POST EMBRYONIC MIDLINE GLIA OF *DROSOPHILA*

THE MIDLINE GLIAL CELL LINEAGE IN THE POST EMBRYONIC FRUIT FLY *Drosophila melanogaster*

By

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ABSTRACT

This study investigated the position, ultrastructure and life history of glia in the midline of the *Drosophila melanogaster* Central Nervous System (CNS) by using enhancer traps (AA142, X81, *argos*^{w11}, *pointed*^{1277}) and reporter constructs (EE1, *slilacZ 1.0, slilacZ 4.5*) as glial-specific markers. Previous work has established that glia are necessary for proper formation and morphogenesis of longitudinal and commissural axon tracts, and axon ensheathment (Jacobs, 1993; Klämbt *et al.*, 1991; Jacobs and Goodman, 1989). By the end of embryogenesis there are three midline glial (MG) cells remaining in each segment (Sonnenfeld and Jacobs, in press) which this study verifies. In the third instar larval MG cells proliferate to 24 cells per segment as followed with the *E. coli lacZ* expressing *pointed*^{1277} strain. These *E. coli lacZ* expressing *pointed*^{1277} MG cells begin dividing 57 hours after hatching as seen with 5 -bromodeoxyuridine and hydroxyurea treatment. Some MG genes cease midline expression before MG proliferation (seen with EE1, X81), others (seen with AA142) continue to be expressed until the beginning of MG proliferation. Only the *argos*^{w11}, *slilacZ 1.0, slilacZ 4.5,* and *pointed*^{1277} expression strains continue *E. coli lacZ*

expression to the end of the larval stages. In the first larval stage a few perineuropilar glia begin to express the *E. coli lacZ* gene and increase to 400 cells per CNS in the third instar as seen in the *pointed*^{1277} marker strain. *pointed*^{1277} EM micrographs show that *E. coli lacZ* labeled cells have a glial-like ultrastructure. There was no co-localization of the *E. coli lacZ* expression in *pointed*^{1277} and an anti-RK2 (*repo*) antibody in third instar larvae. In *pointed*^{1277} pupae the MG cell *E. coli lacZ* expression stops after 48-72 hours and the prerineuropilar signal stops after 24 hours. In newly hatched *pointed*^{1277} adults perineuropilar *E. coli lacZ* expression is present with a cluster of 12 cells in the center of the neuropil. To summarize, after embryogenesis, in the *pointed*^{1277} marker strain, the MG cells begin dividing after 57 hours and the *E. coli lacZ* gene expression ends after the second day of the pupal stage. In the first instar, perineuropilar glia begin to label for the *E. coli lacZ* product and this expression ends by one day into the pupal stage, with re-appearance in the adult CNS.

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INTRODUCTION

Nervous systems require the support of glia. Glia ensheath neuronal axons and participate in the establishment of the three dimensional cytoarchitecture. Young neurons can also migrate along glia, an example is that of the Bergmann glia along which young neurons in the embryonic mammalian cortex migrate (Gilbert, 1991; Raisman, 1993; Stitt *et al.*, 1993). Glia also mediate transfer of metabolites, ions, and neurotransmitters between the neurons and the rest of the organism (Rakic, 1993; Williams and Deber, 1993; Bouvier *et al.* 1992). They provide structure for axonogenesis (Steindler, 1993), and perform macrophage-like functions similar to phagocytes (Galea *et al.*, 1992). Degeneration of glial cells causes diseases like multiple sclerosis (MS), in humans, which can be fatal in extreme cases (Davidson, 1991; Williams and Deber, 1993). The cause of this disease is the destruction of the myelin sheath in the white matter of the brain. The *dropdead* mutation in *Drosophila* (Buchanan and Benzer, 1993) has a similar phenotype to MS. The glial cells in the mutant adult contain "stunted processes" and what appears to be signs of apoptosis (programmed cell death), which leads to degeneration of the brain and death.

The first three glial lineages that develop in *Drosophila* are the longitudinal glia, midline glia (MG), and segment boundary cells. The

segment boundary cells are required for pathfinding of the intersegmental nerve (Goodman and Doe, 1993). The longitudinal glia are important in longitudinal axon tract formation (Jacobs, 1993) and later ensheath multiple axons (Jacobs and Goodman, 1989). The MG cells guide commissural tract axons (Jacobs and Goodman, 1989), help establish the cytoarchitecture through commissure separation (Klämbt *et al.*, 1991; Jacobs and Goodman, 1989), and ensheath multiple axons (Jacobs, 1993; Jacobs and Goodman, 1989). Disruption in the embryonic functions of the MG cells has the ultimate effect of fusing the longitudinal tracts or fusing the anterior and posterior commissures (Sonnenfeld and Jacobs, 1994). The above constitute the early functions of the MG cells. This thesis will examine position, ultrastructure and number of MG cells after embryogenesis.

Midline Glia in Embryonic Development

Nervous System Structure:

The structure of the fully developed *Drosophila* nervous system consists of 14.5 segmental ganglia (Campos-Ortega and Hartenstein, 1985). In each segment there are two longitudinal axon tracts, and an anterior and a posterior commissure which connect the two longitudinal tracts. The midline glia anterior (MGA) cells are situated ahead of the anterior commissure, the midline glia median (MGM) cells are situated between the commissures, the midline glia posterior (MGP) cells are situated on the dorsal surface of the

posterior commissure (Nambu *et al.*, 1991; Jacobs and Goodman, 1989).

Development of the Midline Glia:

The MG cells are derived from two ventrolateral single cell-wide stripes along the anterior/posterior axis of the embryo, termed the mesectoderm (Noll *et al*, 1993; Nambu *et al.*, 1991). These mesectodermal bands are situated between the mesodermal and neuroectodermal cell regions along the blastoderm embryo (Nambu *et al.*, 1993; Rao *et al.*, 1991). The two strips of mesectoderm join during gastrulation and divide synchronously to form 16 cells per segment by stage 10 (Nambu *et al.*, 1991; staging is defined by Campos-Ortega and Hartenstein, 1985). These midline cell precursors then move from the exterior into the interior of the embryo and are positioned in the middle of the invaginated neural lineages to form the midline. The midline lineages form three pairs of glia (MGA, MGM, MGP), the ventral unpaired median neurons (VUM), median neuroblast (MNB), a pair of precursor 1 neurons (MP1), and the unpaired median interneurons (UMI) (Bossing and Technau, 1994; Klämbt and Goodman, 1991).

At late stage 12 the MGP glia move anterior into the next segment and the MGM glia move posteriorly over the dorsal side of the MGA glia to separate the anterior commissure from the posterior commissure. One of the functions of the MG cells is to provide a permissive substrate for axonogenesis and to direct commissural axon growth (Klämbt and Goodman, 1991; Jacobs and Goodman,

1989). The growth cones change their behavior and direction when they cross the midline. Jacobs and Goodman (1989) state that the growth cones that will pioneer the anterior commissure contact the MGA cells (stage 12). It is later in development (one hour; stage 13) that the MG cells begin to ensheath axons (Jacobs and Goodman, 1989). Electron micrographs show that the glial cells contain numerous mitochondria and vacuoles. These glia also have more extensive endoplasmic reticulum than neurons, extend their cytoplasm to ensheath surrounding axons, and are irregular but cuboid in shape (Hoyle, 1986; Jacobs and Goodman, 1989; Klaes *et al.*, 1994; Sonnenfeld and Jacobs, 1994).

As has already been mentioned, the MGM glia migrate to separate the anterior and posterior commissures. Initially the two commissures develop in close association, with the anterior commissure (AC) developing straight across the midline and the posterior commissure (PC) developing bilaterally towards the anterior commissure to touch the AC at it's midpoint (Klämbt *et al.*, 1991). The MGM cells travel along the medial VUM axon and position themselves between the two commissures to separate the PC from the AC (end of stage 12). Therefore it can be said that the MG cells guide the formation of the anterior and posterior commissures in each segment.

By the end of embryogenesis (Stage 17), the six MG cells are reduced, through apoptosis, to three cells per segment (Sonnenfeld and Jacobs, in press). The usual order of the remaining cells is to

have the three cells in some combination of the MGA and MGM lineages, with the combination containing at least one MGM cell. What are their patterns from this time onward? Do they die, divide, or remain at three cells per segment? This becomes important in the context of metamorphosis. Do the MG cells perform similar functions during metamorphosis as they did in embryogenesis? These are the questions that initiated the work reported in this thesis.

Metamorphosis

Larval Growth:

During the larval developmental stages (called instars), the CNS of *Drosophila* does not change much with respect to it's basic structure (Kankel, 1980). However, growth during the larval stages prepares the system for the transition into adulthood. The cortex and neuropil grow thirty fold during these stages. It is during the larval stages that the remaining neuroblasts begin to divide to form immature neurons that will become functional in the adult fly (Levine, 1986; Truman, 1990a; Truman *et al.*, 1994).

90% of the above noted neuroblasts are located in the three thoracic segments (300 neuroblasts per neuromere) of the larval CNS (Truman and Bate, 1988). The first thoracic neuroblasts begin dividing at twenty-six hours post hatching (Truman, 1990a; Truman and Bate, 1988).

Pupal Stage:

As the organism reaches the pupal stage, massive remodeling of the CNS begins with regulation from hormones. Not only does neurogenesis and apoptosis occur, but many of the larval neurons (like the serotonergic and motor neurons) are re-used for different functions in the adult (Truman, 1992; Truman, 1990a; Levine, 1986). One round of apoptosis occurs at the beginning of metamorphosis and a second round occurs at the end of the pupal stage (Truman, 1990b). At 12 hours into pupation the larval cytoarchitecture has begun to be removed (Truman, 1990a). A constriction between the subesophageal and thoracic neuromeres appears which will eventually separate the brain from the ventral nerve cord (Truman, 1990a). Most of the increase in size of the CNS occurs in forming the brain and the thoracic segments, with a reduction in the size of the abdominal section of the ventral nerve cord. The largest expansion of the neuropil occurs between 18 and 36 hours after pupation and corresponds to the peak period of axonogenesis (Truman, 1990a).

Three hormones are involved in the regulation of metamorphosis. These are ecdysteroids, juvenile hormone (JH), and the eclosion hormone (Karim *et al.*, 1993; Truman *et al.*, 1990b and c; Levine *et al.*, 1986). It is the decreased concentration of ecdysteroids and absence of the JH that directs the organism toward metamorphosis (Truman and Schwartz, 1984). If JH were to be present, then the larva would go through another larval molt instead of pupating. As the concentrations of ecdysteroids drop the eclosion

hormone (EH) concentration increases in the hemolymph. It is the EH (produced in only two cells of the CNS) that begins ecdysis and directs the fate of the cells of the CNS (Truman, 1992).

However, it is not only the hormones that regulate all of the apoptosis in pupation. There is also an element of neuron-neuron interactions that control some of the cell death. For example, if the wings are prevented from being inflated in newly emerged adults, some neurons will not be triggered to die (Truman *et al.*, 1990b).

Glia in Metamorphosis:

During the second day of the third larval instar the MG cells begin to divide (Truman *et al.*, 1994). At this time, the MG cells express the B1 isoform of the EcR (Ecdysone receptor). At the onset of metamorphosis the cells switch to the A isoform of the EcR. Structurally, are the *Drosophila* glia also changing position and numbers to adjust to the changes occurring with the neurons? In Lepidopterans (Butterflies, Moths, and Skippers) the perineuropilar glia dissociate during metamorphosis (Lane and Treherne, 1980). There is also glial separation and reorientation associated with the axonal re-arrangements in the Lepidopterans (Lane and Treherne, 1980).

What is the numerical increase in the glia? Are the glial functions the same in metamorphosis as in embryogenesis? Does an increase in the number of midline glia occur to ensheath new neuronal growth? Where are the glia positioned during metamorphosis and the adult? When do the glia die? A gene

expression visualization technique is needed to identify the MG specific genes that are expressed in all stages of development.

Strains Studied

P-element insertions:

AA142 (enhancer trap), *argos^{w11}* (enhancer trap), *pointed^{1277}* (enhancer trap), *X81* (enhancer trap). *EE1* (reporter construct), and *slilacZ 1.0 and 4.5* (reporter constructs) are strains which express the *Escherichia coli lacZ* gene in the MG cells. The *lacZ* gene (within a P-element) has been inserted into the *Drosophila* genome so that it's expression is responsive to enhancer regions close to the point of insertion.

An enhancer trap is a method of generating cell specific markers. A P-element (transposon) containing the sequence for the *E. coli lacZ* gene (ß-gal), and the *Drosophila rosy* (Rubin and Spradling, 1982) or *white* gene (Klämbt and Goodman, 1991; Rubin, 1988; Bier *et al.*, 1989b; Karess and Rubin, 1984) is injected into a precellular egg. A plasmid with the transposase gene is injected along with the above construct. The transposase allows the P-element to insert randomly into the genome of the germline (Rubin, 1988; Karess and Rubin, 1984; Rubin and Spradling, 1982). When the P-element integrates near the 5' controlling region of gene X it will be active in the same cells and at the same time that the fly gene X is active. When gene X is transcribed, the *E. coli LacZ* gene will produce ß-galactosidase in the same cell (Rubin and Spradling, 1982).

From these flies homozygous expression strains are obtained through further crosses with *rosy*⁻ or *white*⁻ flies that contain a functional transposase gene to allow *Drosophila* transfer of the P-element to other locations on the genome (Rubin, 1988). Visualization of the *E. coli lacZ* gene product may proceed with antibody labeling or by assaying for the enzyme's activity using the X-Gal or Bluo-Gal chromogens as seen in Sonnenfeld and Jacobs (1994).

Reporter constructs are similar to the enhancer trap with respect to the observable effect. The P-element has the promoter of the gene X fused to the *E. coli lacZ* gene (Nambu *et al.*, 1990). It is then introduced into the fly genome as described for the enhancer trap screen. When the original gene X of the fly becomes active, ß-gal is produced.

Description of the Used Strains:

AA142: This enhancer trap maps to the 66D chromosomal region (Klämbt *et al.*, 1991; Klämbt and Goodman, 1991) and is next to a gene that has not been characterized. Therefore the specifics of the gene's structure and it's possible functions are unknown. This particular enhancer trap labels the MGA and MGM cells strongly and the MGP cells weakly (Klämbt *et al.*, 1991; Klämbt, 1993). It should also be noted that the MG cells are the only lineage to express *E. coli lacZ* in the embryonic CNS in this strain.

argos: The enhancer trap for this gene (*argos*^{w11}) is expressed in cells that express a secreted EGF-like protein (Freeman, 1994; Freeman *et al.*, 1992; Sawamoto *et al.*, 1991). *argos* contains

nucleotide sequences which suggest that the encoded protein is secreted into the extracellular matrix. Twenty-two hydrophobic residues at the N-terminus is similar to a signal sequence (Freeman *et al.*, 1992). In addition it contains no sequence for a peptide region that could span a membrane (Freeman *et al.*, 1992). In Monkey COS cells, *argos* protein has been demonstrated with Western blots to accumulate in the tissue culture media (Freeman 1994). There is also the possibility that the *rhomboid* gene product and the *argos* gene product work in the same developmental pathway (Freeman *et al.* , 1992) since both *argos* and *rhomboid* mediate wing vein development (Sawamoto *et al.*, 1991).

The tissues that are effected by a loss of function in the *argos* gene are the eyes of the adult, axonal guidance of the retinal projections into the brain, wing vein patterns, and the head and ventral cuticle of the embryo (Freeman *et al.*, 1992; Sawamoto *et al.*, 1991). The expression of this gene is also found in the MG cells (Freeman *et al.*, 1992) of the embryo. The gene is initially expressed in the cellular blastoderm followed by expression in the ventral mesoderm. The study of the loss of function mutations suggest a role of repressing cells from developing along certain developmental routes (Freeman *et al.*, 1992). Evidence includes that wild-type *argos* protein can rescue an extra photoreceptor cell phenotype, in the adult eye (Freeman *et al.*, 1992).

pointed : This gene (represented by the $point^{1277}$ enhancer trap expression strain) is a member of the spitz family, which

includes *rhomboid, spitz, star,* and *faint little ball* . These genes are not related by their genetic sequences but by the phenotypic expression of the null alleles of these genes (Mayer and Nüsslein-Volhard, 1988; Klämbt, 1993). The *pointed* (*pnt*) gene encodes two ETS-like proteins (Klaes *et al.*, 1994; O'Neill *et al.*, 1994; Klämbt, 1993) which are called P1 and P2 (important for the proper functioning of the MG cells) (Klämbt, 1993). ETS stands for the ETS domain present in all members of the family, and this domain binds DNA (Chen *et al.*, 1992; Klämbt, 1993; O'Neill *et al.*, 1994). The *pnt* gene itself is therefore suggested to be a transcription factor.

The function of the *pnt* and other spitz group genes is in the dorsal-ventral patterning of the epiderm and proper segmentation of the embryonic CNS (Klämbt, 1993). The null mutant phenotype displayed includes fused right and left head parts (pointed head) and the reduction of the width of the ventral band (Bier *et al.*, 1989a). When this gene is mutated the anterior and posterior commissures are fused due to the failure of the MGM cells to migrate since they do not recognize the VUM cell surface and do not travel along the median VUM axon to the proper position (Klämbt, 1993; Sonnenfeld and Jacobs, 1994). Also, the eyes of the adult lack some photoreceptor cells (O'Neill *et al.*, 1994).

rhomboid/veinlet : This gene belongs to the spitz gene family and the encoded protein has a molecular weight of 39,356 (Bier *et al.*, 1989a). The protein is a putative transmembrane molecule containing three to seven transmembrane regions and may

be part of a signal receiving pathway (Sturtevant *et al.*, 1993; Rutledge *et al*, 1992; Bier *et al.*, 1989a).

During embryogenesis the *rhomboid (rho)* gene expression is first seen, by using the enhancer trap strain *X81*, in the ventrolateral strips of mesectodermal precursor cells and later in the ventral midline, stretch receptor progenitors, and segment boundary cells (Sturtevant *et al.*, 1993; Bier *et al.*, 1989a). Adult wing vein formation is also regulated by this gene (Sturtevant *et al.*, 1993). In the adult female, *rho* is expressed in follicle cells and is important for proper dorsal-ventral axis-formation of the oocyte (Ruholoa-Baker *et al.*, 1993).

In the CNS of the *rho* null mutant the commissures are reduced by 25% to 50% in length (Mayer *et al.*, 1988), leading to a partial collapse in the CNS (Sonnenfeld and Jacobs, 1994). Specifically, the null mutant phenotype has a dorsal displacement of the midline glial cells with fused commissures (Sonnenfeld and Jacobs, 1994). Double mutant analysis of *rho* and *point* shows similar severity as that of the single mutants, such that when one or both of the proteins is not functional the entire pathway is not functioning. Therefore, it may be suggested that the two proteins are present and functioning in the same pathway (Bier *et al.*, 1989a).

single-minded : This gene, represented in this study by the *EE1* reporter construct, was first described by Crews *et al*, 1988 and Thomas *et al* , in 1988 as a midline cell specific nuclear localized gene product. It was originally classified as a *spitz* group gene

(Mayer and Nüsslein-Volhard, 1988; Bier *et al.*, 1989a; Muralidhar *et al.*, 1993) because of the mutant phenotype. The amino acid sequence is similar to that of the *period* gene that controls biorhythms in *Drosophila* (Crews *et al*, 1988). *sim* functions as a transcription factor which is consistent with the fact that the protein is localized to the nucleus. The structure of the *single-minded (sim)* protein is a bHLH (basic Helix-Loop-Helix) transcription factor (Menne and Klämbt, 1994; Nambu *et al.*, 1991).

It has been established that the *sim* protein is required for the proper expression of other genes such as *slit, toll, rhomboid,* and *engrailed* in the mesoderm (Kim and Crews, 1993; Nambu *et al* 1990). Therefore, this protein regulates the expression of midline glial-specific genes that are expressed after *sim* is expressed (Franks and Crews, 1994). However, unlike *rhomboid,* the mutant does not have a fused commissure phenotype but does have the collapsed longitudinal axon tracts. This mutant phenotype places the *sim* gene in the same family as the *slit* gene (Sonnenfeld and Jacobs, 1994).

slit: The *slit* product, represented in this study by the *slilacZ 1.0* and *slilacZ 4.5* reporter constructs, is an extracellular protein that has epidermal growth factor (EGF) and LRR (leucine rich repeats) domains (Rothberg, *et al* ., 1988 and 1990). It is 52kd in size and has differential intron splicing of the mRNA transcript at the end of the seventh EGF repeat (Rothberg, *et al* ., 1988 and 1990). The *slit* protein is secreted (Rothberg *et al.*, 1990; Rothberg and Artavanis-Tsakonas, 1992) and deposited on the longitudinal tracts and the

commissures. The expression pattern begins in all of the midline precursor cells and only later becomes restricted to the midline Glia (Wharton and Crews, 1993). This protein is also produced by the cardioblasts that form into the embryonic heart (Rothberg *et al.*, 1990).

Sonnenfeld and Jacobs (1994) classified *slit* mutations with the *sim* mutations as causing collapsed longitudinal axon tracts. The midline glia remain the same as the wild type MG cell number count but some become displaced ventrally or dorsally (Sonnenfeld and Jacobs, 1994). A null mutation of the *slit* gene also causes the heart to be disrupted such that the cardioblasts cell rows are broken apart without loss of cells (Perz, unpublished data).

Objectives

The objective of this thesis is to investigate the nature of the midline glial lineage and the morphology of these glia beyond embryogenesis. How do the CNS glia change during development, especially during metamorphosis? To what stage do these cells live? Which expression strain(s) express the *E. coli lacZ* protein for the full life time of the MG cells? What does the MG cell pattern look like in the adult, if the MG cells live to this stage?

These questions were investigated by antibody and histochemical labeling of the seven fly strains (representing six midline glial specific genes). To further characterize the *E. coli lacZ* labeled cells in *point*^{1277}, anti-RK2 antibody (RK2 is present in non-

midline glial lineages in the embryonic CNS (Campbell *et al.*, 1994)) labeled cells were studied. This was done to investigate the possibility that the cells that begin to label around the neuropil are glial and are of the midline glial lineage. An EM study on post-embryonic glial morphology was used to determine the ultrastructure of the labeled cells of the third instar larva and to address the question of cellular identity. Hydroxyurea and 5-bromodeoxyuridine treatment was also done to determine the period of mitotic activity of the MG cells in the third instar larva.

These experiments demonstrated that the number of glia of the embryonic and first instar larva is approximately three cells per segment and these cells increase (57 hours after the larva hatches) to 24 cells per segment as described with the *pointed*^{1277} strain. Also in the *pointed*^{1277} strain, perineuropilar glial (PG) cells began to label during the first instar larval stage with the number increased to 400 cells per CNS in the late third instar larva. An EM study on the third larval instar of the *pointed*^{1277} strain demonstrated that all of the labeled cells are glial. In the *pointed*^{1277} strain the PG cell *E. coli lacZ* expression stopped 24 hours into the pupal stage and the MG label ended between 48 and 72 hours into this stage. Upon adult emergence of the *pointed*^{1277} *E. coli lacZ* excpression re-appeared in cells positioned perineuropilarly but; it was not determined whether these adult cells were the PG cells visualized in the larval samples.

MATERIALS AND METHODS

Fly Strains

All of the expression strains are either a promoter fusion or an enhancer trap. The enhancer traps are *argos*^{w11} (w;P[w+]) created by Dr. Freeman, *rhomboid [X81]* (P[*rho/lacZ*]), *AA142* (P[w+, *lacZ*] created by Klämbt *et al.* 1991), *pointed*^{1277} (P[*lacZ*; w+]). As previously noted, enhancer trap strains have a P-element with the *E. coli* lacZ gene being incorporated into the *Drosophila* genome near or in a gene. The *E. coli lacZ* gene is active in the same cells which shows gene expression.

The reporter constructs are *single-minded* (*EE1*) (P[*sim/lacZ*] located on the X chromosome, created by Dr. Crews as reported in Muralidhar *et al.*, 1993), *slilacZ 1.0* (p[*slit 1.0lacZ*] on the X chromosome; Rothberg *et al.* 1988), and *SlilacZ 4.5* (p[*slit 4.5lacZ*]; Rothberg *et al.* 1988). As previously noted, reporter constructs are P-elements which incorporate the *E. coli* lacZ gene and the whole or part of a gene's regulatory region. The Transposon is randomly inserted into the *Drosophila* genome and when the gene is active the *E. coli lacZ* is also activated in the same cell.

Reagents

The primary antibody utilized was an antibody to *E. coli* ß-galactosidase (Sigma, chemical company, St. Louis, MO) and the secondary antibody was goat anti mouse conjugated to Horseradish Peroxidase (HRP) from Jackson ImmunoResearch laboratories Inc. to bind to the *E. coli LacZ* gene product. The anti-RK2 antibody which was a gift from Dr. A. Tomlison (Columbia University) was used in the double labeling study with the *pointed*^{1277} marker strain. The secondary antibody used with the anti-RK2 was a goat anti rat conjugated to HRP (Sigma). Brdu (from Boehringer Mannheim) labeled cells were labeled using anti-Brdu from (Sigma).

Histochemicals used include X-Gal (5-Bromo-4-Chloro-3-Indolyl-ß-D-Galactopyranoside) from Diagnostic Chemicals LTD (160 Christian St., Oxford, Conneticut, USA) and Bluo-Gal (5-Bromoindolyl ß-D-galactopyranoside) from Gibco-BRL. These two chemicals are substrates for the *E. coli LacZ* protein and form blue crystals that are visible with light and electron microscopy (Sonnenfeld and Jacobs, 1994; Jacobs, 1993).

Embryo Collection

Embryo collection began by placing the adults on apple juice agar plates with a smear of yeast (Hiroma *et al.*, 1985; Jacobs, 1993). After 12 hours, the plates were removed and the embryos were dechorionated by violently spraying 50% bleach onto the plates and letting the embryos stay in the bleach for 10 minutes. This was

followed by collection on nitex mesh, and rinsing with dH_2O and blotting dry.

Light Microscopy Fixation and Labeling with Antibodies

The light microscope fixation and antibody labeling procedure was adapted from Patel *et al* ., (1989), Grenningloh *et al.*, (1991), and Jacobs (1993). The dechorionated embryos were placed into a vial containing 5 mL heptane, 4.5 mL phosphate buffered saline (PBS, pH 7.4) and 0.5 mL 37% formaldehyde for 30 minutes, for fixation. The lower aqueous fixative layer was removed and methanol was violently added, using a pasteur pipette to devitellinize the embryos. The devitellinized embryos sunk to the bottom of the test tube. These embryos were then washed three times with methanol to remove any traces of heptane. The methanol was then replaced with PBS with 0.5% Triton X (PBT). Two washes for five minutes each in PBT and one wash for thirty minutes in PBT on a rotator were performed.

Following the washes, the embryos were incubated for thirty minutes in 110 mL of PBT+N (100 µl PBT and 10 µl normal goat serum). After this, 1.5 mL of anti-ß-Gal antibody (1:200 dilution) was added and the samples incubated overnight at 4 degrees Celsius. The samples were then washed three times for five minutes each and then twice for thirty minutes in PBT. The samples were then incubated for thirty minutes in 100 mL of PBT+N, with addition of 0.5 mL goat anti-mouse antibody (1:200) conjugated to horseradish

peroxidase (HRP) for two hours at room temperature. The embryos were then washed three times for five minutes each in PBT and twice for 30 minutes each in PBT. If high background was a problem, incubation overnight at 4°C with rotation in PBT was performed to achieve better signal to background ratio on the labeling.

The samples were then suspended in 200 µL PBT plus 100 µL DAB (3,3'-Diaminobenzidine Tetrahydrochloride made to a stock concentration of 1mg/ml of PBS) for ten minutes. Then 3 mL of 3% H_2O_2 were added to allow the HRP to interact with the DAB to form the brown label. The reaction was stopped with the addition of PBT. To preserve and clarify the samples, dehydration with increasing concentrations of ethanol (50%-75%-90%-95%-100%) for five minutes each was completed. Then the ethanol was replaced with methyl salicilate. To view, the samples were placed on a glass slide and mounted in permount. The microscope used was a Zeiss Axioskop with a 12V 50W halogen lamp and Nomarski optics. Photographs were taken with a Zeiss MC 100 camera using Kodak technical pan film or Fuji RTP slide film.

Electron Microscopy Fixation and Labeling

The post embryonic nervous systems were dissected out of the organisms and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). This method was used to preserve the enzymatic activity of the ß-galactosidase protein (Hiroma *et al.*, 1985;

Ashburner, 1989; Jacobs *et al.*, 1989; Jacobs, 1993). The samples were then transferred with a Pasteur pipette to a test tube and fixed at 4°C for thirty minutes. After fixation, the samples were washed in several changes of PBT for two hours at room temperature. Once this was accomplished, X-Gal (10 mM PO_4 buffer(pH = 7.2), 150 mM NaCl, 1 mM $MgCl_2$, 3.1 mM $K_4Fe[(CN)_6]$, and 3.1 mM $K_3[Fe(CN)_6]$) buffer solution was added. The buffer was removed after ten minutes and the labeling solution was added. The labeling solution contained the above noted X-Gal solution with the addition of 25μl of 8% w/v Bluo-Gal (EM level work) or X-Gal for light microscope work. The samples were incubated from two hours to overnight at room temperature or 37 degrees Celsius depending on the level of *E. coli lacZ* expression. After the samples had been labeled, the labeling solution was replaced with several washes of PBT.

The samples were then treated with several changes of 0.1 mM sodium cacodylate. The tissues were placed into 1% Osmium tetroxide in the cacodylate buffer for 30 minutes. After washing with cacodylate buffer and several changes in distilled water, the samples were placed into 5% uranyl acetate for 30 minutes. After removal of the uranyl acetate with several washes of distilled water the samples were dehydrated and embedded (Jacobs *et al.*, 1989). To orient the EM sections, light level thick sections were cut and counter stained in Basic Fuscin (Jacobs *et al.* 1989). This procedure consisted of heating the gelatin coated slide and placing a coating of water saturated with Basic Fuscin on it with a few drops of acetone

saturated with Basic Fuscin. After about 30 seconds the excess stain was washed off and slide was dried and mounted with permount.

Embedding

This protocol was described by Jacobs, 1993 (adapted from Jacobs *et al.*, 1989). Samples were dehydrated with a series of washes with 50%, 70% and 90% ethanol respectively for ten minutes each. Then washed with 95% and 100% ethanol each twice for ten minutes with a final wash in 100% ethanol for thirty minutes. The samples were then incubated in 50% absolute ethanol 50% plastic (4.4g Araldite, 6.6g Epon resin, 12g DDSA) with catalyst (0.3 ml DMP 30) for thirty minutes. This was followed by replacement with 25% absolute ethanol:75% plastic with catalyst for thirty minutes. The samples were then placed in100% plastic with catalyst (overnight in a desiccator and replaced with new plastic for incubation for another day in the desiccator. The next day, the mold wells were filled half way with the plastic plus catalyst and baked in the oven for one to three hours at 75°C. This has the result of hardening the plastic enough to have the embryo lay in the middle of the plastic and not along the bottom edge. The mold wells were then filled completely and the sample placed and oriented in the plastic.

Cell Counts

When the midline glial cells in embryonic and first instar samples were counted, the material was labeled using the above

noted anti-ß-galactosidase protocol and preserved using methyl salicylate. Cell counts were taken using an oil immersion lens (60X) and a sagittal sample orientation. Mean, median, mode, and standard deviation were calculated by standard methods.

Larval stages were determined by the physical differences of the three stages in conjunction with growth of a cohort of samples at 25°C. Larvae were selected by collecting eggs over a three hour period with eventual collection of the larvae that hatched within one hour of each other (adapted from Sokolowski *et al.*, 1984). This was called a cohort. At 25°C the eggs hatch in 22-24 hours with the first and second instar larval stages lasting for one day each, the third instar larva lasting for two days, prepupa for 4.5 hours, and the pupal stage for 4.5 days (Graf *et al*, 1992; Rubin, 1988). In conjunction with this temperature control specific developmental traits were used to determine the instar of a specific larva. The first instar larva has mouth hooks with usually one tooth, the second has two or three teeth, and the third instar larva has nine to twelve teeth (Demerec, 1965).

Third instar larvae were also chosen and identified by the fact that they were in the wandering phase (Roberts, 1986), and the presence of a thoracic ventral bulge on the ventral surface of the CNS (Truman, and Bate, 1988). Samples were either mounted in permount or placed in 100% ethanol. The samples placed into permount were used as a reference as to what the intact CNS looked like.

An alternative method was used to count the number of immunolabeled cells. After reimmersion into 100% ethanol the samples were embedded in plastic as described above. Serial sections were taken at 2.5 microns and mounted with permount. The sections were photographed and enlarged . The enlarged negative was traced onto sheets of overhead plastic. The sheets were then super-imposed onto each other to reconstruct the nervous system. From these sheets the numbers of cells could be most accurately counted. Cell counts were performed for the ventral nerve cord with separation of midline glia from perineuropilar glia. Separation of the two glial types were done on the basis of position and cell size The GM glia were inside the neuropil on the midline and larger in size with respect to the PG cells, the PG cells were located outside of the neuropil and smaller in size with respect to the MG cells.

5-Bromodeoxyuridine Labeling

5-Bromodeoxyuridine (Brdu) was added at a concentration of 1 mg/ml autoclaved yeast paste and fed to larvae at the desired time of development (Truman and Bate protocol of 1988) for three hours. Brdu is incorporated into the genome during replication as an analog of thymine (Rizki and Rizki, 1969). Brdu at a concentration of 0.5 mg/ml of food will not kill the labeled cells, it causes increased growth with developmental modifications (Rizki *et al.*, 1972) caused by nucleotide mispairings (Rizki and Rizki, 1969). Two samples were taken, the first occurred directly after the three hour incubation of

the staged cohort and the second was taken as the samples developed to the wandering stage in late third instar Both sample types were fixed for one hour at 4°C in 4% formalin in PBS. The antibody protocol used was essentially the same way as described by Truman and Bate(1988) and Ashburner(1989). Incubation with anti-Brdu primary antibody at 1/2,000 dilution at 4°C for 36 hours followed by several washes in PBT and incubation at 4°C for 36 hours in goat antimouse HRP (1:200) antibody. After several washings in PBT, the samples were treated with DAB and dehydrated in ethanol to be placed in methyl salicylate.

Hydroxyurea Ablation

50 mg/ml Hydroxyurea (HU) in autoclaved yeast paste (1 part baker's yeast: 2 parts water) was made up as described by DeBelle and Heisenberg, 1994. A four hour pulse of 50 mg/ml of HU in the media of the newly hatched larvae was given. This resulted in the total ablation of the MBs in 93.5% of the adults and reduced MBs in the rest. The HU treatment did not seem to effect the function or structure of the rest of the brain. HU is used as an anti-leukemia and solid tumor drug, a potent DNA synthesis inhibitor which is anti-mitotic and cytotoxic depending on the concentration and exposure time that is used, and increases the chances of chromosomal aberrations by two times. HU is readily absorbed and distributed throughout the body (Timson, 1975).

At the appropriate time larval cohorts were starved in water for one hour. The starved larvae were placed in HU treated paste for four hours. The larvae were removed by placing them on a nitex sieve and washed several times in water. The larvae were then placed in fresh fly food to develop into wandering stage third instar larvae. At this time the nervous systems of the larvae were removed using the above noted protocol for X-Gal labeling.

Fixation of the Pupal and Adult Nervous Systems

Pupal casings were broken open the with forceps, surgical scissors, and a scalpel in cold1X Schneider's medium (Truman and Bate, 1988). They were then fixed for 30 minutes at 4°C in several changes of 2% Glutaraldehyde in 0.1M Sodium Cacodylate buffer. The samples were then labeled with X-Gal and the tissue was removed, washed, and viewed with a microscope.

For the adults, the head, wings, and legs were cut off with surgical scissors. The dorsal surface of the thorax was then dipped into cyanoacrylate glue and placed on a glass slide rimmed with silicone cement. Glutaraldehyde was placed on the samples and the ventral surface was ripped open with forceps to expose the nervous system. The CNS was fixed at 4°C for 30 minutes in 2% Glutaraldehyde in 0.1M Sodium Cacodylate buffer, and the same was done for excised brains. Labeling with X-Gal was done as previously described.

RESULTS

Embryonic Morphology of the Used Strains

Comparisons between the *AA142, argos (argos^{w11}), rhomboid (X81), pointed (pointed^{1277}), single-minded (EE1), and slit (slilacZ 1.0, slilacZ 4.5)* expression strains revealed that for stage 17 embryos and first instar larvae, differed in the non-CNS expression patterns and also in the CNS expression patterns. This is seen in Figures 1.1 and 1.2. All but *argos^{w11}* and *pointed^{1277}* have the same pattern in the CNS.

The *AA142, X81, EE1, slilacZ 1.0,* and *slilacZ 4.5* strains had approximately 3.2 glial cells per segment that express the *E. coli lacZ* gene in both the embryonic and larval samples. The above numbers reflect a measured average of at least 8 samples for each stage of each expression strain. The number of cells in *argos^{w11}* samples were 4.5 midline cells per segment in the late embryo and 4.3 cells per segment in the first instar larva. This number is inconsistent with what is known about the larval CNS which has a maximum of 3 midline glial cells remaining at the end of embryogenesis. The number of cells in the *pointed^{1277}* expression strain were 2 glial cells per segment in both the late embryo and first instar larva. The difference in the number of cells per segment between the late embryos and the larval stage of all of the strains were not

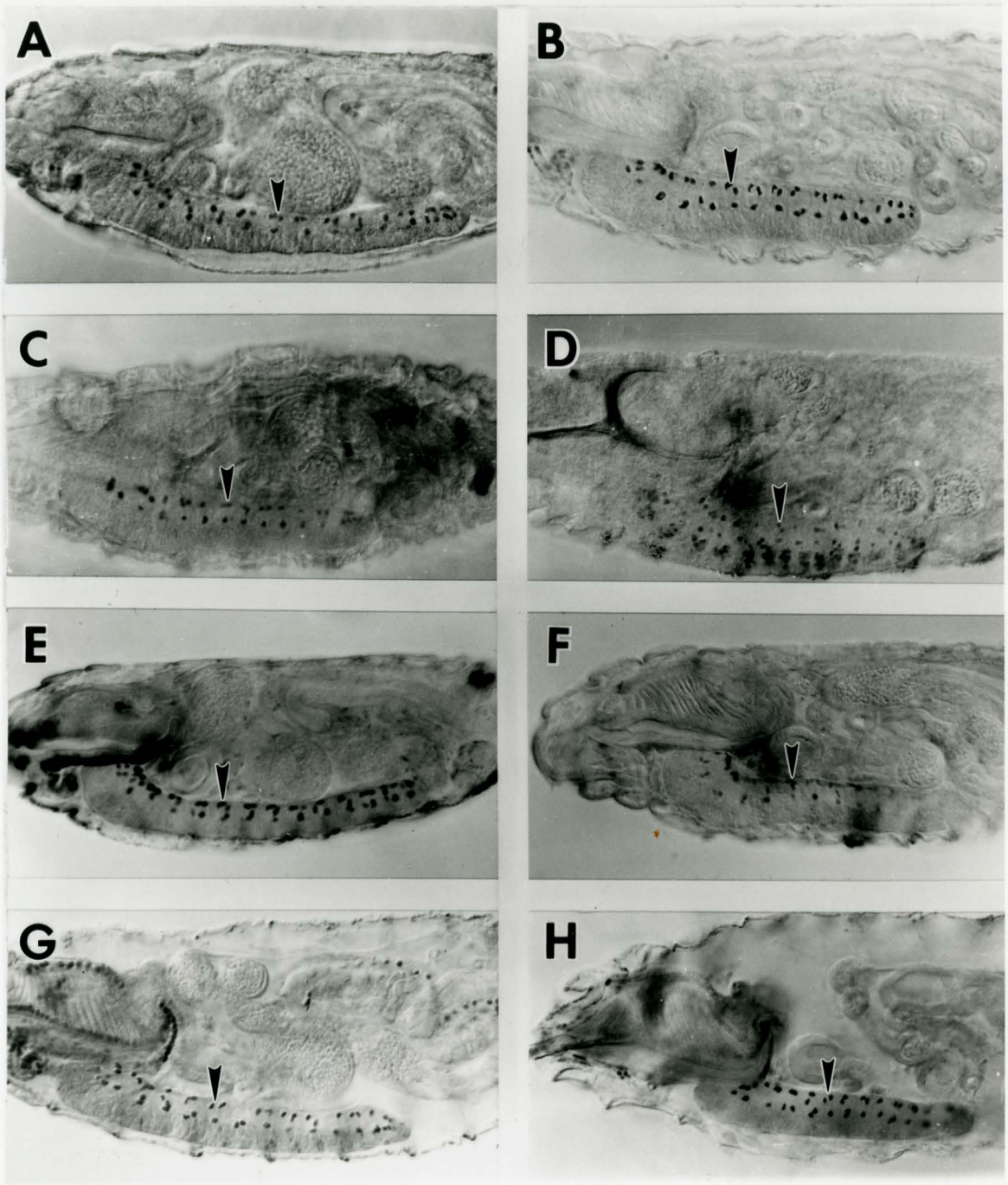
significant. These numbers were summarized in Table 1.

Along with the midline glial labeling there were other cell lineages in the larval CNS that labeled in each strain. In the *single-minded* expression strain, there were a cluster of cells positioned just ventral of the midline that began to label at stage 17 of embryogenesis. This can be seen in Figure 1.1 panel D. It is not known what lineage these cells belonged to. In the *argos*^{w11} strain the number of midline cells far exceeded the number of midline glia that survive to the end of embryogenesis. The lineage of the extra cells is not known. Non MG cell lineages can be seen in some of the embryonic and larval samples in Figures 1.1 and 1.2.

There were also some cells that began to label during the first instar larva, in all of the strains, that were in a perineuropilar position. However, these perineuropilar cells were characterized in the *pointed*^{1277} expression strain in Figure 2.

Other than the CNS expression pattern, there were other tissues/cells which can be seen to express the *E. coli lacZ* gene at the stages that were viewed. In the *AA142* strain, the supraoesophageal ganglia, gut, posterior spiracles, ring gland, some muscle cells, and the posterior spiracles labeled. In the *argos*^{w11} strain the denticle bands, posterior spiracles, the ventral half of the esophagus which could be the pharyngeal ridges, cells at the anterior tip, and cells lining the gut all labeled. *X81* labeled the midgut, proventriculus, denticle belts, region called the frontal sac, cells located in the tracheal tree, and a cluster of cells located at the

anterior tip of the organism. In the *pointed*^{1277} expression strain, the proventriculus, lymph gland, some cells around the mouth hooks, midgut, posterior spiracles label with the antibodies. *EE1* has anti *E. coli* ß-galactosidase antibody labeled in the midgut and posterior spiracles. In *slilacZ 1.0* the posterior spiracles, the ring gland, and the anterior tip labeled. *slilacZ 4.5* labeled the posterior spiracles, some of the midgut, and the mouth hooks. The difference between the two *slit* strains is that the 4.5 strain has a larger piece of the promoter region than the 1.0 strain and it is possible that this difference accounts for the difference non-CNS expression patterns. Due to the different areas that labeled on the samples, not all of the labeled tissues/cells can be seen in Figures 1.1 and 1.2


A
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H

FIGURE 1.1: Labeling patterns of various *E. coli lacZ* expressing strains for stage 17 embryos and early first instar larvae.

This figure presents the anti-ß-Gal labeling patterns of the glial marker strains 250 times magnification. The left column (A, C, E, G) present stage 17 embryos, the right column (B, D, F, H) present the first instar larvae. A and B) are the embryo and first instar, respectively, of *AA142.*, C and D) present *EE1 (single-minded).*, E and F) are the *argos*^{w11}. samples, and G and H) are for *X81 (rhomboid).* Arrow heads are the midline glia.

FIGURE 1.2: Labeling patterns of various *E. coli lacZ* expressing strains for stage 17 embryos and early first instar larvae

As in Figure 1.1 the right side (I, K, M) present stage 17 embryos, the right side (J, L, N) present the first instar larvae. I and J) present *slilacZ 1.0* with K and L) presenting *slilacZ 4.5*., and M and N) are of the *pointed*^{1277} expression strain. Arrow heads are the midline glia. All pictures are at 250 times magnification

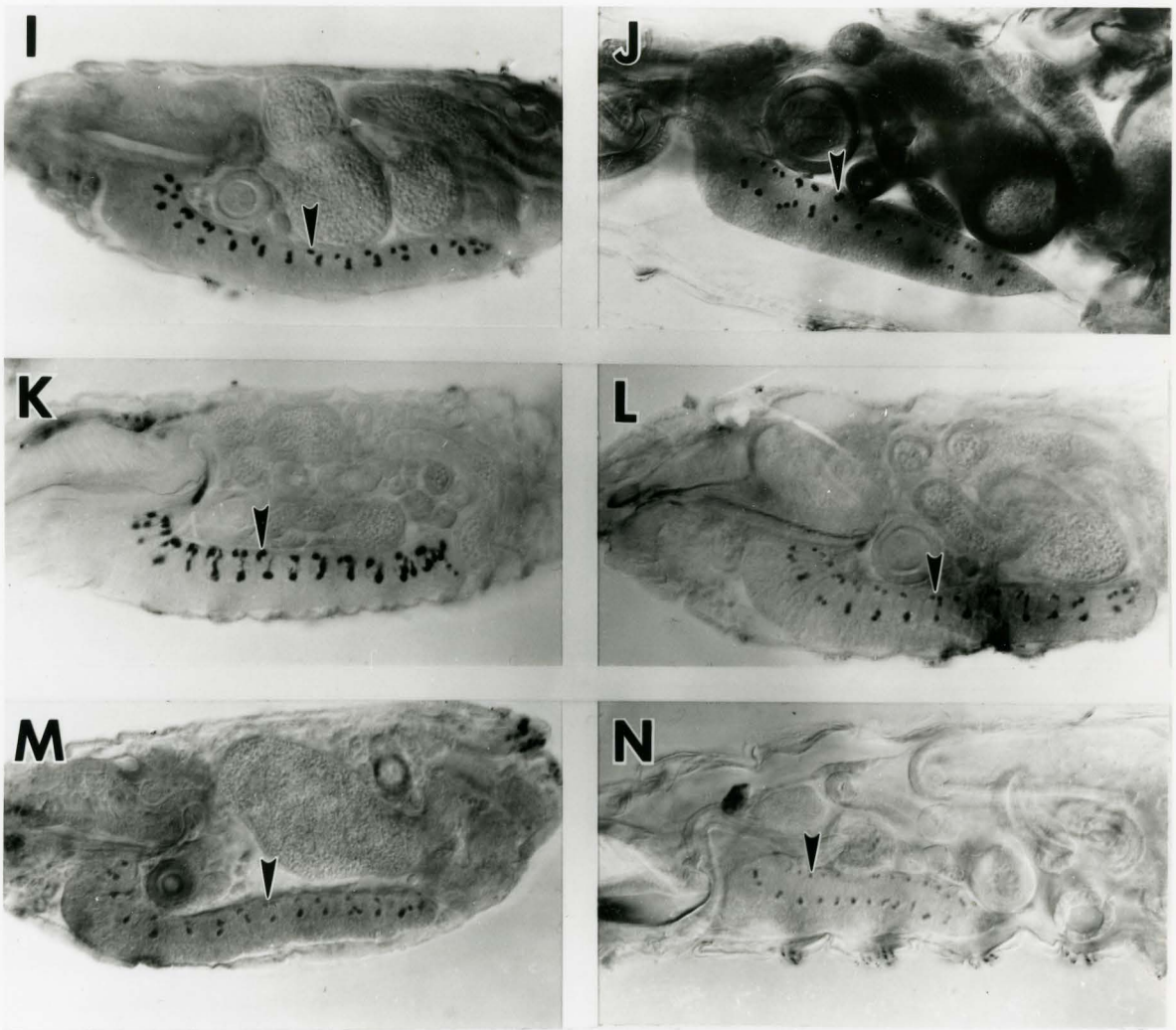

I
J
K
L
M
N

TABLE 1: Embryonic stage 17 and first instar larval MG cell counts of the seven strains used with mean and standard deviation.

This table represents the accumulated cell counts for all seven strains for the stage 17 embryos and early first instar larvae. Included are the average, mean, and standard deviation of the number of cells per segment.

MG Cell Counts

STRAIN	AVG	SD	# OF COUNTED SEGMENTS
AA142			
Embryo	3.2	±0.14	64
First Instar	3.2	±0.23	48
EE1			
Embryo	3.1	±0 .3	48
First Instar	3.0	±0.2	48
SlilacZ 4.5			
Embryo	3.0	±0.3	64
First Instar	2.8	±0.2	64
SlilacZ 1.0			
Embryo	3.2	±0.2	64
First Instar	3.1	±0.2	64
X81			
Embryo	3.2	±0.2	64
First Instar	2.8	±0.2	64
Pointed^{1277}			
Embryo	2.2	±0.3	63
First Instar	2.1	±0.3	48
Argos^{w11}			
Embryo	4.5	±0.26	63
First Instar	4.3	±0.52	57

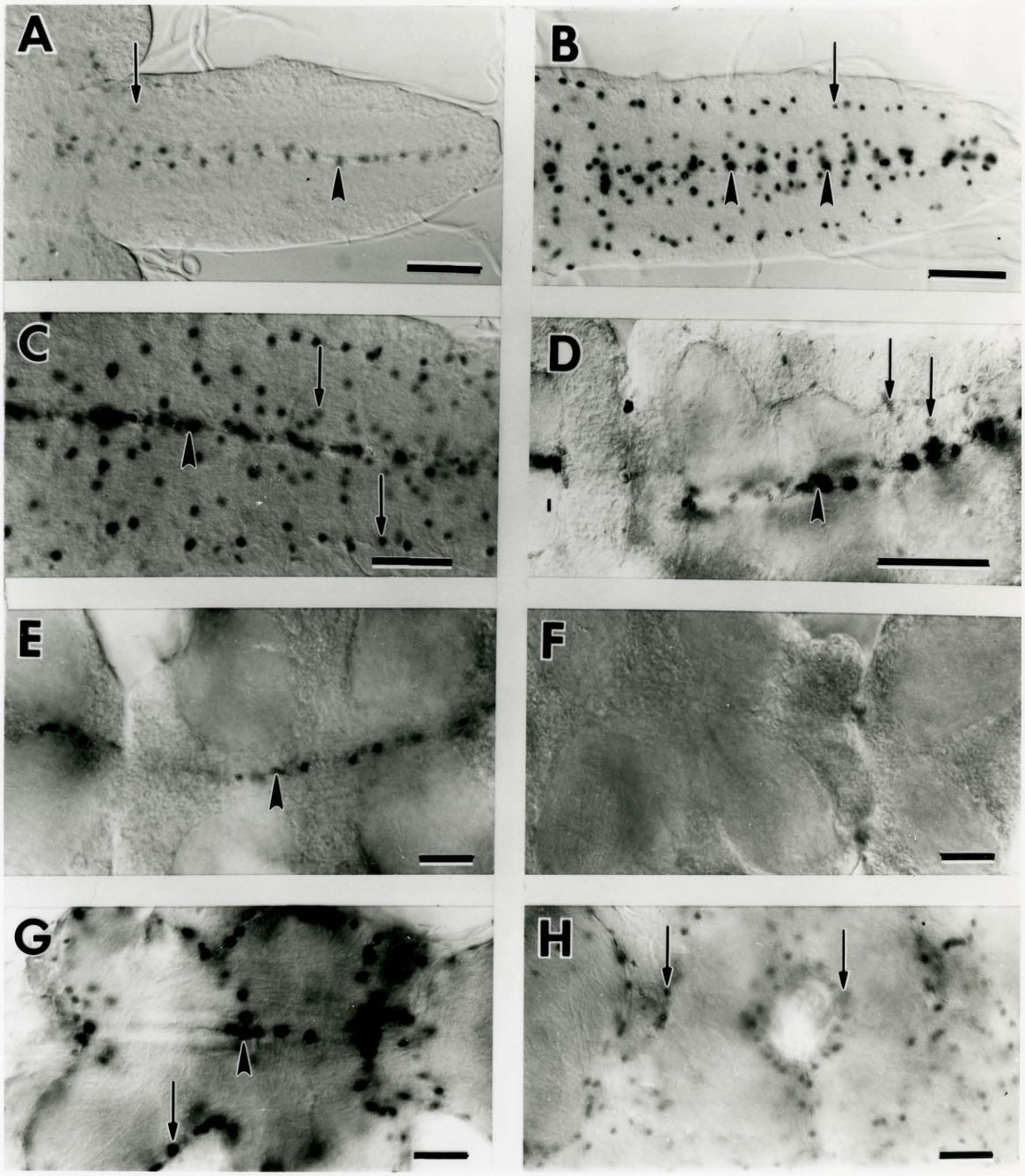
Post Embryonic Patterns

After dealing with the embryonic and early larval labeling patterns, it was decided that the *pointed*^{1277} *E. coli lacZ* expression strain would be the strain that most of the rest of the project should focus on. The reasoning behind this is that the *pointed*^{1277} strain had the most clear, and longest expressing *E. coli lacZ* gene protein in the midline in comparison to the other six strains. However, work on the expression patterns was done on all of the strains up to the time of metamorphosis.

One feature that was seen immediately was the presence of perineuropilar cells in all of the expression strains except *argos*^{w11}. Figure 2 shows that the perineuropilar cells began to label during the first instar stage for the *pointed*^{1277} strain. From this stage on, they divided to form a large number of cells as seen in panels B and C of Figure 2. These cells remained *E. coli LacZ* positive till the first day into pupation. During the first 24 hours of pupation, these cells ceased their expression as seen in panel D of Figure 2. In panel D, reduced perineuropilar labeling remained; but it is light and not nearly as many cells were labeled with respect to the third instar larva. However, no apoptotic bodies have ever been noticed, therefore programmed cell death cannot be established from this data. Perineuropilar cells labeling for *E. coli lacZ* expression returned upon the emergence of the adult fly as seen in panel G and H of Figure 2.

FIGURE 2: Developmental profile of the *pointed*^{1277} expression strain from the first instar larva to the adult using X-Gal.

A) First instar larva (270 times magnification) showing perineuropilar cells and midline glia; B) Second instar larva (220 times magnification) showing more perineuropilar cells but still contain 2 midline glia per segment; C) wandering third instar larval CNS (260 times magnification) showing the increased number of midline cells per segment and the large number of perineuropilar cells; D) 24 hours after the onset of metamorphosis (260 times magnification) showing the disappearance of the labeling of the perineuropilar cells but the midline glial labeling is still intact. E); 48 hours after the onset of metamorphosis (200 times magnification); F) 72 hours after the onset of metamorphosis showing the absence of all labeling (200 times magnification); G and H) are the adult labeling patterns with G being that of the ventral nerve cord and H being the brain. Both G and H (200 times magnification) show abundant perineuropilar cells labeling. Arrow heads refer to the midline glia and the arrows refer to the perineuropilar cells. The bar equals 50 μm.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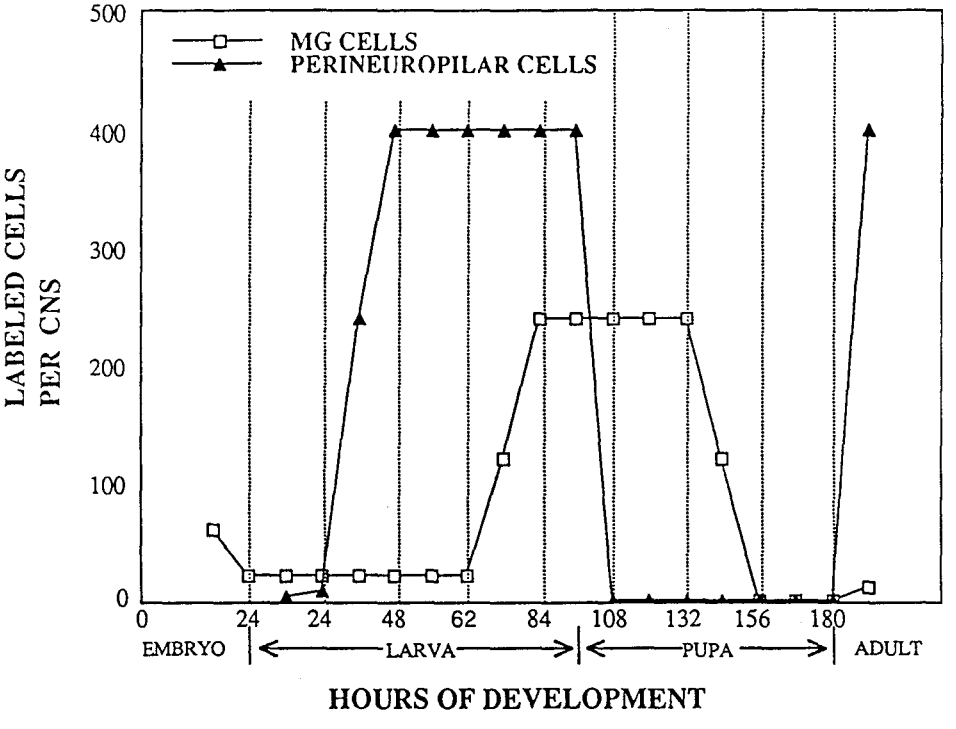
The presence of these cells has not been discussed in the published literature until now. Are these cells glial or not? If they are glial are they of a midline lineage? The first question can be addressed with an electron microscopic study to determine the cell's morphology. Therefore the perineuropilar were unlikely to be of the midline glial lineage because of their smaller size and parassagital location.

As with the perineuropilar cells, the midline glia varied in number during development of the fly. In *pointed*^{1277} the number of midline glial cells remained the same till the middle of the third larval instar, when the number of cells increased from 2 to 24 midline glial cells per segment. The *argos*^{w11} strain had a similar increase in midline cell numbers (24 cells per segment) but no perineuropilar labeling was evident. The *slit* strains showed a similar increase up to 20 cells per segment, at which time the midline expression stops. With *AA142* a small increase in the number is seen but the midline expression also ceased. With both of the *EE1* and *X81* strains, the midline expression stopped during the feeding stage of the third larval instar. These results were placed in Chart 1. In panel B the chart shows the number of glial midline cells at different times for all strains. Panel A represents the change in overall *E. coli lacZ* expressing CNS cells in the *pointed*^{1277} strain.

CHART 1: *E. coli lacZ* expressing cell numbers during the development of *Drosophila.*

A) Representation of the total number of *E. coli lacZ* expressing cells in the CNS of The *pointed*^{1277} strain B) Representation of the change in labeled glial cell numbers per segment during development into the Adult. NOTE: Data for the *slit* and *argos*^{w11} strains are not available after the larval stages.

A


MG CELLS
PERINEUROPILAR CELLS
LABELED CELLS PER CNS
500
400
300
200
100
0
0
24
24
48
62
84
108
132
156
180
EMBRYO
LARVA
PUPA
ADULT
HOURS OF DEVELOPMENT

B

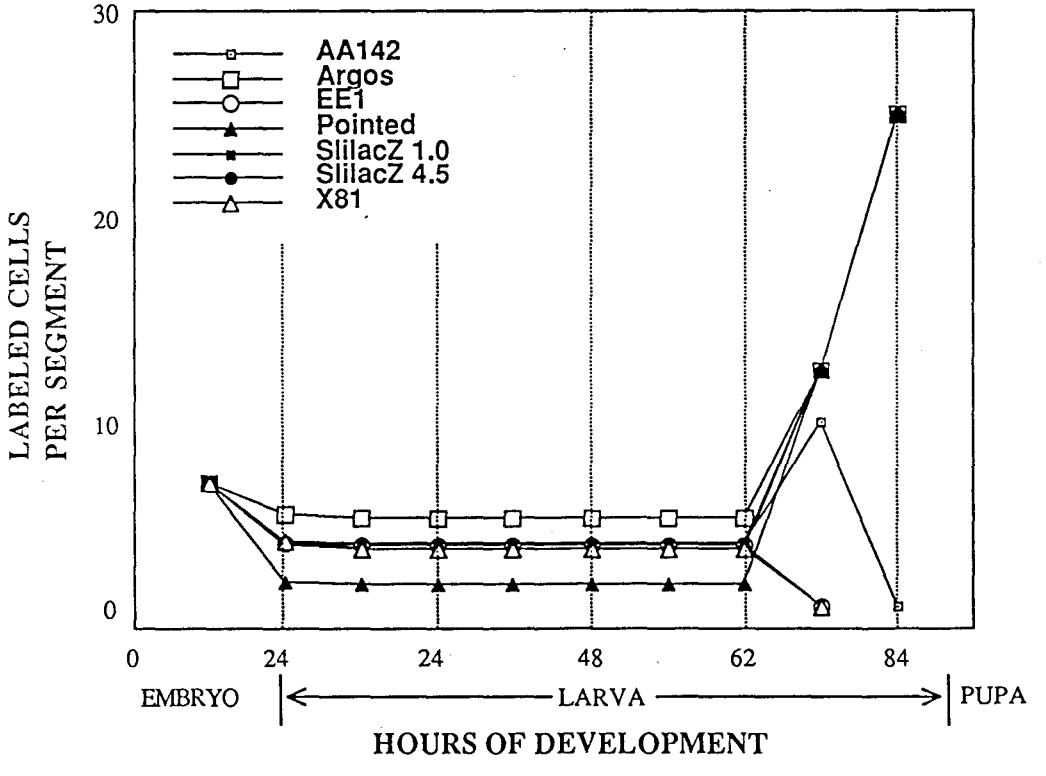
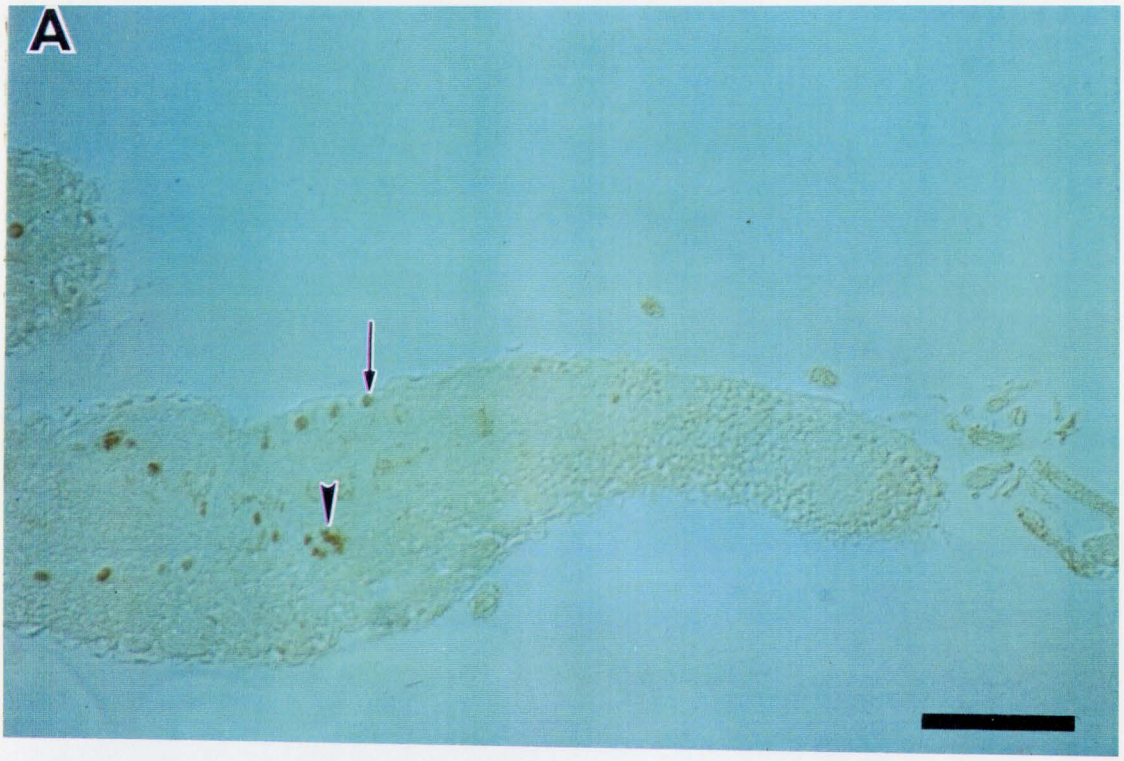
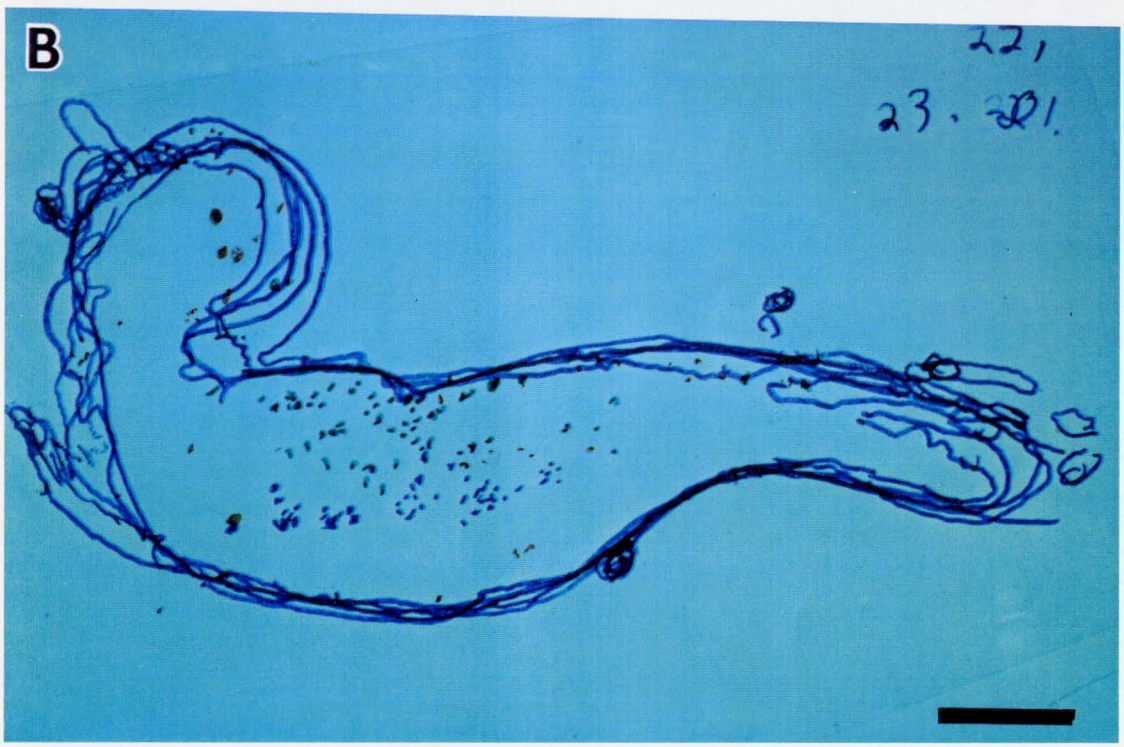

AA142
Argos
EE1
Pointed
SlilacZ 1.0
SlilacZ 4.5
X81
LABELED CELLS PER SEGMENT
30
20
10
0
0
24
24
48
62
84
EMBRYO
LARVA
PUPA
HOURS OF DEVELOPMENT

Figure 3 shows the method employed to count the increase in cell numbers in the late third instar larva. Anti-ß-Galactosidase labeled third instar larval nervous systems were embedded in plastic and sectioned for light microscopy. Serial saggital sections were photographed as seen in panel A of Figure 3. The black and white negatives were enlarged as would normally be done in the printing process, but were then traced onto cellulose acetate sheets. These sheets were then stacked to reconstruct the CNS and recording of the cell numbers proceeded accurately. This is seen on panel B, which was a composite of five of these acetate sheets with the blue and green marks representing the midline cells and the red marks representing the perineuropilar cells which increased to 400 cells in the entire ventral nerve cord.

FIGURE 3: Sectioned material from a wandering third instar larva.

Panel A) is one of the sections (2.5 mm thickness) of antibody labeled samples showing midline cells and perineuropilar cells being labelled (400 times magnification). B) represents the technique used to reconstruct the sections to count cells (360 times magnification). There are 5 sections represented in this reconstruction showing midline cells as blue and perineuropilar cells as green, the arrow heads refer to the midline glial cells, and the arrow refers to perineuropilar cells. The bar equals 50 μm.


A

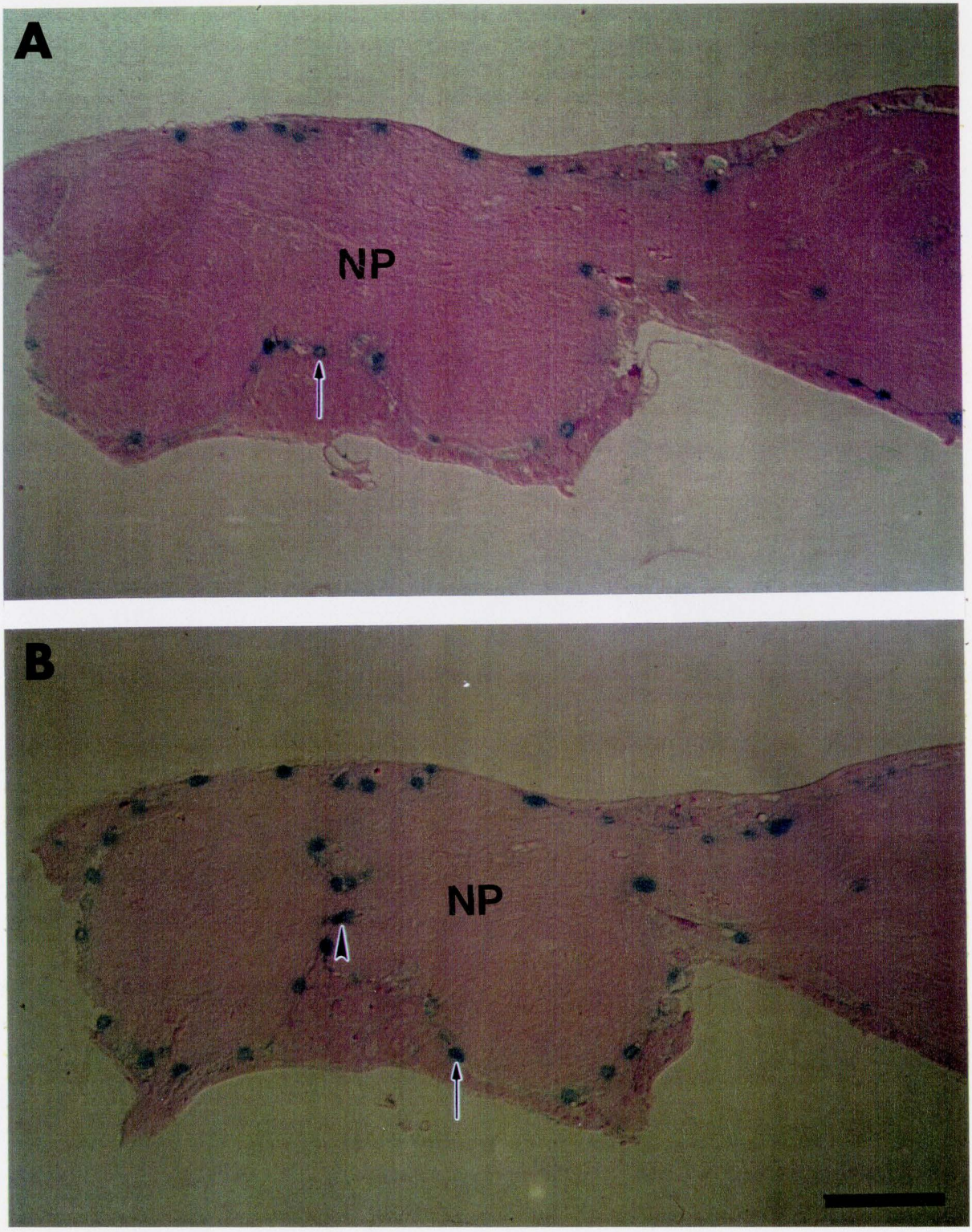

B
22,
23. 21.

It is not known if the increase in midline cells number was due to mitotically reactivated midline glia or another lineage which were dividing and expressing the marker in the midline. After all, there are neuronal lineages present in the midline (i.e., VUM, MNB, MP1). Without knowing the morphology of all of the labeled cells it is possible that some of the cells were not glial.

In the *pointed*^{1277} expression strain, the midline glia continued to label 48 hours into metamorphosis as seen in panel E of Figure 2. In panel F (72 hours into metamorphosis) there no longer was any labeling in the CNS. As with the perineuropilar cells, no apoptotic bodies were noted. In the adult CNS there was a cluster of midline cells in the abdominal section of the ventral nerve bundle. Were these glial cells that have survived metamorphosis or some other lineage? An X-gal labeled adult thorax of the *pointed*^{1277} strain was sectioned and stained with basic fuscin. In the serial sections it was observed that the neuropil was mostly devoid of labeled cells (Figure 4). Two exceptions were a few cells that were found in the middle of the neuropil and a cluster of cells in the thoracic region of the adult CNS. The cell cluster was possibly a large invagination into the center of the neuropil which contained perineuropilar cells. A small channel was seen to be connecting the cluster with the ventral edge of the neuropil. The rest of the labeled cells were in a perineuropilar location and likely represent the same glia that labeled in the larval and pupal preparation. Verification that the labeled cells were actually glia must come from an electron

FIGURE 4: Sections of an Adult *pointed*^{1277} CNS Labeled with X-GAL.

This figure contains saggital sections (290 times magnification) of the adult nervous system of this expression strain at different levels. A) is a section that would be found near the midline of the CNS. B) is a section that is from the midline of the CNS. For most of the cells the designation of perineuropilar cells should be given. Arrow heads refer to possible midline glia and arrows refer to the perineuropilar cells. Anterior is to the left and dorsal is at the top. The bar equals 50 μm.

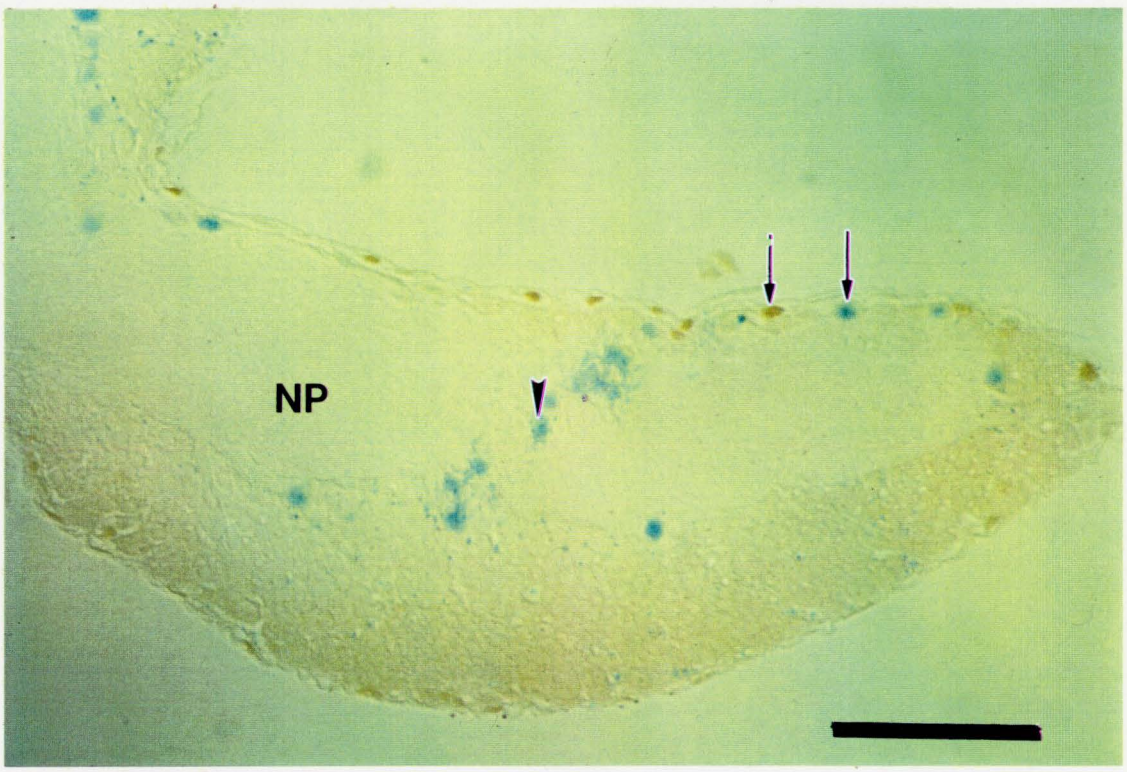

A
NP
B
NP

microscope study.

To establish the origins of the *E. coli lacZ* expressing cells of the *pointed*^{1277} strain, an anti-RK2 antibody was used (Campbell *et al.*, 1994). The antibody binds to all of the glia in the embryonic CNS except for those of the midline lineage. A double labeling experiment was done in an attempt to better define the lineages of the various *E. coli lacZ* expressing cells of the CNS of *pointed*^{1277}. The result is seen in Figure 5. In this figure the blue labeled cells were the cells that were expressing the *pointed*^{1277} marker and the cells that were labeled brown were those that had labeled with the RK2 antibody. In this section and in all of the other sections of the sample there were no double labeled cells. The two labeling patterns were mutually exclusive.

FIGURE 5: RK2 antibody/X-Gal double labeled *pointed*^{1277} expression strain third instar larval section.

This figure (460 times magnification) shows the RK2 label as the brown colour and the cells expressing *E. coli lacZ* in blue from the X-Gal label. Arrow heads refer to the midline glial cells and arrows refer to the perineuropilar cells. The bar equals 50 μm.


NP

Ultrastructure of the Labeled Cells

In Figure 6 a thick (2.5 nm) cross-section of a wandering third instar larval CNS was stained with basic fuscin. This allowed the ability to note where exactly the cells that were labeling were located in the CNS. A transmission electron micrograph (Figure 7) of the wandering third instar larva of the *pointed*^{1277} expression strain, showed the morphology of all of the labeled cells was consistent with the known morphology of embryonic glial cells (Jacobs and Goodman, 1989). Bluo-Gal was used to label the *E. coli lacZ* expressing cells for ease of visualization of the crystals in the micrographs. The Bluo-Gal crystals showed up as long very electron dense structures located peripheral to the nucleus (Jacobs, 1994; Sonnenfeld and Jacobs, 1994). All of the labeled cells were elongated, had well developed endoplasmic reticulum, and were sending cytoplasmic extensions out to encompass axons (Jacobs and Goodman, 1989). All of the labeled cells that were viewed, were glial in their morphology. However, there were cells that looked glial but did not label with the Bluo-Gal (data not shown). It is possible that the glial cells that did not label corresponded to the cells that labeled with the RK2 antibody that was seen in Figure 5.

FIGURE 6: Cross sectioned 2.5 mm third instar *pointed*^{1277} larva labeled with Bluo-Gal.

The section (640 times magnification) shows three cells labeling in the midline (arrow head) with one cell (arrow) expressing the lacZ gene on the left neuromere. The bar equals 50 μm.

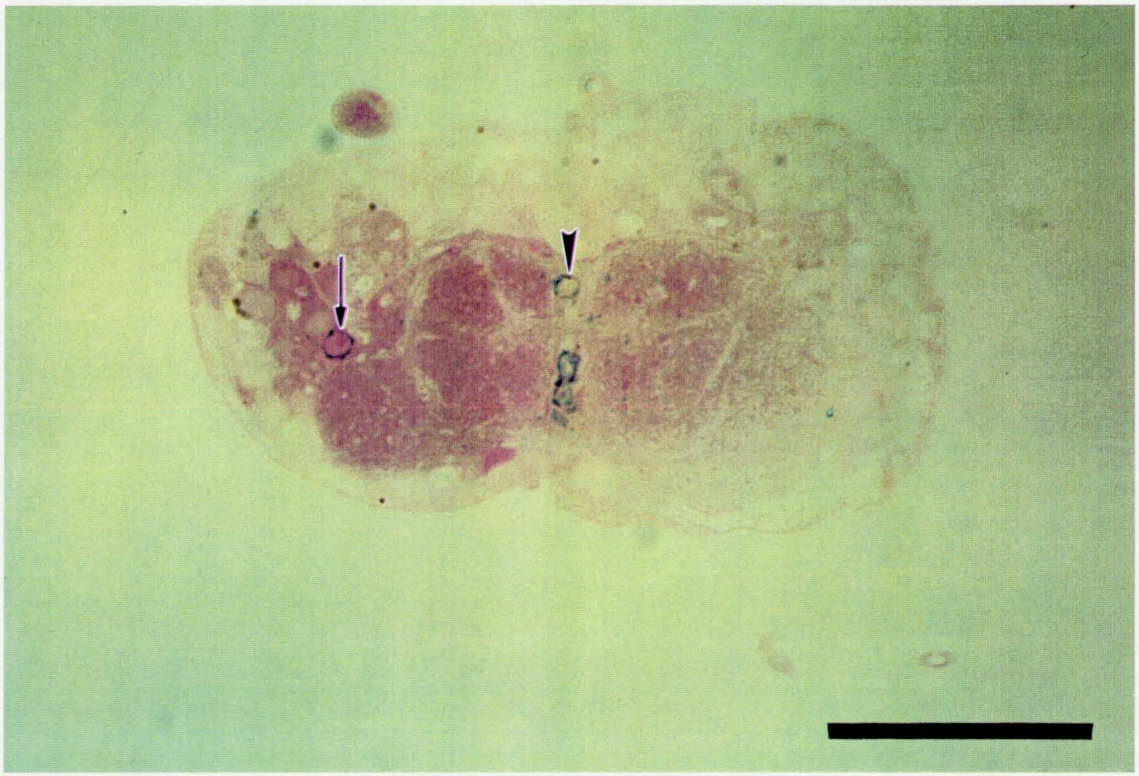
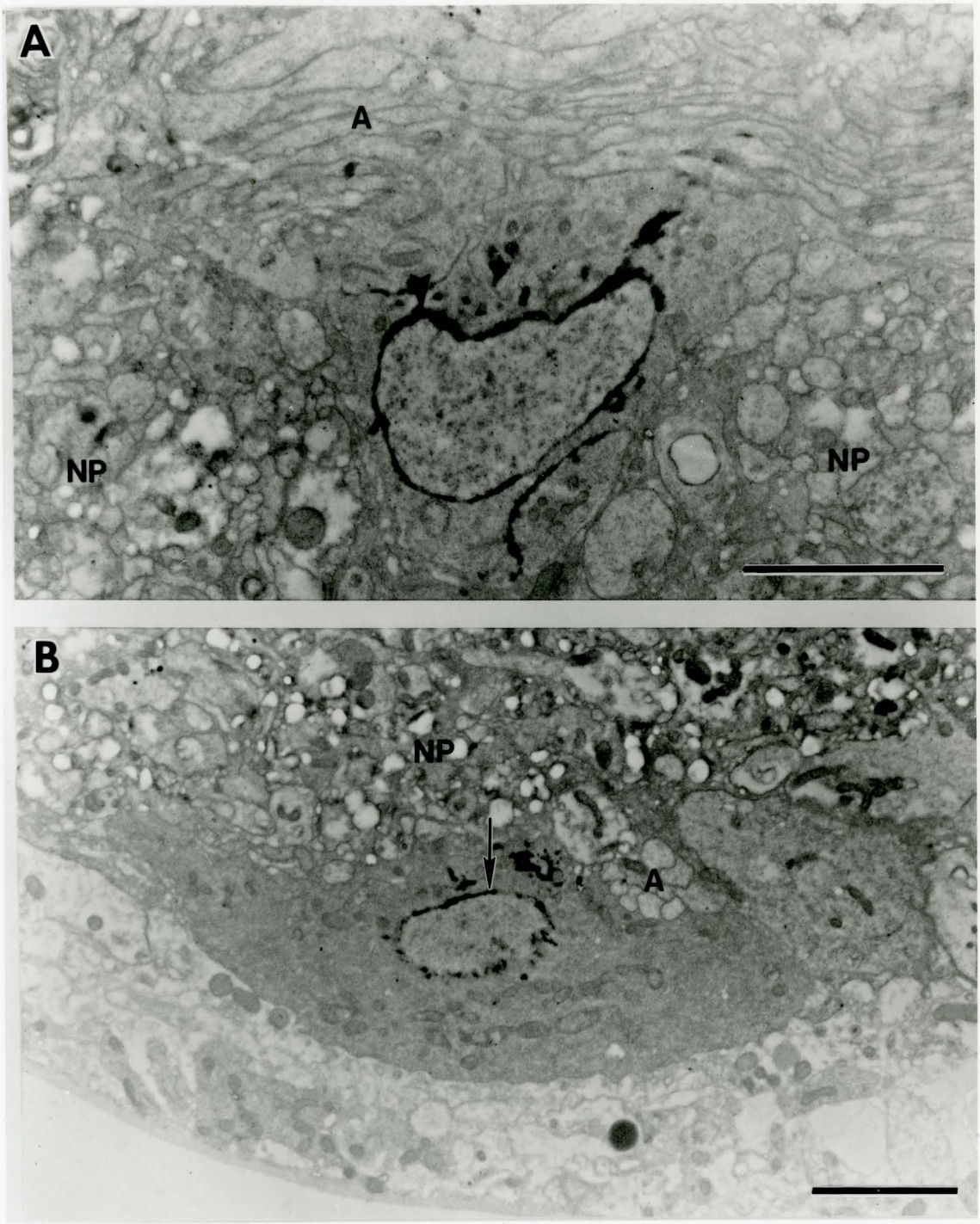


FIGURE 7: Electron micrographs of the same sample as represented in Figure 5.

Panel A) represents a cell labeling in the midline at 7 500 times magnification. B) represents a Bluo-Gal labeled PG cell at 5 500 times magnification. The arrow head refers to the midline glial cell, the arrow indicates the labeled PG cell, NP indicates the neuromere, and A indicates axons. The bar equals 2 mm.


A
A
NP
NP
B
NP
A

Chemical Labeling of the Midline Glial Cells

An experiment using Brdu was done to determine the exact time in which the glial cells became mitotically active during the third larval instar. The Brdu (1 mg/ml) was administered by adding it to yeast paste and feeding this mixture to the larvae at the desired time. The time period used started at 57 hours after hatching and went to 66 hours after hatching. The results (Figure 8) revealed that midline labeling started at 60-63 hours after hatching and is strong once the larva is at 63-66 hours. It was sensible to assume that the cells remain mitotically active up to the time in which the organism stops wandering to become a prepupa. Panel A, C, E, and G of Figure 8 show larvae that were dissected immediately after exposure to the Brdu at 57, 60, 63, and 66 hours, respectively. The panels show cells which were mitotically active but only one or two cells (at most) in a segment labeled. This suggests that the cells were ready to divide but may have not done so. Panels B, D, F, and H show the same time frame of Brdu labeling but the larva were dissected while in the wandering stage. These panels show the number of Brdu labeled daughter cells formed from the theoretical one cell per segment that labeled during the treatment. Large numbers of neuroblasts labeled in most of the treatment times but no perineuropilar glia (PG) stained during the treatment times and can also be seen in Truman m*et al.*, 1994.

Cell ablation was attempted with use of Hydroxyurea (HU) to determine if the dividing glial cells were from the three glia that

remained after embryogenesis or if there were glial precursor cells located in the midline of the *pointed*^{1277} strain. As seen in Figure 9 (labeled with X-gal) the only treatment which achieved ablation was 60 hours after hatching. Many of the PG cells seemed to be dead and the number of midline cells went down slightly. The reason for the perineuropilar death with HU but no Brdu labeling of these glia at the same time is not known. There is the possibility that the X-gal staining did not penetrate the PG cells for reasons such as improper fixation. If the sample were over fixed the PG *E. coli lacZ* expression would not be seen. Unfortunately, at this time period in development, there were many neuroblasts that also were dividing in the nervous system and many cells in other tissues were also dividing. Therefore it can never be clear what the effect of no glial division is, due to the unwanted ablation of neuroblasts which could stop some sort of signal from telling the glial cells to divide or even to stay alive.

FIGURE 8: Anti-Brdu antibody labeling of four hour Brdu treated CNS whole mounts.

A) Brdu labeled CNS of a larva treated at 57 hours and fixed at 60 hours. B) same as in panel A except fixation occurred at the wandering larval stage. C) is a sample treated at 60 hours after hatching and fixed immediately after treatment. D) is the same as in panel C except that this sample was fixed at the wandering third instar larval stage. E) is a sample treated at 63 hours and fixed at 66 hours. F) is the same as panel E except that the fixation took place during the wandering larval stage. G) is a sample treated at 66 hours and fixed at 69 hours after hatching. H) same as panel G with fixation at the wandering larval stage. Small arrow heads refer to the midline glial cells and the large arrow heads refer to the neuroblasts. The bar equals 50 μm. Magnification is at 210 times.

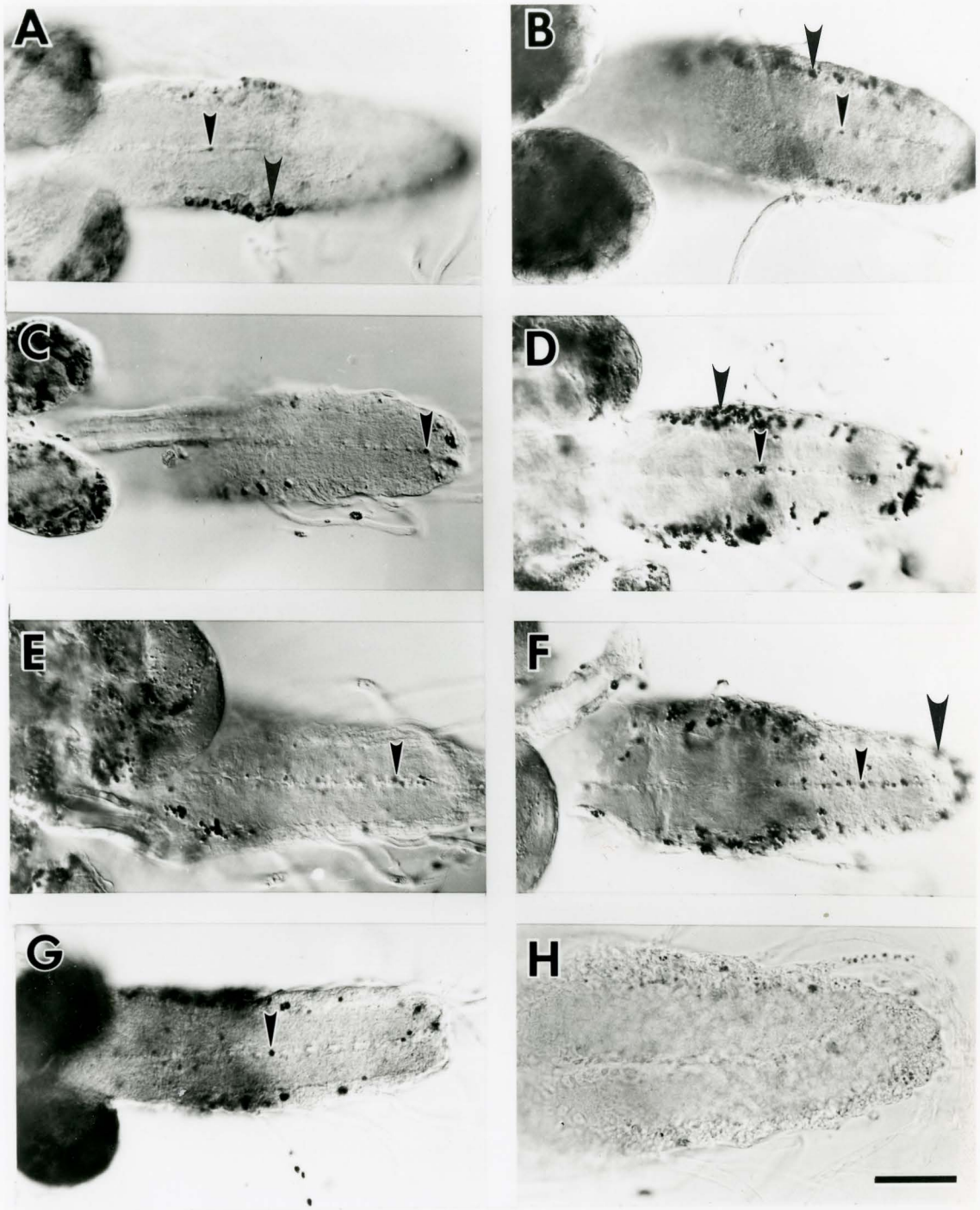
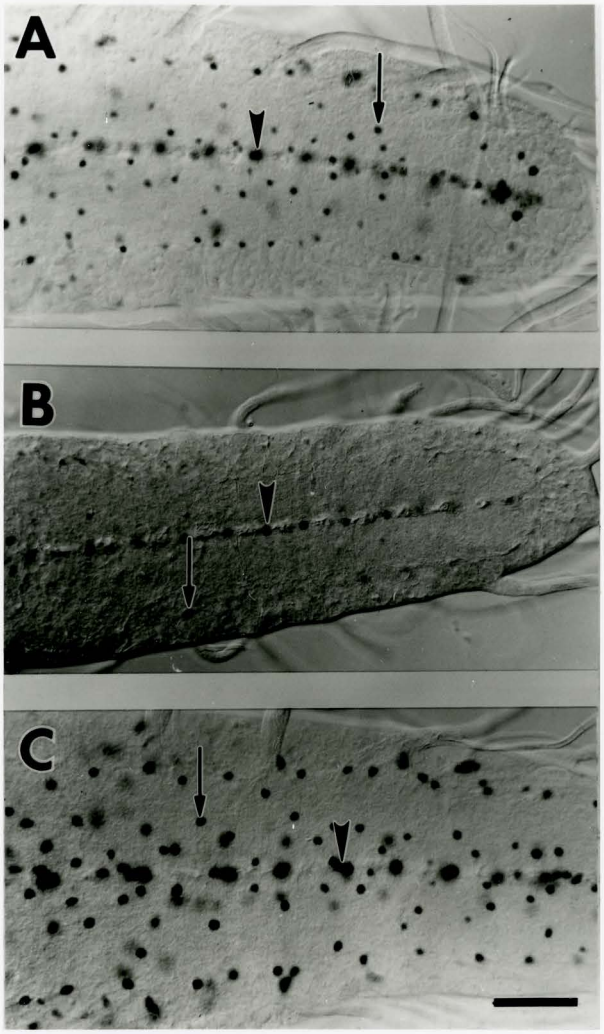

A
B
C
D
E
F
G
H

FIGURE 9: X-Gal labeled samples of three hour hydroxyurea treated larvae of the *pointed*^{1277} strain.

A and C) represents the lack of disruption to the glial labeling pattern during the 52 and 72 hours (after hatching) treatments and B) represents a 60 hour treatment that shows disruption to the increase of glial cells. Arrow heads refer to the midline glial cells and the arrows refer to the PG cells. The bar equals 50 μm. Magnification is at 210 times.


A
B
C

DISCUSSION

Embryonic Development of the Used Strains

MG cells contribute to the cytoarchitecture of the CNS midline. When the nervous system is formed the glia are important in the tasks of axon guidance, commissure morphogenesis, and ensheathment of axons (Jacobs 1993; Klämbt et al., 1991; Jacobs and Goodman, 1989). To properly complete these glial functions the embryo has six MG cells at stage 12. By the end of embryogenesis (stage 17) this number has been reduced to three cells per segment. This study verifies the count on the number of MG cells per segment in wild type embryos (Sonnenfeld and Jacobs, in press). In the *AA142*, *rhomboid*, *single-minded*, and *slit* expression strains the number of labeled MG cells at the end of embryogenesis was 3.2 cells per segment. For the *pointed*^{1277} expression strain there were 2 cells per segment and *argos*^{w11} had 4.5 cells per segment. The reasons for the discrepancy in the *pointed*^{1277} and *argos*^{w11} strains cell counts will be examined later.

First and Second Larval Instar Development

In the *AA142 slilacZ 1.0*, *slilacZ 4.5*, *EE1* and *X81* strains there were 3.1 cells per segment in the first instar larva as seen in Figures 1.1 and 1.2. Table 1 is a summary of the collected numbers.

The difference between the larval number and the number of MG cells in the embryonic samples was not statistically significant. It was also noted that the number of MG cells varied randomly between segments which was also seen in Sonnenfeld and Jacobs (in press). However, *pointed*^{1277} labeled 2 cells segment and *argos*^{w11} labeled 4.3 cells per segment. *pointed*^{1277} appeared to label the MGM but not the MGA, with evidence from the position of the labeled cells. In the *argos*^{w11} strain the extra cell per segment remained unidentified. This extra cell was also reported in the paper by Freeman et al. (1992, Figure 6 panel I) which examined the role that *argos*^{w11} played in the *Drosophila* eye. Even though the embryonic midline was not the focus of the paper it was dealt with briefly. Since there were only three MG cells per segment, as represented by *AA142*, *pnt*^{1277}, *EE1*, *slilacZ 1.0*, and *slilacZ 4.5*, the extra cell in the *argos*^{w11} expression strain CNS may have be a neural midline lineage like the VUM cells.

The *EE1* expression strain labeled a cluster of cells along the ventral side of each segment in the ventral nerve cord first detected at the beginning of the first instar stage. The position of these unidentified cells suggests that they were a cluster of MNB progeny. However, no experiments were undertaken to test this supposition. As with the *argos*^{w11} expression strain, the identity of these cells will be determined after ultrastructural and antibody studies.

MG cell numbers during the first and second instar stages remained the same in all of the strains. Cells located on the neuropil

started to label in all strains except *argos*^{w11} during the first instar stage. The number of the PG cells increased into the third instar larva as seen in the *pnt*^{1277} expression strain in Figure 2. The extra cells were called perineuropilar because they appeared to be in immediate contact with the neuropil as seen in Figures 2 through 5.

Third Larval Instar Development

By the end of the third larval instar the number of midline cells increased (Truman *et al.*, 1994) and the increase stopped in the prepupal stage. It has been established by using the Brdu and Hydroxyurea chemicals that the onset of mitotic activity within the *pointed*^{1277} strain began at 57 hours after hatching. The PG cells continued to label in the third instar stage. The number of these cells increased to approximately 400 cells per CNS from zero cells per CNS in the first instar, as seen in the *pointed*^{1277} expression strain.

AA142 showed an increase (12 cells per segment) in MG cell numbers, however the *E. coli lacZ* signal ceased in the midline before the end of the third larval instar with the perineural signal persisting. The *slit* expression strains showed similar increases in the MG cell number as the *pointed*^{1277} expression strain but *E. coli lacZ* expression was weak. The PG cell labeling also persisted in these two *slit* expression strains. Both the *EE1* and the *X81* midline signal ended before the increase in cellular number, but the PG cell labeling persisted during this stage. The midline signal stopped between the second and third larval instars in the *EE1* and *X81*

expression strains. The *argos*^{w11} expression strain also showed an increase in the labelled midline cell numbers similar in magnitude to the *pointed*^{1277} strain, however, the perineuropilar signal ceased.

It is not known if the same PG cells were labeling in each strain but the pattern of labeled cells appeared to be similar in all of the lines. The identity of the labeled PG cells were investigated and will be discussed below.

Cellular Identity in the *pointed*^{1277} expression strain

The number of midline cells in the late third instar increased to twenty-four cells per segment from three cells per segment in the first instar larva. Both the labeled midline cells and the PG cells required an ultrastructural study to determine their structure. In Figures 3, 4, and 6, the PG cells were observed to be in close proximity to the neuropil.

To determine the relationship of the PG cells to the MG cells, *pointed*^{1277} samples were labeled with the anti-RK2 antibody and also treated with X-gal. RK2 is a glial specific homeodomain protein that was reported to be present in non-midline glia in the embryonic CNS (Cambell *et al.*, 1994). Xiong *et al* (1994) reported and described a gene called *repo* whose pattern of expression matched that of RK2. It has been confirmed that RK2 and *repo* are the same gene (postscript to Cambell *et al.*, 1994). There were no cells in the CNS of the third instar larvae that labeled with both the RK2 and the lacZ labeling systems. If the RK2 protein was present in all non-

midline glia in the larva as it was in the embryo (Campbell *et al.*, 1994), then the *E. coli lacZ* expressing PG cells were of the midline lineage. But the expression pattern of the RK2 antigen in the CNS of post-embryonic samples has not been reported. Therefore it is possible and probable that there were non-midline glia in larvae that do not express RK2.

The PG cells started to label in the first instar larva and were non-midline cells. There were no *E. coli lacZ* labeled cells noted to be migrating away from the midline. If PG cells were migrating from the midline then it stands to reason that there should be more than three labeled midline glia per segment in the first and second larvae.

An EM study was then done to determine the cellular identity of the labeled cells both inside and outside of the midline of the CNS of the *pointed*^{1277} expression strain. Were the labeled cells neuronal or glial in ultrastructure? *E. coli lacZ* expressing cells were identified with an electron dense ß-gal reaction product, Bluo-gal. All cells that labeled with Bluo-gal were seen to be glial in ultrastructure. This was true for both cells in the midline and perineuropilar positions.

Mitotic Activity of the Midline Glia

The data discussed thus far (summarized in chart 1) show that the midline glia increased in number during the third instar larval stage, but the time of mitotic onset remains unknown. For this reason the Brdu (a nucleotide analog to label cells) and hydroxyurea

(HU, a cytotoxic agent to mitotically active cells) experiments were undertaken. It was hoped that destruction of the mitotically active glial cells with HU would allow us to determine the development of MG cells in the CNS during later larval development.

As has already been noted that at 57 hours into larval development cells in the *pnt*^{1277} CNS midline are seen to be mitotically active with Brdu and HU. The Brdu experiments labeled the midline cells that correspond to the position of midline glia since the same midline cells were ablated at 60 hours in the HU experiment in which the *E. coli lacZ* gene product was labeled with X-gal. When treated samples were fixed and labeled immediately, only approximately one cell per segment labeled. When the samples were fixed at the wandering stage a cluster of cells were seen in the midline. The Brdu labeling ended by 67 hours after hatching. In the *pointed*^{1277} expression strain the midline glia formed clusters similar to what was observed with the Brdu labeling and the HU ablated these clusters at 60 hours after hatching.

The Brdu results were supported with HU studies in which mitotically active cells were destroyed. Treatment at 60 hours, but not at 50 or 70 hours, resulted in fewer *E. coli lacZ* labeled cells in the midline. This coincided with the period in which the midline labeled with Brdu.

What is not known is if there was a glioblast in the midline that was dividing, or if the MG cells that labeled at the end of embryogenesis were the dividing cells? One method to answer this

question would be to ablate the MG cells before the time of mitosis. A system such as lacZap (Nirenberg and Cepko, 1993) would ablate the labeled MG cells and determine the identity of any new labeling in the late third instar as originating from a glioblast. It is not known what the effect in the CNS would be if all of the midline glia were to be destroyed by the lacZap procedure in the larval stages. The difficulty with the lacZap procedure is that PG cells would also label. An exception to this is the *argos*^{w11} strain, but this strain has an extra cell that labeled in the embryonic and first instar larval stages.

Another approach is to feed larvae a lower concentration of Brdu than was used in this study, from the time of hatching to the time of fixation of the CNS samples. The sampling times would have to be at 60 hours to view mitotically active cells and 96 hours after hatching to see the actual increase in cell number. The tissue could then be labeled with both X-gal and the anti-Brdu antibody. If all of the X-gal labeled cells in the midline were also labeled with the anti-Brdu then no glioblast was present. If there remained three cells per segment that only labeled with the X-gal and the rest labeled with both, then the glioblasts were present.

Pupal Stage

Figure 2 shows that *E. coli lacZ* expressing MG cells of the *pointed*^{1277} strain lived into the pupal stage. It is during the pupal stage that metamorphosis occurs (Truman, 1990a) and it appears

very probable that many axons need to be guided and later ensheathed by glia. The same embryonic glial functions are needed for metamorphosis as a new system with a new structure, sensory inputs, and behavioral laws is being formed. However, the *pointed*^{1277} expression ceased somewhere between 48-72 hours into the pupal stage for the MG cells and ceased at 24 hours into pupation for the PG cells, with no apoptosis being noted in any of the viewed samples. This leaves the question "when do the cells die, if they die?" An EM study is required during this pupal stage to search for the presence of apoptotic bodies with X-gal labeling to determine the time period for programmed MG cell death. An alternative idea is that the *E. coli lacZ* expression ceased before the death of the MG cells, therefore apoptosis would never be seen with bluo-gal and EM micrographs.

PG cell expression abruptly returned at end of pupation as seen in panel G of Figure 2. The reason for this cessation and re-introduction of *E. coli lacZ* expression has not been investigated for *Drosophila*. Dr. Lane's (1980) studies on lepidopterans may be the key to understanding what is occurring to these PG cells. The PG cells must dissociate from the neuropil to accommodate the growth of the adult structure. During this time the *pointed*^{1277} *E. coli lacZ* protein is not being produced and at the time of re-association of the PG cells to the neuropil the protein was once again being expressed.

Adult

Many PG cells in the *pointed*^{1277} expression strain labeled. They were called PG cells with the assumption they were the same PG cells that labeled in the larva. Some of these labeled cells occurred in the midline. There were approximately twelve cells in the thoracic midline. It is unknown if these cells were MG survivors or PG cells that had invaginated into the neuropil. As with other questions that have not been addressed in this thesis, the identity of the labeled cells in the adult ventral nervous system must be investigated with the use of an electron microscope. No study was undertaken to establish the labeling patterns of the other strains in the adult CNS.

Glia After Embryogenesis

One possible reason for the MG and PG label would be for the ensheathment of the third instar neuropil as seen from the EM study. This ensheathment may be needed since the neuropil is physically larger with an increased number of axons. There is the possibility that this occurred to form a complete "blood-brain" barrier to accommodate the increased size of the neuropil (Lane and Treherne, 1980). During metamorphosis the ultrastructure of the glia can only be speculated about due to the absence of experimental data. The MG cells may ensheath parts of the neuropil since not all of the axons degenerated. Along with these remaining larval structures were the

many new neurons and axons that were generated and need to be guided.

Future Investigations

As an extension of the data presented in this work several studies are suggested. The adult glial CNS pattern in all of the used expression strains needs to be studied to establish which genes are being expressed. Once the strains with expression are found, the labeled cells should be counted. Following these counts, an EM study should be completed to verify the ultrastructure of the labeled cells as being glial. If the labeled cells are glia then the ultrastructure of the cells may demonstrate an ensheathment function on the adult neuropil. The ultimate fate of the MG cells will also be dealt with in these experiments. Do the glial cells in the midline and around the neuropil die sometime during adulthood or with the death of the adult? In other words, is the expression of midline glial genes required throughout the life of the adult or are they eventually turned off without any observable effect on the adult.

Another question is, do the adult *E. coli lacZ* labeled glia of the *pointed*^{1277} strain originate from the glia that labeled in the larva? If not, where do they originate from? A study of the *pointed*^{1277} adult that was labeled with a Brdu treatment during 60-63 hours after hatching could address this question. This procedure may proceed with fixation of the adult CNS within one day of emergence. The tissue could then be labeled with X-gal and with the anti-Brdu

antibody as was done in the larva of Figure 4. This will demonstrate if any of the larval MG cells survive to adulthood. A similar procedure can be carried out for the PG cells with Brdu treatment at an earlier time. Also, the larval data in this thesis may be supported by using mutants of at least one of the six genes used in this study to perturb the function(s) of glia during and after metamorphosis. A researcher could use a temperature sensitive mutant of the *slit* gene, for example, and disrupt midline glial function at the third larval instar stage. The problem with this experiment is that other cell lineages will be perturbed, even if the other lineages are not present in the CNS. The larva must also remain viable for the duration of the experiment. In addition to the above experiment, it would be beneficial to study what the effect on metamorphosis would be without the midline glia, using the previously noted LacZap approach.

Other experiments that will clarify the described data include, investigating the identity of the extra midline cells labeled by *argos* and *sim* reporters in the embryo. An EM study on the *pointed*^{1277} pupal stages of 0 hour, 24 hour, 48 hour and 72 hour time points to find the presence or absence of apoptosis in the labeled glial cells needs to the completed. This EM study will also reveal the ultrastructure of these cells which can uncover the functions of the glial cells during pupation.

Conclusions

This study has shown that midline cell numbers did not change between the end of embryogenesis and the first instar larva. Midline glial numbers began to increase at approximately 60 hours after hatching probably in response to the increased size of the neuropil and for preparation for pupation. During larval growth the number of labeled PG cells increased from just a few cells to around 400 cells in the CNS.

Within one day of pupation the PG cells ceased to express *pnt* possibly because of the need of these cells to dissociate from the neuropil. This dissociation may be required to allow the restructuring events in the neuropil to occur. Along with this, sometime between the second and third days into pupation the midline *E. coli lacZ* glial expression of the *pointed*^{1277} strain ceased. No apoptosis was noted, therefore the fate of these cells is not known.

Upon emergence of the adult fly the *E. coli lacZ* expression PG cells re-appeared in the CNS. If these cells were glia then they were probably ensheathing and supporting the neurons and axons. A group of about twelve labeled cells also re-appeared in the thoracic midline, but their lineage (PG or MG) remain undetermined.

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