

THE EFFECTS OF THE TROL⁴ AND TROL^{S1} MUTATIONS ON NEURAL STEM
CELL DEVELOPMENT AND PROLIFERATION IN *DROSOPHILA*

MELANOGASTER EMBRYOS

A Senior Honors Thesis

By

MARDELLE RENEE ATKINS

Submitted to the Office of Honors Programs
& Academic Scholarships
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In partial fulfillment of the requirements of the

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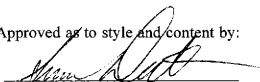
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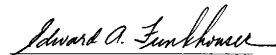
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ABSTRACT

The Effects of the *trol*^f and *trol*^{pd} Mutations on Neural Stem Cell Development and Proliferation in *Drosophila melanogaster* Embryos. (April 2000)

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The locus *terribly reduced optic lobes* or *trol*, is an X-linked homeobox gene involved in the control of neuroblast proliferation in *Drosophila melanogaster* larvae. It is known that *trol* acts in a pathway to counteract cell cycle repressors via the induction of cyclin E expression. The goal of the project is to use immunohistochemical methods to attempt to establish a phenotype for *trol* in the embryonic stage of development.

Using the anti-engrailed and anti-fasciclin III antibodies, the developing nervous system can be examined for physical differences between different alleles and the wildtype embryos.

This work is dedicated to my family, and especially my grandfather Edward J. Lutczyn

for always encouraging my interest in the sciences.

Acknowledgements

I would like to thank Dr. Sumana Datta for all of her help, support, and patience ever since she let me in the door two and a half years ago. Also my sincerest thanks go to my lab mates Dr. Youngji Park and Sean McDermott for all their help and encouragement throughout this project.

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Introduction

Background and significance:

Normal development occurs through the interaction of many signals. These interactions direct cells to divide at appropriate times and to divide the appropriate number of times. *Terribly reduced optic lobes (trol)* is a developmental signal found in *Drosophila melanogaster* that causes the precursor cells of the nervous system to begin division (Datta, 1995). Mutations in *trol* interfere with normal nervous system development (Datta, 1995).

Every system of an organism has precursor, or stem, cells. Stem cells are undifferentiated cells that undergo a process of asymmetric cell division. With each division, a stem cell reproduces itself and gives rise to a daughter cell. This daughter cell differentiates during subsequent divisions to form specific cell types. For example, in the *Drosophila* nervous system the stem cells, or neuroblasts, give rise to daughter cells called ganglion mother cells (GMC). The GMC's divide to give rise to either neurons or glial cells (Campos-Ortega, 1993). This ability of stem cells to give rise to several different cell types within their systems is referred to as multipotence.

This thesis follows the style of the journal *Development*.

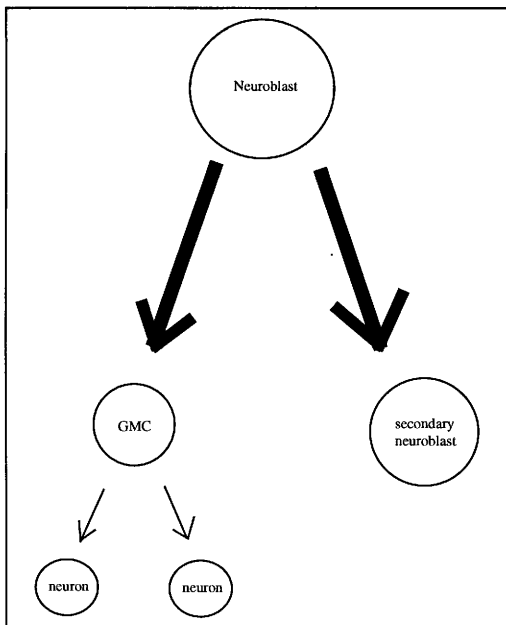


Fig1: Neuroblasts undergo asymmetric division to form neuroblasts and ganglion mother cells (GMC's). The GMC's divide to form neurons.

The ability of stem cells to divide indefinitely to regenerate specific kinds of tissues is creating hope for their usage in treatment of severe tissue damage and some degenerative disorders, including Tay Sachs (Flax, et. al., 1997, Bjornson, et.al. 1999). In order to be able to effectively use these kinds of cells in treatments, we must be able to regulate when they divide, how often they divide, and what cell types are formed. In order to control these activities we must be able to use developmental signals correctly. *Drosophila melanogaster* provides a well-characterized system for studying development and has been shown to have close homologies to higher organisms with respect to early developmental signals. *trol* is one signal in a pathway that controls division of distinct neuroblast populations in *D. melanogaster*, and its study will be helpful to better understanding the mechanisms which control stem cell division.

Characterization of *trol*:

trol has been shown through genetic analysis to be a locus on the X chromosome of *Drosophila melanogaster* (Datta and Kankel, 1992). It is proposed that *trol* is a homeobox gene. Homeobox genes are a class of developmental genes that function as transcription factors helping to regulate development. The function of a homeobox gene may change depending on the region of expression or the stage of development at which the gene is being expressed.

Mutations in the *trol* locus are homozygous lethal, with the exception of the *trol*^{rb22} allele which is only semi-lethal (Datta, 1992, Caldwell and Datta, 1998). *trol* has been found to induce the initiation of neuroblast proliferation in the larval stages of development (Datta, 1995). Mutations within the *trol* locus result in larval lethality or severe structural brain defects in adults (Datta and Kankel, 1992).

trol works in a developmental pathway to oppose the actions of a cell cycle repressor gene *anachronism (ana)* (Datta, 1995). The proposed mechanism for this induction is that *trol* increases cyclin E expression. This hypothesis is supported by the ability to rescue *trol* mutants by induced cyclin E expression (Caldwell and Datta, 1998). Examination of different *trol* mutant (*trol*^{mf}) alleles shows a high level of lethality in embryogenesis with few to none of the larvae hatching (Caldwell and Datta, 1998).

Hypothesis:

This project proposes that *trol* is acting as a regulatory signal not only during larval development but also during embryogenesis; and that mutations in *trol* somehow disrupt the pathways that lead to normal embryonic nervous system development. As a result of this disruption there is a high level of lethality witnessed in severe loss of function mutants between the embryonic and larval stages. To test this hypothesis, CNS and PNS development in wildtype and mutant embryos were examined using fluorescent immunohistochemistry. Using these methods, a phenotype may be

established to show that mutations in the *trol* locus cause a proliferation defect in *D. melanogaster* embryos. A more direct analysis is not possible at this time because *trol* has not yet been cloned.

If *trol* function proceeds by the same mechanism in embryos and larvae, then it is expected that the wildtype number of neuroblasts will be present in early stage *trol* embryos. Also, the number of neurons formed, and neuroblasts formed in subsequent divisions, will be decreased because *ana* repression will not have been overcome and proliferation will not have been initiated. If this is not the case, the results may indicate that the role played by *trol* in embryonic development may be different from that in the larval stages. This would not refute the role of *trol* in *D. melanogaster* development, because it is congruent with the theory that *trol* is a homeobox gene. It is not uncommon for homeobox genes to be expressed at different stages of development and to have different target cells, or different functions within the same cell subset dependent upon their temporal expression.

Materials and Methods

Alleles:

Three different mutant alleles were examined: *trol^l*, *trolnd*, and *trol^{l1}*. *trol^l* is the least severe of these mutants and *trolnd* is the most severe. *trolnd* is a naturally occurring severe partial loss-of-function mutation identified by Sumana Datta (Datta, 1992). *trol^{l1}* was induced by radiation (Datta, 1992). *trol^l* was induced in 1998 by a chemical mutagenesis using diepoxybutane (Caldwell and Datta, 1998).

Stocks and crosses:

There are two markers being used in this cross to help distinguish the mutant flies at all stages of development. *Yellow (y)* causes a phenotype in mutant larvae of golden brown mouthhooks as opposed to black in their wildtype siblings. The β -galactosidase (*lacZ*) insert in the *fushi tarazu (ftz)* locus gives a clear, easily distinguishable banding pattern in the wild type embryos (see Fig 2) when stained with anti- β -galactosidase, while the mutant shows no staining.



Fig. 2 *lacZ* staining of a wildtype embryo

The crosses used were *ytrol^{mt}w/fm7eftzlacZ* females X *fm7eftzlacZ* males. This cross gives four progeny classes: *fm7eftzlacZ/fm7eftzlacZ* females, *fm7eftzlacZ* males, *ytrol^{mt}w/fm7eftzlacZ* females, and *ytrol^{mt}w* males. Only this final class, lacking the normal X chromosome with the *lacZ* marker will exhibit the mutant phenotype. All other progeny classes will show wildtype patterns of development.

Table 1: Resulting Phenotypic Classes.

Only the *ytrol^{mt}w* males will show a mutant staining phenotype.

<i>fm7eftzlacZ</i>	<i>ytrol^{mt}w/fm7eftzlacZ</i>	
	females	
males	<i>ytrol^{mt}w/fm7eftzlacZ</i>	<i>Fm7eftzlacZ/fm7eftzlacZ</i>
	<i>fm7eftzlacZ</i> male	<i>ytrol^{mt}w</i> male

Collection of embryos:

Embryos were collected from egg lay plates made with apple juice agar and sprinkled with yeast. The egg lays were incubated at 25°C. Initially embryos were collected after 12 to 14 hours of incubation. In later stages of the experiment they were incubated with the parents for 2 hours, then removed from the parent stock and allowed to age at 25°C to 3-5 or 7-9 hours before being collected. Embryos were transferred to small, mesh-bottomed collection baskets with 1X Triton/NaCl and then dechorionated in 50% bleach for 2-4 minutes. Embryos were then washed with water and transferred to Eppendorf tubes for fixation. The protocols for collection, fixation and staining were obtained from Brad Jones in Bill McGinnis's lab (personal correspondence).

Fixing of embryos:

Embryos were fixed for 20 minutes on a shaker in 1:1 heptane: paraformaldehyde fix. Replacing first the paraformaldehyde fix (aqueous) layer with methanol, shaking 30 seconds, then replacing the heptane with methanol devitellinized the embryos. The methanol wash was repeated three times. Embryos were allowed to then set at room temperature in methanol for 20-30 minutes before being stored at -20 °C or labeled with antibodies.

Antibody labeling procedure:

The primary antibodies were incubated with the embryos in PBNT (1X Phosphate Buffered Saline + 0.1% TWEEN 80 + 1% Normal Goat Serum) either overnight at 4°C or 4 hours at room temperature. Monoclonal mouse anti-4D9 (*anti-engrailed/ invected*) was used at a 1:1 dilution (Patel, et.al., 1989); monoclonal mouse anti-7G10 (*anti-fasciclin III*) at 1:1000 (Patel, et.al., 1987) and rabbit anti- β -galactosidase were added to all preps at a 1:5000 dilution. The primaries were removed by washing three times for fifteen minutes each in 1X PBS. After completing the third wash, wash once with PBNT at 4°C for 30 minutes, and then return the samples to 1X PBS for 15 minutes. The secondary antibodies were incubated for an identical time period and that was followed by the same wash protocol. The secondary antibodies used were Alexa 488 goat anti-mouse conjugated monoclonal antibody (a FITC analog), and Alexa 594 goat anti-rabbit conjugated monoclonal antibody (a Texas Red analog). Whole mount preparations were then made of the embryos using a Dako Corporation aqueous mounting media for fluorescence containing NaN_3 .

Microscopy:

Slides were viewed using a Zeiss compound microscope and photographed using a Hamamatsu camera system, then transferred to and processed using Adobe PhotoShop 5.0 for Macintosh.

Results**Antibody Specificity:**

BP106, 4D9 and 7G10 were all obtained from Corey Goodman at the University of California at Berkeley. 22C10 was obtained from Seymour Benzer at the CalTech.

BP106: *anti-Neurotactin* recognizes neurotactin, a surface glycoprotein with homology to serine esterases. This antigen is expressed throughout embryogenesis in different tissues. It is expressed in restricted region during the blastodermal phase, and heavily stains the ventral furrow. Expression includes nearly all cells when gastrulation is complete, and expression is later confined to the developing nervous system (Hortsch, et al., 1990). My initial work with this antibody was unsuccessful. The fluorescence shown was expressed overwhelmingly in the gut and ectoderm. This fluorescence did not suit my purposes for identification because it very strongly resembled autofluorescence.

4D9: *anti-engrailed/invected* recognizes the engrailed/invected gene products.

These two genes are homeobox genes expressed throughout development. In the embryo, 4D9 stains the nuclei of all row 6 and 7 neuroblasts, and one neuroblast of row 1 as well as the nuclei of a small subset of neurons (Patel, et. al., 1989).

7G10: *anti-fasciclin III* recognizes the fasciclin III antigen. This antigen is a surface glycoprotein expressed on several cell types transiently over the course of *Drosophila* development. The 7G10 antigen is found to be regionally expressed on a subset of neurons and axon pathways during 10-13 hours of development (axon outgrowth), can be used to trace neuroblast lineages, and in late embryonic development can be used to examine morphogenesis through its epidermal staining pattern (Patel, et. al., 1987).

MAb22C10: 22C10 stains a subset of the CNS neurons and all PNS neurons by staining the cytoplasm and the inner surface of the cell membrane. (Patel, 1994)

My initial screens across 0-14 hours of development were done primarily on 22C10.

The antibody worked well, but is not expressed at the early stages that the project ended up focussing on. As a result I have no data to show for the 22C10 staining.

Table 2: Summary of Antibody functions

<i>Antibody</i>	<i>Numerical abbreviation</i>	<i>Reactive against</i>	<i>Expression pattern</i>
Anti-Neurotactin	BP106	Drosophila neurotactin	Most cells by end of gastrulation, strongly in nervous system later in development
Anti-engrailed/ invected	4D9	Engrailed and invected gene products	CNS neuronal subset, median neuroblast progeny, and small set of PNS neurons
Anti-fasciclin III	7G10/ 2D9	Fasciclin III	Subset of neurons and axons in CNS
MAb 22C10	22C10	Cytoplasm and inner surface of cell membrane of neuron sets	Stain a subset of CNS neurons and all PNS neurons

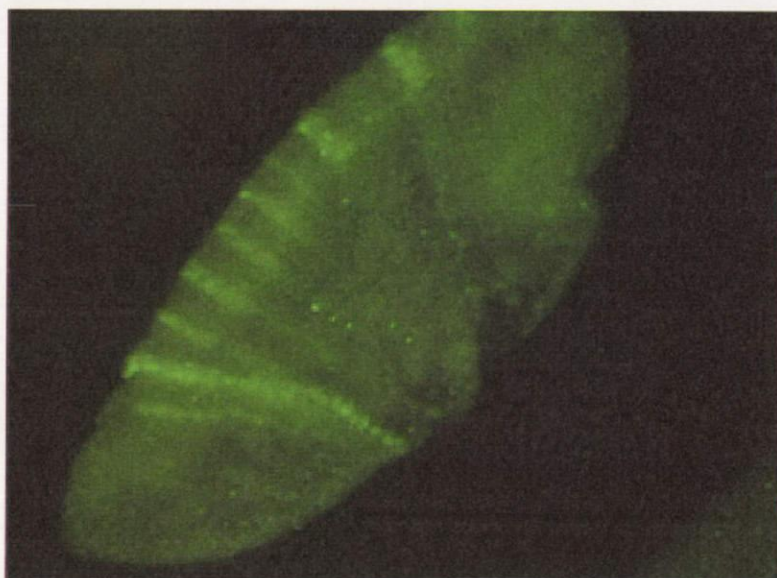


Fig. 3 Wildtype staining with 4D9 in 0-2 hours development. Staining shows only 1-2 rows of cells present in segments 1-5.



Fig. 4 Wildtype expression of 4D9 in a 3-5 hour old embryo, dorsal view. Observe in this image that in segments 4 and 5 no more than 2-3 rows of neuroblasts show staining.

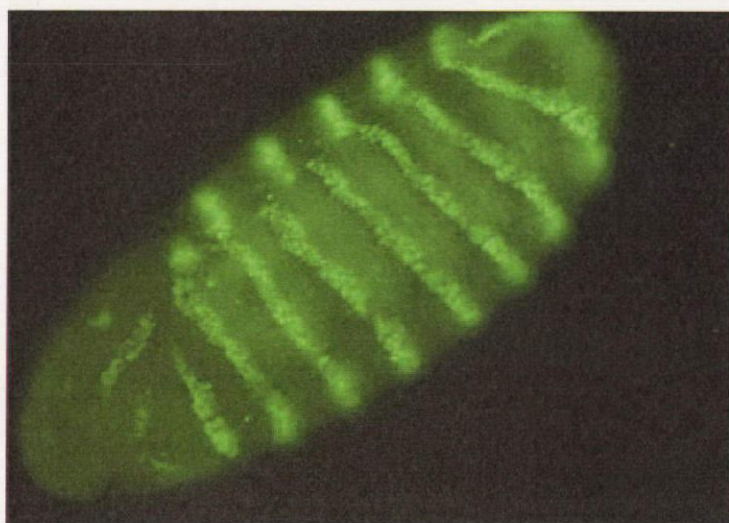


Fig. 5 *tro4* staining in 3-5 hour old embryo. The pattern of 4-5 rows of cells staining in rows four and five has been observed repeatedly for this allele.

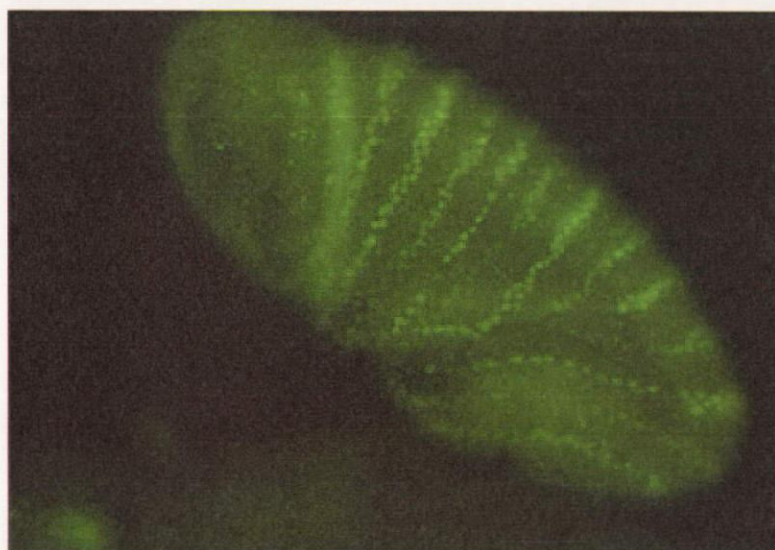


Fig. 6 *tro4* staining in 0-2 hour old embryo. Although the first segment is not in focus, it is clearly broader than the single cell row of the wildtype sibling. Also, in segments 3 and 6, rows 2-3 cells thick can be seen. This width exceeds the 1-2 cell width of the wildtype rows.

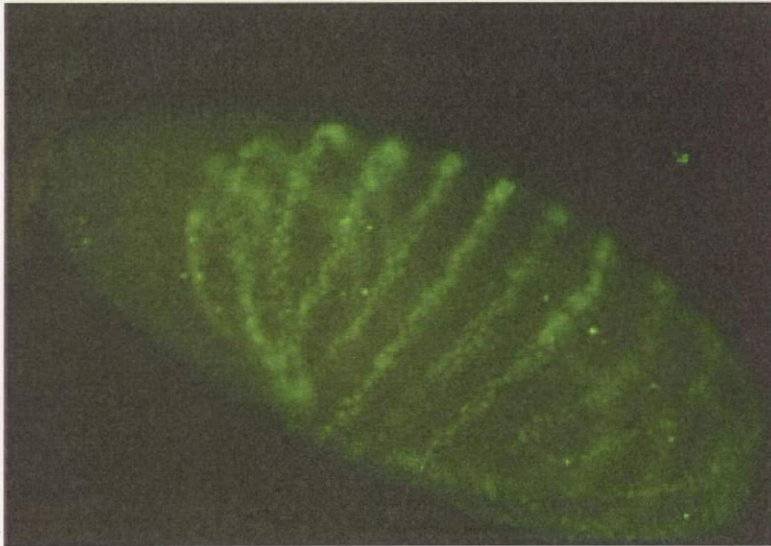


Fig. 7 This is a dorsal view of a *trots1* embryo at 0-2 hours development. While from a slightly different angle than the wildtype image (Fig 3), the presence of 3 cell rows or more uniformly through out the embryo show a more severe defect than the corresponding *trots4* staining pattern (Fig 6).

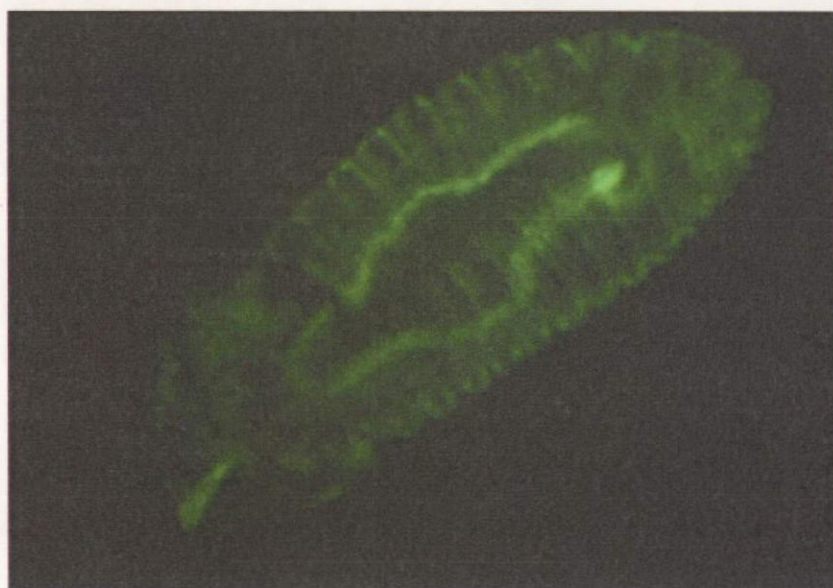


Fig. 8 7G10 staining of wildtype (top) and *trol4* (bottom) embryos ages 3-5 hrs. In these images, the reproducible staining pattern is observed. In the wildtype embryo the involution of the body segments stains smoothly producing a curved line, whereas the mutant has a break in the staining at the most interior point of each involution.

Discussion

Most of the work done in this project has been on the 0-5 hours stage of development. At this time, repeatable results have been obtained using the 4D9 and 7G10 MAb's. Further work needs to be done to characterize the development throughout the embryonic development. Also, staining experiments with *trolrd* need to resume. Due to difficulty with my stocks, the results from this allele were too few to establish any repeatability.

In embryos stained with 4D9 it appears that mutations in the *trol* locus do generate a phenotypic difference between wildtype and mutant embryos. In embryos 0-5 hrs old, the number of rows of neurons per segment stained in the embryo by the antibody increase in number from the wildtype number of 1-2 through 2-5 for *trol^r* and 3-5 for *trolrd*. It is encouraging that we are seeing this increase in severity of the phenotype as the severity of the allele increases. At the present time, the result is repeatable, but the number of high quality images obtained is too small to give a good statistical analysis. However, at this point it should be easier to get images conducive to this analysis because I now know what segments are of more importance, and which focal plane must be clear.

These results suggest an excess of neurons is being formed. These preliminary results may indicate one of two possible explanations for the defect. There may be a defect in neuroblast proliferation, i.e. for some reason the neuroblasts are dividing excessively. The other option is that *trol* is acting in the embryo to cause a more generalized initiation of mitosis, and as a result mutations in *trol* lead to generalized cell cycle defects. To determine which of the hypotheses are correct, first, the number of neuroblasts being formed must be carefully examined. Careful sampling during neuroblast delamination may provide an answer when stained with either *anti-engrailed* (*anti-eng*) or *anti-horse radish peroxidase* (*anti-HRP*). If neuroblast proliferation appears normal, it may become practical to establish an *in vitro* culture system to directly measure the change in rates of division between wildtype and mutant cell populations.

The staining observed in the 7G10 embryos initially appeared to contradict these findings, because a loss of cells staining is observed. Upon closer examination of the staining function of 7G10 however, it became clear that this is not the case. According to Patel, Snow and Goodman, during the early stages of development and neurogenesis, most central nervous system (CNS) staining is obscured by staining of the ectoderm in patches which align with the segmental grooves (1987). Apparently we are seeing the

loss of ectodermal cells, and an increase in neurons, and as a result it is likely that observation of the number of neuroblasts being formed is going to increase. This would indicate that *trol* is having some effect on the fate of the neuroectodermal progenitor cell proliferation and differentiation so that there is a change of fate taking place. Once again, initial experiments in determining if this is actually what we are observing should center on determining the number of neuroblasts being formed in the early divisions of neurogenesis.

Conclusions

Mutations in the *trol* locus do appear to be having an effect on the development of the embryonic nervous system, and at this point preliminary evidence indicates a more generalized cell cycle defect, or a potential change of fate for neuroectodermal cells is occurring. Work should continue at this point to first establish the statistical validity of the phenotypes observed, and then work should focus on the numbers of neuroblasts found at different stages of embryogenesis. Increases or decreases in the numbers of neuroblasts formed between wildtype and mutant strains will further clarify the role of *trol* in the developing embryo.

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