventral nerve cord segment and by stage 17, two MG were found between the anterior and posterior commissures. These results are consistent with our finding that the MGM have a greater probability of survival than the MGA and MGP.

Variable migration or random displacement of MG nuclei during development may contribute to variations in MG nuclei position between ventral nerve cord segments. However, there is no evidence for migration of MG between segments or of random displacement during development by *in vivo* labeling techniques (Bossing and Technau, 1994). It is also possible that position of MG nuclei is irrelevent as long as the processes of the MG ensheath the axons.

Do variable numbers of midline precursors contribute to later variations in MG number? There are variations in the number midline precursors expressing P[*sim/lacZ*] in ventral nerve cord segments and an average of 7.5 midline precursors was found in contrast to 8 progenitors previously reported (Bossing and Technau, 1994; Klämbt et al., 1991). We have also observed similar variations in midline precursors expressing β-gal in embryos carrying the P[*sim/lacZ*] reporter and the X81 enhancer trap (Sonnenfeld pers. obs.). Analysis of the midline precursors with P[*sim/lacZ*] and X81 did not reveal the presence of dead cells in wholemount embryos before stage 13 suggesting that midline precursor variability is not due to cell death. Furthermore, variations in the number of midline glia precursors were not observed in stage 11 embryos carrying the AA142 enhancer trap (Sonnenfeld and Jacobs, 1995a). It is therefore unlikely that variations in the number of precursors to the MGA and MGM contribute to later variations in their number.

How does midline precursor variability arise? To determine whether there is a genetic basis to the segment-specific variability in MP, a search for genes that have segment-specific differences in expression can be performed. Bossing and Technau (1994), propose that both positional information and interactions between midline precursors are involved in midline precursor specification in a ventral nerve cord segment.

How does lineage variability occur in other developmental systems? In grasshopper embryos the MNB progenitor is multipotent and generates glia and neurons at different developmental stages (Condron and Zinn, 1994). Inhibition of *engrailed* expression in the MNB lineage, by injection of antisense oligodeoxynucleotides, results in the conversion of glial precursors into neuronal

GMCs (Condron et al., 1994). In antisense-injected embryos there are no progeny that migrate anteriorly into the adjacent segment in the midline and there are no cells that express the glial specific protein annulin. In these same embryos there is an increase in neuronal progeny of the MNB. These results suggest that *engrailed* is required for glial fate specification in the MNB lineage. The results of Condron et al., (1994) are the first to show a molecular mechanism that controls a glial/neuronal cell fate decision in a multipotential cell lineage.

It has been suggested that the MGP arise from the MNB in *Drosophila* embryos (Bossing and Technau, 1994). Using the P[*sim/lacZ*] reporter, one to two cells were found in a dorsal position posterior to the posterior commissure in stage 16 embryos suggesting that they were the MGP originally characterised (Jacobs and Goodman, 1989a). However, in contrast to the MGA and MGM the cytoplasmic processes from these cells did not stain and the cells migrated ventrally with the MNB progeny (Bossing and Technau, 1994). Three observations suggest that these may not be the MGP we have analysed with X55: (1) anti-β-galactosidase and anti-engrailed do not co-localize to the cells designated as MGP (Sonnenfeld and Jacobs, pers. obs.) (2) the MGP identified with X55 do not migrate ventrally with the neurons (Sonnenfeld and Jacobs, 1995a) (3) the MGP are no longer present at stage 16 while X55 expression persists in the ventral neurons (Sonnenfeld and Jacobs, 1995a). To confirm their existence, the morphology of the MGP can be investigated by electron microscopy of X-gal labeled embryos carrying the X55 enhancer trap.

Although enhancer trap expression is not clonely restricted (Bossing and Technau, 1994) our results show that it can reveal important aspects of lineage

development such as cell death. It is therefore important to use multiple techniques to study cell lineages.

2b) Why do MG die? Is there a relationship between glia survival and axon contact?

The temporal consistency of MG death subsequent to their function in commissure morphogenesis prompted us to investigate the relationship between the MG and commissural axons. Ultrastructural and molecular evidence, in conjunction with mutational analysis, suggests there is a functional relationship between the MG and the commissural axon tracts in *Drosophila* embryos (Jacobs and Goodman, 1989a; Klämbt et al., 1990). In addition, expression of D-netrin, a recently discovered chemotropic factor, in the midline glia suggests that these cells are involved in axon guidance (Mitchell et al., 1994).

Given the morphological relationship between the MG and commissural axons (Jacobs and Goodman, 1989a) we proposed that MG death results from competition of filopodia for axon space (Sonnenfeld and Jacobs, 1995a). We used available tools to study MG survival after alterations in axon-glia contact. In embryos with mutations in the *comm* gene, decreases in axon-glia contact correlated with decreases in MG survival and an earlier onset of MG death. In *sli* mutant embryos maintanence of axon-glia contact correlated with wildtype MG survival. After ectopic *rho* expression there was an increase in the initial number of MG followed by their death and an eventual restoration of wildtype MG numbers. We therefore proposed that the ratio of midline glia to axon is important for MG survival in a manner similar to oligodendrocytes (Raff et al., 1993).

The mutant backgrounds used in the above experiments raise some questions about the validity of our MG-axon competition proposal. The genes *sli*, *comm* and *rho* are expressed in the midline and are involved in MG development (Rothberg et al., 1990; Seeger et al., 1993; Bier et al., 1990; Sonnenfeld and Jacobs, 1994). Therefore, the effects attributed to altered axon contact in the mutant embryos cannot be distinguished from absence of gene function in the case of *sli* and *comm* and from ectopic functions of *rho*. In *rho* and *S* mutant embryos the MG are initially present but ultimately die despite the presence of commissures (Sonnenfeld and Jacobs, 1994). It is possible however that the MG produced in these mutants are not capable of establishing contact with commissural axons. Alternatively, the altered MG survival may be autonomous due to loss of gene function. A clonal analysis of the MG in these mutant backgrounds would show whether more homozygous *comm* mutant MG die in the presence of axon contact than the surrounding wildtype MG. In this case the effects of MG death would be autonomous. Although technically difficult, one could also ablate the neurons producing the commissural axons and assay for MG survival.

While migration of MG between segments was not observed by *in vivo* dye labeling in *Drosophila* embryos, it occurs in grasshopper embryos (Condron et al., 1994). Glia from uninjected segments migrate into adjacent antisense-injected segments and assume normal positions around the commissures that, due to lack of *engrailed* expression, have no glia. It was proposed that midline glia number is regulated by the number of axons requiring ensheathment and that there is an increase in glial proliferation in response to available space (Condron et al., 1994). Although there was no evidence of glial proliferation, the number of

glia in control-injected segments was not reduced suggesting that the extra glia may have been generated. This proposal is similar to our MG-axon competition theory (Sonnenfeld and Jacobs, 1995a).

In embryos deficient for the *reaper* gene (Df(3l)WR10), almost all programmed cell death that normally occurs is blocked (White et al., 1994). In these mutants the cell death program is intact but fails to activate. To determine whether the reduction in MG numbers occurs in the absence of cell death we examined the expression of P[*sli/lacZ*] and X55 in *reaper* mutant embryos (Sonnenfeld and Jacobs, 1995a). There is an excess of MGA, MGM and MGP in embryos deficient for the *reaper* gene (Sonnenfeld and Jacobs, 1995a). In addition to their expression of MG markers, the extra cells were identified as glia by their dorsal location in the CNS and their apparent proximity to the anterior and posterior commissures in wholemount embyros. Electron microscopy would confirm whether the additional cells ensheath the commissural axons.

We then investigated whether the MG are destined to die only after axon contact occurs. Embryos carrying the AA142 enhancer trap were double labeled with anti-β-gal and terminal transferase (Tunel) to identify when DNA fragmentation occurs in MG (Gavrieli et al., 1992; White et al., 1994). Tunel labeled apoptotic glia were found in stage 13 embryos in macrophages outside the VNC. It was therefore concluded that DNA fragmentation was detectable at the same time that apoptotic cells were identified by immunocytochemistry in wholemount embryos.

The MG-axon contact theory would be refuted if excess glia are present in stage 12 (or earlier) *reaper*-deficient embryos and if *reaper* transcripts are expressed in the MG before stage 12. In addition, an ultrastructural investigation

of axon-glia contact in *reaper* mutant embryos will reveal whether there is space for the processes of extra glia on the axons. On this basis, we have observed a decrease in MG nuclei size from wildtype in *reaper* mutant embryos at the wholemount level (Sonnenfeld and Jacobs, pers. obs.).

If competition for available axon space is a general mechanism to regulate glial cell numbers in *Drosophila* we would expect variations in the number of other ensheathing glia including longitudinal glia and exit glia. Variations in the number of glia in embryos have been reported in the dorsal roof glia and peripheral glia (Nelson and Laughon, 1993).

The extra MG in *reaper*-deficient embryos may be derived from midline cells that die before glial specific markers detect them. The absence of dead cells during early stages in embryos expressing P[*sim/lacZ*] and X81 does not support this hypothesis (Sonnenfeld, pers.obs.). Alternatively, the absence of apoptosis may change cell interactions during glial determination and therefore divert extra cells to the MG fate. Interestingly, there were variations in the number of additional MG in both HS-*rho* and *reaper* mutant embryos in ventral nerve cord segments. This may reflect a variability already installed (ie. restriction in cell fate) in the number of precursor cells responsive to a putative `determining' signal. *In vivo* labeling of the midline cells in *reaper*-deficient embryos by the technique of Bossing and Technau (1994) may be useful to determine whether there are early alterations in the midline lineages. Enhancer trap analysis can be used to investigate whether there is a depletion in neuronal lineages at the expense of the MG in *reaper* deficient embryos. However, in *reaper*-deficient embryos there are no alterations in the number and position of neuronal lineages located at the ventral midline (VUM's and MNB progeny) expressing the X55 enhancer trap. In

addition, cell death may be involved indirectly in proliferation and investigating whether there is more cell division with Brdu in *reaper* mutants and after ectopic *rho* expresssion will address this issue (*reaper* AA142/Brdu double label).

What is the contribution of MG death to CNS midline development? This question can be addressed by studying effects of an excess of MG on midline development. In embryos containing additional MG such as HS-*rho* and *reaper*, the axon tracts develop as in wildtype embryos (Sonnenfeld and Jacobs, 1995a; Sonnenfeld and Jacobs, pers. obs.). Therefore, MG death and the death of other CNS cells is apparently not necessary for proper wildtype axon development. In contrast, a decrease in the number of MG in ventral nerve cord segments is detrimental to axon tract formation and possibly to embryogenesis as determined by studying *spitz* group mutants. It is therefore possible that the rate limiting event is establishment of axon-glia contact which appears, in most cases, to involve two midline glia (Sonnenfeld and Jacobs, 1995a; Bossing and Technau, 1994).

Our results are the first to document cell death in an identified cell lineage during wildtype *Drosophila* embryogenesis. We have specifically shown that the MG follow a *reaper*-mediated pathway to apoptosis (Sonnenfeld and Jacobs, 1995a). Therefore, despite the variety of triggers, most cells in *Drosophila* embryos appear to follow the same morphological (apoptosis) and genetic (*reaper*) pathway to death.

Cell death is a common event during development but is it essential? The available evidence suggests that development of the embryo proceeds in the absence of cell death. Mutations in ced-3 and ced-4 are not essential for viability

of *C. elegans* embryos (Yuan et al., 1993). Mutants in the *Drosophila* cell death gene *reaper* are not embryonic lethal (White et al., 1994).

2c) Are glia involved in the cell death process of neurons in the embryonic nervous system?

A guidance role has been proposed for glia in the embryonic CNS of *Drosophila* (Klämbt et al., 1990). Given the morphological and molecular diversity of glia additional functions may exist for these cells. An ultrastructural examination of the embryonic CNS was performed to explore the function of glia beyond commissure morphogenesis. This study revealed that ensheathing glia including longitudinal and midline glia are capable of phagocytic activity.

However, there were more subperineurial glia than ensheathing glia containing phagocytic material in the *Drosophila* embryonic CNS. These results reveal the plasticity of glia in the embryonic CNS. Cells in late stages of apoptosis are found inside the glia suggesting that glia are capable of degradation. Since the *Drosophila* CNS is avascular, haemocytes do not enter and subperineurial glia may substitute for their function in the developing CNS. Therefore, it appears that the CNS is designed (or has adapted) to accomodate the phagocytic activity of glia.

To further document the role of subperineurial glia as phagocytes it would be of value to determine how they respond to increases in cell death during *Drosophila* embryogenesis. An experiment using X-irradiation and electron microscopy of wildtype and *Bic-d* embryos will allow one to determine how

subperineurial glia respond to increases in cell death (Abrams et al., 1993). Proliferation of subperineurial glia in response to increased cell death can be detected using Brdu labeling of RK2 immunostained embryos. However, the number of subperineurial glia labeled with the antibody RK2 in *reaper*-deficient embryos was similar to wildtype. Although the size of the subperineurial glia was not measured, they contained increased numbers of apoptotic cells. In addition, increased numbers of macrophages do not occur in mutations with increased amounts of cell death (Tepass et al., 1994); instead, macrophages grow to a larger size. Likewise, in the absence of cell death, as in *reaper*-deficient embryos, macrophages decrease in size from wildtype (White et al., 1994).

In *Drosophila* embryos haemocytes are derived from the mesoderm and migrate along invariant migratory paths at the beginning of germ band retraction (Tepass et al., 1994). Haemocytes containing apoptotic material are referred to as macrophages and this conversion is dependent on cell death; in mutations with increased amount of cell death including *Bic-D*, the number of haemocytes turning into macrophages increases over wildtype (Tepass et al., 1994). During stages 13 and 14 of embryogenesis, haemocytes identified with anti-peroxidasin migrate between the dorsal surface of the ventral nerve cord and the mesoderm and between the ventral epidermis and the ventral nerve cord (Tepass et al., 1994). We have also found by electron microscopy that macrophages are consistently located outside the ventral and dorsal surfaces of the CNS. There are two lines of evidence supporting the requirement for macrophages in these regions during stage 13: (1) macrophages containing labeled apoptotic CNS neurons are found in these regions during early stage 13 as shown by electron microscopy (Sonnenfeld and Jacobs, 1995b) and (2) apoptotic MG were first

observed in macrophages outside the dorsal CNS during stage 13 (Sonnenfeld and Jacobs, 1995a). Similarly, in wildtype embryos, macrophages first appear in large numbers in the head where there are large amounts of cell death. In addition, haemocytes located around the gut do not become phagocytic until late in embryogenesis when cell death occurs in the gut (Tepass et al., 1994). Therefore, the time and place that haemocytes become phagocytic is determined by the pattern of cell death.

Initiation of the cell death process and phagocytosis are independent in *Drosophila* embryos; cell death occurs in the absence of macrophages in mutations in *Bic-D*, *tor*^{4021} and *twi/sna* double mutant embryos (Sonnenfeld and Jacobs, 1995b; Tepass et al., 1994) and macrophages can resume their function after the absence of cell death (White et al., 1994). This is in contrast to the requirement of macrophages for cell death in the developing mouse eye (Lang and Bishop, 1993). The disruption of macrophages in transgenic mice results in the persistence of two transient ocular tissues, the hyaloid vasculature and the pupillary membrane.

The studies describing midline glial death in this thesis provide the basis for determining factors which control cell survival. This knowledge will contribute towards understanding cell survival during neurodegenerative diseases and cancer. Studies of subperineural glia function in Drosophila may be a basis for understanding the microglial-immune connection in the vertebrate brain.

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