A HOMEOTIC GENE EXPRESSION MAP IN THE CENTRAL NERVOUS SYSTEM OF LARVAL *DROSOPHILA MELANOGASTER*: A QUEST FOR THE HOLY *ENGRAILED*

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ABSTRACT

A Homeotic Gene Expression Map in the Central

Nervous System of Larval *Drosophila melanogaster*:

A Quest for the Holy *engrailed*. (April 2004)

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The expression of homeotic genes during early animal development determines the identity of specific populations of cells and thus the regions of the developing body. Furthermore, their expression is regulated so that it occurs at specific developmental time points. Previous studies demonstrated that the expression of homeotic genes could be used as molecular markers for identifying individual sets of neuroblasts within the embryonic central nervous system of *Drosophila melanogaster*. In this study cellular maps of the expression of the homeotic gene *engrailed* within the thoracic ventral ganglion of *Drosophila* larvae were created. Additionally, a staining technique used to label both *engrailed* expressing cells and dividing neuroblasts within the thoracic region was optimized. This double staining technique will allow for the identification of individual populations of dividing neuroblasts with the *engrailed* expressing cells acting as landmarks within the brain.

To Frodo S. C. M. Baggins
whose love and support I cannot live without
Veni. Vidi. Scribi.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

All of the tissues in an organism's body trace back to the complex, highly regulated division and differentiation of a unique subset of embryonic cells called stem cells. These undifferentiated cells arise early in embryogenesis, and studies have shown that some stem cell populations persist throughout the organism's life and are responsible for the maintenance of tissues, such as blood and epithelium, that have high turnover rates (Marshak et al., 2001). Stem cells divide in either an asymmetric or symmetric fashion; in both cases they either increase or maintain their population level (Marshak et al., 2001; Panicker and Rao, 2001). The division of stem cells does not take place at one time during embryonic development, however, but occurs in precise stages where specific populations of stem cells are targeted for cellular division. Many extracellular factors, such as hormones and growth factors, dictate when and which stem cell populations divide. Furthermore, these factors influence the daughter cells to become more highly differentiated than their parent cells (Marshak et al., 2001). These daughter cells continue to divide where each successive division restricts their differentiation plasticity and thus causes these cells to diverge to form more specialized populations of cells. The end product of these successive divisions in the development of the body from a one-celled zygote to a multicellular organism is the formation of all of the functionally unique tissues that compose the body.

This thesis follows the style and format of *Development*.

Neural development is a highly regulated process that involves the coordination of many cellular events, such as cell division, differentiation, and migration, to form the proper neural connections that innervate an organism. The processes that regulate neural development are conserved across the animal kingdom (Brown et al., 2001). *Drosophila melanogaster* is a powerful model system in which to study neural development since it is not as highly complex as that of human neural development yet the same processes govern both. Furthermore, the genetics of *Drosophila* have been extensively studied and are well characterized. Neurogenesis in *Drosophila* occurs in specific stages during both embryonic and larval development. In embryonic development neuroblasts, neural stem cells, arise from the neuroectoderm (Doe, 1992; Campos-Ortega, 1993; Goodman and Doe, 1993; Jan and Jan, 1993; Broadus et al., 1995; Cui and Doe, 1995). Some of these cells then divide asymmetrically to produce a neuroblast copy and a ganglion mother cell (Doe, 1992; Campos-Ortega, 1993; Goodman and Doe, 1993; Broadus et al., 1995; Cui and Doe, 1995; Datta, 1995; Caldwell and Datta, 1998). Ganglion mother cells continue to divide to produce neurons and glial cells that compose the neural tissue of the embryonic central nervous system (Campos-Ortega, 1993). However, most neuroblasts remain quiescent in the embryo until they are reactivated during larval development (Truman and Bate, 1988; Datta, 1995). For example, neuroblasts that reside in the thoracic ventral ganglion of the larval brain are reactivated to divide 28 hours after hatching, which falls into the early second instar developmental period (Datta, 1995). Extracellular proteins cue specific neuroblasts to divide at the correct developmental time period by activating signaling cascades that regulate the expression of genes

encoding cell cycle participants. In *Drosophila* the growth factors Branchless (Bnl) and Hedgehog (Hh), encoded by *branchless* and *hedgehog* respectively, activate these signaling cascades by binding to receptors located on the plasma membrane (Park et al., 2003). Both Bnl and Hh have human homologues. Bnl is a homologue of Fibroblast Growth Factor 2 (FGF-2) (Klambt et al., 1992), and Hedgehog has three different homologues in humans (Ingham and McMahon, 2001). Not surprisingly, Bnl binds to the human FGF receptor homologue Breathless (Klambt et al., 1992); and Hh binds to the Hh-receptor encoded by *ptc* (Ingham and McMahon, 2001). Additionally, the heparan sulfate proteoglycan co-receptor Trol, encoded by *trol*, is needed for both Bnl and Hh binding to their appropriate membrane receptors and for the perpetuation of the signaling cascade activating division (Park et al., 2003). Studies performed by Park et al. have shown that mutations in either *bnl* or in *hh* coupled to a mutation in *trol* causes a decrease in the amount of neuroblast division in the brain lobes of first instar larvae. This effect was not seen, however, in larvae that had a mutation in only one of these genes. It is not known whether this decrease in the amount of dividing neuroblasts in these double mutants is a stochastic event where all dividing neuroblasts are inhibited from dividing or whether specific populations of neuroblasts are targeted for inhibition due to the mutated growth factor's inability to activate signaling cascades. In order to address this question, specific populations of dividing neuroblasts must be identified at various times within the brain.

Previous studies used the expression of developmental genes, such as homeotic and proneural genes, as molecular markers for identifying individual neuroblasts within

the developing *Drosophila* brain (Doe, 1992; Urbach et al., 2003; Urbach and Technau, 2003). By using primary antibodies raised against the protein products of these genes, specific neuroblasts were stained and were thus identified. The expression of homeotic genes was used since these early developmental genes segment the embryo into unique regions and determine the developmental fate of these various segments (Duboule, 1994; Snustad and Simmons, 2000). Additionally, these genes are expressed at fixed times during development. *Drosophila* homeotic genes include gap genes, pair-rule genes, and segment-polarity genes. In this study, the homeotic genes *wit*, *enabled*, *Nervana*, *rho1*, *Abd-B*, and *engrailed* were evaluated for their potential use as molecular markers for identifying individual cells within the thoracic ventral ganglion of early second instar Canton-S (CS) larvae. These genes were used since the genes characterized in the studies mentioned previously did not work in second instar larvae. Expression of *engrailed* was determined to be the best homeotic gene marker for the identification of individual cells within the thoracic ventral ganglion. Furthermore, maps of *engrailed* expression were developed and were used as cellular landmarks in this region. Additionally, a double staining technique whereby cells expressing *engrailed* and neuroblasts undergoing cellular division are stained together was evaluated and optimized. Preliminary results from the use of a visible-fluorescent double stain indicate that this technique can be used to identify specific populations of dividing neuroblasts since the *engrailed* expression maps provide landmarks within the brains for determining the relative positions of these neuroblasts.

MATERIALS AND METHODS

Developmental staging

Canton-S (CS) larvae were raised at 25°C on apple juice plates containing yeast; larval collection was synchronized in one hour windows where newly molted second instar larvae, determined by the appearance of spiracle buds, were isolated and allowed to grow by themselves for either 1, 2, or 3 hours on apple juice plates with yeast.

BrdU incorporation

Food media containing 1 mg/mL of BrdU (5-bromodeoxyuridine), a reagent that incorporates into replicating DNA, was made. Developmentally staged larvae were raised in this media for 1 hour at 25°C.

Immunohistochemistry

Brains dissected from CS larvae were fixed for 10 minutes at room temperature in Enhancer Trap fix; after which they were washed three times in 1X PBST. Brains where BrdU had been incorporated were denatured in 2N HCl in 1X PBST for 30 minutes at room temperature and were then washed. Brains were incubated for 1 hour at room temperature in 1X PBNT. The brains were then split into separate groups where each group was exposed for 12 hours at 4°C to a specific dilution of a primary antibody (listed in Table 1) against the protein product of one of the homeotic genes. After primary antibody incubation, the brains were then washed three times in 1X PBST and

were then incubated at room temperature in a secondary antibody solution for the times and dilutions listed in Table 2. Upon completion of the secondary antibody incubation, the brains were washed three times in 1X PBST. A solution of 0.05% diaminobenzidine with 0.03% H_2O_2 was then added to the brains labeled with the HRP-conjugated secondary antibody; these brains were left to incubate in the dark for up to 10 minutes or until signal began to appear. The alkaline phosphatase signal was developed after labeling with the alkaline phosphatase-conjugated secondary antibody by incubating the brains in a 1:50 NBT/BCIP solution for 5 minutes at room temperature. All brains were then washed 3 times with 1X PBST and were mounted in 50% glycerol or DAKO fluorescence mount for observation under a Zeiss axiophot compound microscope.

Table 1. Primary antibodies, dilutions, and sources

Primary Antibody	Dilution	Source
Mouse anti-rho1 IgG	1:100, 1:500	Developmental Hybridoma Bank
Mouse anti-Nervana IgG	1:100	Developmental Hybridoma Bank
Mouse anti-enabled IgG	1:100	Developmental Hybridoma Bank
Mouse anti-wit IgG	1:100	Developmental Hybridoma Bank
Mouse anti-Abd-B IgG	1:200, 1:300, 1:500	Developmental Hybridoma Bank
Mouse anti-engrailed supernatant IgG	1:5	Developmental Hybridoma Bank
Mouse anti-engrailed concentrate IgG	1:25, 1:50	Developmental Hybridoma Bank
Mouse anti-engrailed Ascites IgG	1:200, 1:300, 1:500	Developmental Hybridoma Bank

Table 2. Secondary antibodies, dilutions, incubation times, and sources

Secondary Antibody	Dilution	Incubation Times (hours)	Source
Goat anti-mouse horse radish peroxidase (HRP)-conjugated IgG	1:200	5	Molecular Probes
Goat anti-mouse alkaline phosphatase-conjugated IgG	1:100	4	Molecular Probes
Alexa Fluor 488 goat anti-mouse IgG	1:1000	1, 2, 4	Molecular Probes
	1:5000	2, 4	
Alexa Fluor 594 goat anti-mouse IgG	1:1000	4	Molecular Probes
	1:5000	1, 4	
Anti-mouse biotinylated anti-mouse IgG	1:100	12	Amersham
Alexa Fluor 488 streptavidin-conjugated IgG	1:1000	2	Molecular Probes
Alexa Fluor 594 $F(ab')_2$ rabbit anti-mouse IgG	1:1000	2	Molecular Probes

RESULTS AND DISCUSSION

Evaluation of homeotic gene expression as specific cellular markers

In order to identify and characterize specific populations of actively dividing neuroblasts within the thoracic ventral ganglion of *Drosophila* larvae, cellular landmarks in this region had to be found. Previous studies showed that the expression of developmental genes acted as molecular markers for identifying specific sets of neuroblasts in the developing *Drosophila* brain (Doe, 1992; Urbach et al., 2003; Urbach and Technau, 2003). The expression of the homeotic genes ***rho1***, ***Nervana***, ***wit***, ***enabled***, *Abd-B*, and *engrailed* were evaluated as potential markers for the identification of specific cellular populations within the thoracic region of the ventral ganglion of early second instar CS larvae. Mouse primary antibodies raised against the protein products of these genes were used as indicators of which cells were expressing which of the genes; goat anti-mouse HRP-conjugated secondary antibody was used to label the mouse primary antibodies.

The first set of primary antibodies evaluated for labeling of individual cells were those that targeted protein products of *rho1*, *Nervana*, *wit*, and *enabled*. Anti-rho1 primary antibody at either 1:100 or 1:500 dilutions was used to stain cells expressing *rho1*. As seen in Figure 1A and 1B, staining was limited to the margins of the ventral ganglion and was very diffuse so that no particular sets of cells could be identified even when the primary antibody concentration was lowered five fold from 1:100 (Figure 1A) to 1:500 (Figure 1B). Anti-Nervana primary antibody at 1:100 was used to label

Nervana expressing cells; however, no staining appeared throughout the entire brain (Figure 1C). Labeling with anti-wit primary antibody yielded a lack of any visible staining, as seen in Figure 1D; thus no *wit* expressing cells are present at early second instar. Additionally, labeling by anti-enabled primary antibody was evaluated and, as seen in Figure 1E, was found to stain a large, diffuse region of the brain where no specific *enabled* expressing cells were selectively labeled.

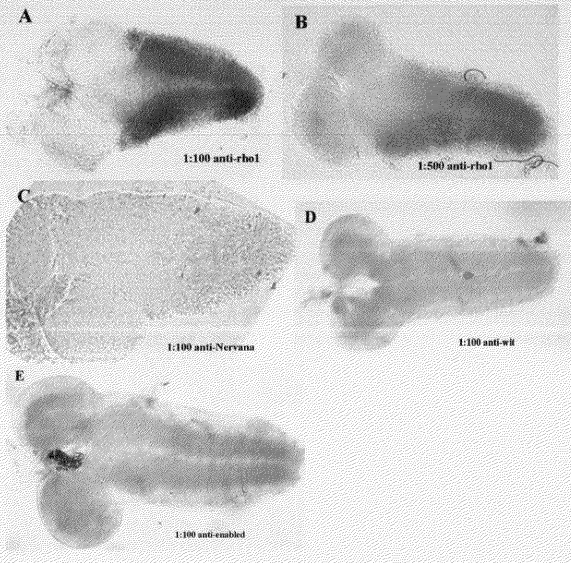

Figure 1. Staining of cells expressing *rho1*, *Nervana*, *wit*, and *enabled*. Brains from second instar CS larvae labeled with (A) 1:100 and (B) 1:500 mouse anti-rho1, (C) 1:100 mouse anti-Nervana, (D) 1:100 mouse anti-wit, and (E) 1:100 mouse anti-enabled; all magnified at 20X.

Though the primary antibodies listed above did not yield staining of individual populations of cells within the brain, individual cells were identified using the primary antibodies anti-Abd-B and anti-engrailed. Anti-Abd-B primary antibody at 1:100 (Figure 2A), 1:200 (Figure 2B), and 1:300 (Figure 2C) dilutions labeled specific populations of *Abd-B* expressing cells in the posterior tip of the ventral ganglion. Furthermore, as the dilution of anti-Abd-B decreased from 1:200 to 1:300, the background staining decreased, thus making individual cells more visible. Even though *Abd-B* expressing cells within the posterior tip of the ventral ganglion were stained, no cells were labeled in the thoracic ventral ganglion. However, when anti-engrailed primary antibody was evaluated for its potential in labeling specific *engrailed* expressing cells, individual cells were identified in the thoracic ventral ganglion. Three different types of mouse anti-engrailed primary antibodies were evaluated; these included anti-engrailed supernatant, anti-engrailed concentrate, and anti-engrailed Ascites. These anti-engrailed primary antibodies differed solely on the concentration of the antibody present in the aliquot whereby supernatant had the lowest concentration of anti-engrailed antibody and Ascites had the highest. As seen in Figure 3A, incubation with 1:5 mouse anti-engrailed supernatant produced a weak labeling of *engrailed* expressing cells in the thoracic ventral ganglion. However, labeling with 1:25 and 1:50 mouse anti-engrailed concentrate or with 1:200, 1:300, and 1:500 mouse anti-engrailed Ascites stained individual *engrailed* expressing cells within the thoracic ventral ganglion (Figures 3B-C and Figures 4A-C respectively). These results indicate that the expression of *engrailed* can be used as a molecular marker for identifying specific cellular populations in the

thoracic ventral ganglion. Thus either the concentrate or the Ascites anti-engrailed primary antibodies were selected to be used throughout this study for identifying individual *engrailed* expressing cells within the larval brain.

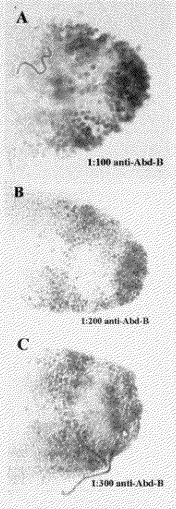

Figure 2. Staining of *Abd-B* expressing cells using various dilutions of anti-Abd-B primary antibody. Brains from early second instar CS larvae labeled with (A) 1:100, (B) 1:200, and (C) 1:300 mouse anti-Abd-B; all magnified 40X.

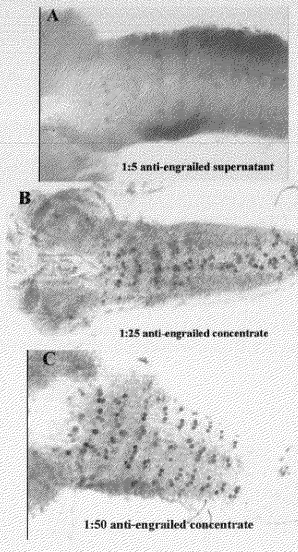

Figure 3. Staining *engrailed* expressing cells using anti-engrailed primary antibodies. Brains from early second instar CS larvae labeled with (A) 1:5 mouse anti-engrailed supernatant, (B) 1:25 mouse anti-engrailed concentrate, and (C) 1:50 mouse anti-engrailed concentrate; all at 20X magnification.

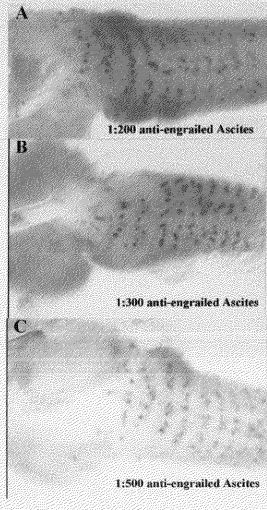

Figure 4. Staining *engrailed* expressing cells using various dilutions of anti-engrailed Ascites primary antibody. Brains from early second instar CS larvae labeled with (A) 1:200, (B) 1:300, and (C) 1:500 mouse anti-engrailed Ascites; all at 20X magnification.

Optimization of anti-engrailed labeling

Labeling cells using anti-engrailed primary antibodies was optimized in that the effects of denaturation were evaluated and the optimal primary antibody dilution was determined in order to yield accurate, reproducible staining for the establishment of cellular maps of *engrailed* expression.

Denaturation by the 2N HCl in 1X PBST solution is necessary in allowing the anti-BrdU primary antibody access to DNA where BrdU has been incorporated; however, it is not known whether the high concentration of HCl in this solution destroys some of the engrailed protein, thus affecting the labeling of cells expressing *engrailed.* To determine denaturation's effects on *engrailed* staining, the placement of this denaturation step was altered so that it occurred either before or after HRP-catalyzed diaminobenzidine staining of *engrailed* expressing cells. One set of second instar brains were fixed and incubated in 1:25 mouse anti-engrailed concentrate. They were then labeled with 1:100 goat anti-mouse HRP-conjugated IgG and were stained with diaminobenzidine. They were denatured in 2N HCl in 1X PBST following these steps. Figure 5A depicts the staining of the *engrailed* expressing cells in the thoracic ventral ganglion of these brains. Another set of second instar larval brains were denatured in 2N HCl in 1X PBST after fixing and were incubated in 1:25 mouse anti-engrailed concentrate; these brains were then labeled with 1:100 goat anti-mouse HRP-conjugated IgG and stained with diaminobenzidine. Figure 5B depicts the staining of *engrailed* expressing cells in the thoracic ventral ganglion of these brains. As seen in Figures 5A and 5B, the same thoracic cells in the ventral ganglion were labeled no matter where the

denaturation step occurred. Furthermore, comparison of the figures reveals that there was more non-specific staining present in brains that were stained with diaminobenzidine before the denaturation step than in brains where diaminobenzidine staining followed denaturation.

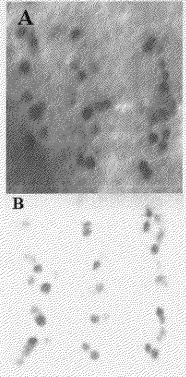

Figure 5. Effects of denaturation on *engrailed* staining. Photographs of the staining of *engrailed* expressing cells in the thoracic ventral ganglion of second instar CS larvae where the denaturation step either (A) followed or (B) preceded the diaminobenzidine staining step. Brains labeled with 1:25 mouse anti-engrailed concentrate primary antibody; all brains magnified 80X.

Since the denaturation procedure does not affect the staining of *engrailed* expressing cells, the type of anti-engrailed primary antibody used and its dilution were optimized so that the brightest and clearest staining could be achieved. Figures 6A-C show the cellular staining of 1-2 hours old second instar brains incubated in either 1:25 mouse anti-engrailed concentrate (Figure 6A), 1:50 mouse anti-engrailed concentrate (Figure 6B), or 1:300 mouse anti-engrailed Ascites (Figure 6C). These photographs reveal that *engrailed* expressing cells were best labeled with either mouse anti-engrailed concentrate at 1:25 or mouse anti-engrailed concentrate Ascites at 1:300; thus either of these two primary antibodies at these dilutions can be used to stain cells expressing *engrailed*.

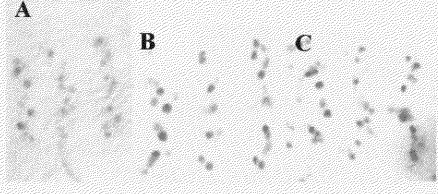

Figure 6. Optimization of *engrailed* staining. Photographs of stained *engrailed* expressing cells in the thoracic ventral ganglion of second instar CS brains labeled with (A) 1:50 mouse anti-engrailed concentrate primary antibody, (B) 1:25 mouse anti-engrailed concentrate primary antibody, or (C) 1:300 mouse anti-engrailed Ascites primary antibody; all brains magnified 80X.

Establishment of age dependent *engrailed* expression maps

The extremely clear and highly reproducible staining of *engrailed* expressing cells using either the concentrate or the Ascites aliquots of the anti-engrailed primary antibody allowed age-dependent maps of *engrailed* expressing cells in the thoracic ventral ganglion to be established.

Brains from developmentally staged second instar larvae that were placed on larval plates for the specific amounts of time previously described were used in these experiments to establish *engrailed* expression maps. The larvae that were raised on the plate for 3, 2, 1, or 0 hours corresponded to 3-4, 2-3, 1-2, 0-1 hours old (postmolting) larvae respectively. These brains were labeled with 1:300 mouse anti-engrailed Ascites primary antibody and 1:200 goat anti-mouse HRP-conjugated IgG secondary antibody. Figure 7 show photographs of the thoracic region of representative brains from 0-1 (Figure 7A), 1-2 (Figure 7B), 2-3 (Figure 7C), 3-4 (Figure 7D) hours old larvae. Seven or more brains were included in each age group. All showed the same cellular staining pattern as depicted in these four photographs, thus demonstrating the highly conserved nature of *engrailed* expression in brains of the same age.

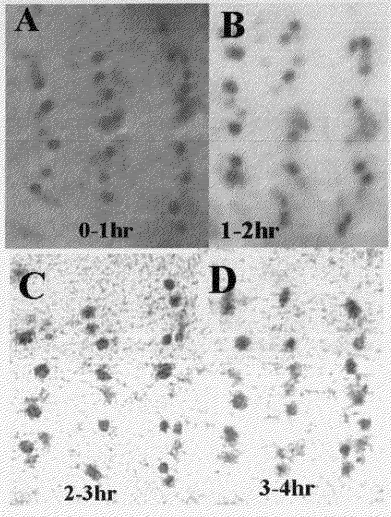

Figure 7. Age dependent *engrailed* expression. Photographs of stained *engrailed* expressing cells in the thoracic ventral ganglion of second instar CS brains from larvae (A) 0-1, (B) 1-2, (C) 2-3, and (D) 3-4 hours old. All brains labeled with 1:300 mouse anti-engrailed Ascites primary antibody and magnified 80X.

The first three rows (from the anterior of the brain) of stained cells shown in the previous figures reside in the thoracic ventral ganglion; thus these cells were selected to represent the *engrailed* expressing cells in the thoracic region. Graphic representations of the age dependent *engrailed* expression maps in the three rows of the thoracic ventral ganglion are depicted in Figure 8. Only the heaviest staining *engrailed* expressing cells from the photographs in Figure 7 were used to create this map. Thus, the establishment of these age dependent maps for *engrailed* expression allows for the use of these stained cells to act as guideposts for identifying the relative positions of actively dividing neuroblasts within the thoracic region of the ventral ganglion.

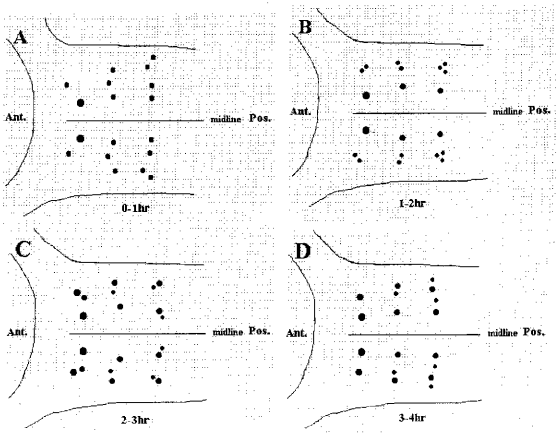

Figure 8. Age dependent *engrailed* expression maps. Brains from (A) 0-1, (B) 1-2, (C) 2-3, and (D) 3-4 hours old second instar CS larvae stained with 1:300 mouse anti-engrailed Ascites primary antibody (Ant. signifies anterior and Pos. signifies posterior).

Optimization of double staining technique

The double staining technique couples the staining of *engrailed* expressing cells and dividing neuroblasts, thus allowing for *engrailed* expressing cells to be used as positional references for the identification of individual populations of dividing neuroblasts. Double staining techniques were evaluated using a series of experiments where the type of double staining was varied from stains that produce two different visible stains to ones that produce stains visible solely under fluorescent light of particular wavelengths.

Double staining using HRP- and alkaline phosphatase-conjugated labels

As shown in previous results, HRP (horse radish peroxidase) catalyzation of the chemical reaction involving diaminobenzidine produces a dark brown stain that is visible under light microscopy. Additionally, alkaline phosphatase catalyzes a reaction that produces a deep purple stain in the presence of the appropriate substrate. Thus, using both of these enzyme-conjugated antibodies produces a double stain of deep purple and brown. The double staining experiments performed in this study used a goat anti-mouse alkaline phosphatase-conjugated secondary antibody to label cells expressing *engrailed* and a goat anti-mouse HRP-conjugated secondary antibody to label neuroblasts that incorporated 5-bromodeoxyuridine (BrdU). These experiments not only assessed the practical value of using the alkaline phosphatase- and HRP-double staining technique but also evaluated the effect of the development of the alkaline phosphatase signal on diaminobenzidine staining of BrdU labeled cells by altering the placement of the

alkaline phosphatase staining step in the procedure. Brains from second instar CS larvae fed BrdU laced media were labeled with 1:50 mouse anti-engrailed concentrate primary antibody and were then split into two separate groups. In the first group the alkaline phosphatase signal was developed after incubation in 1:100 goat anti-mouse alkaline phosphatase-conjugated secondary antibody. These brains were then incubated in 1:200 mouse anti-BrdU primary antibody followed by incubation in 1:200 goat anti-mouse HRP-conjugated secondary antibody. The HRP signal was developed with a diaminobenzidine solution as previously described. The other group of brains was incubated in the mouse anti-BrdU primary antibody following removal of the anti-mouse alkaline phosphatase-conjugated secondary antibody. These brains were then incubated in the anti-mouse HRP-conjugated secondary antibody as described previously. Both the alkaline phosphatase and the HRP signals were developed after this incubation following the same procedure as stated before. Results from these experiments show that developing the alkaline phosphatase signal before BrdU labeling does not have any effect on the staining of neuroblasts where BrdU was incorporated. Furthermore, the brown stain indicating dividing neuroblasts is difficult to distinguish from the deep purple stain indicating *engrailed* expressing cells (Figure 9). Thus, the use of alkaline phosphatase- and HRP-conjugated secondary antibodies for the double staining of cells expressing *engrailed* and actively dividing neuroblasts is not a viable option to use for the identification of individual populations of dividing neuroblasts.

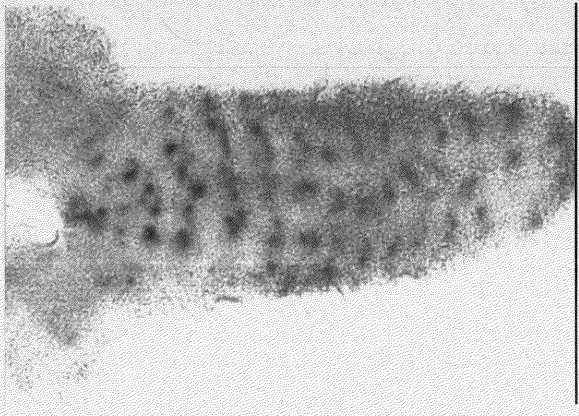

Figure 9. Double staining of using alkaline phosphatase- and HRP-conjugated secondary antibodies. *Engrailed* expressing cells and dividing neuroblasts in the brains of second instar CS larvae were labeled with alkaline phosphatase-conjugated secondary antibody and HRP-conjugated secondary antibody respectively.

Double staining using fluorescent secondary antibodies

Using fluorescent secondary antibodies to double stain both *engrailed* expressing cells and dividing neuroblasts was hypothesized to work well since these antibodies

fluoresce only under certain wavelengths thus decreasing the amount of visual interference that is present in other double staining techniques, such as in the alkaline phosphatase- and HRP-double stain previously described.

In the following experiments the effectiveness of the fluorescent double staining technique was evaluated, and the optimal fluorescent secondary antibody dilutions were evaluated. Brains from early second instar CS larvae that were fed BrdU laced media were incubated in mouse anti-engrailed concentrate primary antibody at a 1:50 dilution. One group of brains was incubated in the dark for 4 hours at room temperature in 1:5000 Alexa Fluor 488 anti-mouse secondary antibody. These brains were then incubated in 1:200 mouse anti-BrdU followed by a 4 hour incubation in 1:5000 Alexa Fluor 594 anti-mouse secondary antibody. Very little staining of *engrailed* expressing cells was seen in the brains labeled with this dilution of Alexa Fluor 488 secondary antibody (Figure 10A), yet the staining of dividing neuroblasts was quite clear (Figure 10B).

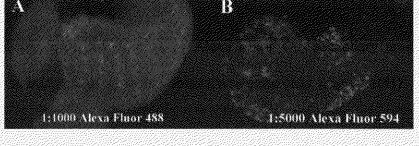

Figure 10. Fluorescent double staining. Early second instar CS brains doubled stained with both Alexa Fluor 488 and 594. (A) Alexa Fluor 488 anti-mouse at a 1:1000 dilution (4 hour incubation) stained *engrailed* expressing cells green; whereas, (B) Alexa Fluor 594 anti-mouse at a 1:5000 dilution (4 hour incubation) stained dividing neuroblasts red.

As seen in the previous experiments, the dilution of Alexa Fluor 488 secondary antibody did not label *engrailed* expressing cells to a high degree; thus engrailed staining was optimized by both increasing the concentration of anti-engrailed primary antibody and by altering either the concentration of the fluorescent secondary antibody or the type of fluorescent secondary antibody (Alexa Fluor 488 or 594) used. Early second instar CS larvae were fed BrdU laced media for an hour; their brains were then incubated in 1:25 mouse anti-engrailed concentrate primary antibody. These brains were split into two groups: one in which the concentration of Alexa Fluor 488 secondary antibody was increased and another where the type of secondary antibody was altered. Brains in the first group were incubated in an Alexa Fluor 488 anti-mouse dilution of either 1:1000 or 1:5000. The incubation times were shortened from 4 hours to 2 hours in order to decrease on the amount of non-specific staining leading to background fluorescence. Brains in the second group were incubated for 4 hours in either 1:1000 Alexa Fluor 594 anti-mouse or 1:1000 Alexa Fluor 488 anti-mouse. Brains in both groups were then incubated in a 1:200 mouse anti-BrdU primary antibody and a 1:5000 Alexa Fluor 594 anti-mouse (except for one group, they were incubated in Alexa Fluor 488) secondary antibody following the procedures stated previously. The staining of both *engrailed* expressing cells and dividing neuroblasts in both these groups did not change from that shown in Figure 10. Thus, these experiments demonstrate that fluorescent staining of *engrailed* expressing cells occurs at extremely low levels, and thus is mostly undetectable, using these two types of fluorescent secondary antibodies.

Since these two fluorescent secondary antibodies did not work well in detecting *engrailed* expression, another fluorescent secondary antibody called Alexa Fluor 488 streptavidin-conjugated IgG was used to enhance the staining of cells expressing *engrailed*. Streptavidin-conjugated secondary antibodies selectively bind to biotinylated antibodies thereby both increasing the staining of specific cells and reducing the non-specific staining of background tissues. Brains from second instar CS larvae were incubated in 1:25 mouse anti-engrailed concentrate primary antibody followed by incubation in 1:100 anti-mouse biotinylated secondary antibody. These brains were then incubated in 1:1000 Alexa Fluor 488 streptavidin-conjugated secondary antibody for 2 hours. As seen in Figure 11, this streptavidin secondary antibody did not work either as characterized by the lack of staining *engrailed* expressing cells and by the extensive non-specific staining of the rest of the brain.

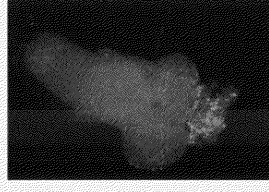

Figure 11. Staining of dividing neuroblasts with Alexa Fluor 488 streptavidin-cojugated secondary antibody. *engrailed* expressing cells in the brains of early second instar CS larvae stained with streptavidin Alexa Fluor 488 secondary antibody at 1:1000 dilution.

Overall, the experiments using fluorescent secondary antibodies to double stain both *engrailed* expressing cells and dividing neuroblasts demonstrate that this procedure is an unviable one since *engrailed* expressing cells were stained very lightly if at all.

Visible-fluorescent double staining

A combination of using both a visible stain to label *engrailed* expressing cells, which worked well in characterizing *engrailed* expression within the thoracic region, and a fluorescent stain to label dividing neuroblasts was hypothesized to yield the best staining results. This double staining technique was used to try to establish age dependent patterns of dividing thoracic neuroblasts where the *engrailed* expression maps were used as reproducible guideposts for determining the relative positions of the dividing neuroblasts in the thoracic ventral ganglion.

Brains were isolated from 1-2, 2-3, and 3-4 hours old second instar larvae and were double stained to try to identify individual populations of dividing thoracic neuroblasts. These brains were fixed and denatured as described previously and were then incubated in 1:50 mouse anti-engrailed concentrate primary antibody followed by incubation in 1:200 goat anti-mouse HRP conjugated secondary antibody. After the *engrailed* expressing cells were stained with diaminobenzidine, the dividing neuroblasts were labeled with 1:100 mouse anti-BrdU primary antibody followed by labeling with 1:1000 Alexa Fluor 488 anti-mouse secondary antibody. Photographs of both the visible stain (Figures 12A, 12C, and 12E) and the fluorescent stain (Figure 12B, 12D, and 12F) of individual brains were taken; these two sets of photographs were then merged

together to produce those shown in Figure 13. The merged photographs of both the visible stain and the fluorescent stain represent the end product of this double staining technique. The clear staining of *engrailed* expressing cells enable the *engrailed* expression maps established earlier in this study to be used as cellular landmarks to identify the relative positions of dividing neuroblasts within the thoracic region. As seen in these merged photographs, there is a clear need to optimize the fluorescent staining of dividing neuroblasts since the staining of these cells appeared diffuse (with a shutter delay of >5 seconds) thus making the identification of individual dividing neuroblasts difficult.

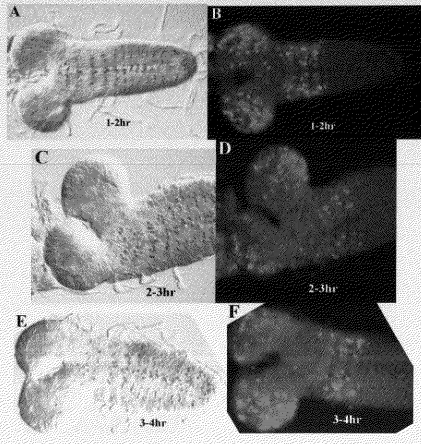

Figure 12. Age dependent visible and fluorescent staining of second instar brains. Visible staining of *engrailed* expressing cells in (A) 1-2, (C) 2-3, and (E) 3-4 hours old brains and fluorescent staining of dividing neuroblasts in the same (B) 1-2, (D) 2-3, (F) 3-4 hours old brains.

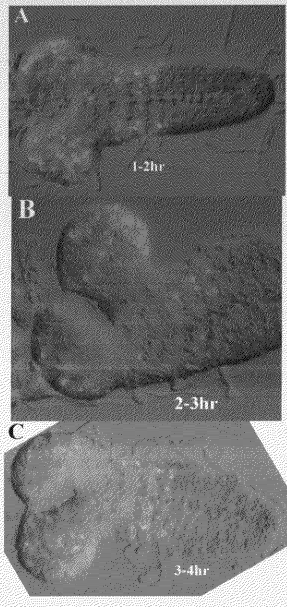

Figure 13. Age dependent visible-fluorescent double staining of second instar brains. Photographs represent the merger of the photographs in Figure 12 and thus depict the double staining of *engrailed* expressing cells (brown stain) and dividing neuroblasts (green stain) in (A) 1-2, (B) 2-3, and (C) 3-4 hours old brains.

Alexa Fluor 594 F(ab')$_2$ optimization

Due to the poor fluorescent staining of dividing neuroblasts using Alexa Fluor 488 anti-mouse secondary antibody, staining with a more specific fluorescent secondary antibody, Alexa Fluor 594 F(ab')$_2$ rabbit anti-mouse IgG, was used to stain dividing neuroblasts in brains from early second instar larvae. BrdU was incorporated into these brains as outlined in the Materials and Methods; the brains were incubated in 1:100 mouse anti-BrdU primary antibody followed by a 2 hour incubation in 1:1000 Alexa Fluor 594 F(ab')$_2$ anti-mouse secondary antibody. Figure 14 shows the photograph of dividing neuroblasts stained with this fluorescent antibody. The bright staining allows for individual neuroblasts to be seen; furthermore, this fluorescent staining was highly visible with a shutter delay of less than a second. The extremely specific and highly visible staining makes Alexa Fluor 594 F(ab')$_2$ rabbit anti-mouse IgG a good candidate to use to re-evaluate the visible-fluorescent double staining technique presented in the previous section for the identification of specific populations of dividing thoracic neuroblasts.

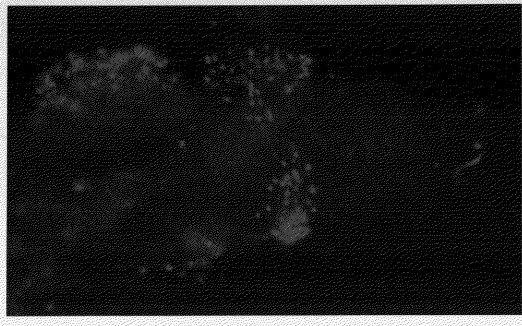

Figure 14. Staining of dividing neuroblasts with Alexa Fluor 594 F(ab')$_2$ anti-mouse secondary antibody. Dividing neuroblasts within early second instar larval CS brains were stained with Alexa Fluor 594 F(ab')$_2$ anti-mouse secondary antibody at a 1:1000 dilution for 2 hours.

CONCLUSION

In previous studies the expression of developmental genes, such as homeotic and proneural genes, were used as markers for the identification of specific populations of neuroblasts within the central nervous system of developing *Drosophila melanogaster* (Doe, 1992; Urbach et al., 2003; Urbach and Technau, 2003). The studies presented in this thesis have further extended the use of homeotic gene expression from their use as individual cell markers to using these markers as cellular landmarks for identifying individual populations of actively dividing neuroblasts within the thoracic ventral ganglion of *Drosophila* larvae. Expression of *engrailed* was shown to reproducibly select specific patterns of individual cells within the thoracic ventral ganglion and to allow for maps of *engrailed* expression in this region to be drawn at different developmental time points during early second instar. By coupling the visible staining of *engrailed* expressing cells to the fluorescent staining of dividing neuroblasts, a process called double staining, one is able to juxtapose the dividing neuroblasts onto the *engrailed* expression map thus allowing for the relative positions of the dividing neuroblasts to be determined and for specific populations of these cells to be identified. The results of this visible-fluorescent double staining technique must be optimized since the fluorescent stain is diffuse and does not allow for individual dividing neuroblasts to be seen; however, the use of Alexa Fluor 594 $F(ab')_2$ anti-mouse secondary antibody as a fluorescent stain holds promise in that it stains dividing neuroblasts both specifically and clearly. Overall, the use of the double staining technique to stain both *engrailed*

expressing cells and dividing neuroblasts allows for the effects of mutations in the components of the signaling cascades that cue neuroblast division to be examined in greater detail.

As stated previously, mutations in the growth factors genes *branchless* and *hedgehog* coupled to mutations in their proteoglycan co-receptor gene *trol* lead to a decrease in the amounts of dividing neuroblasts in both the brain lobes and the ventral ganglion of second instar larvae (Park et al., 2003). It is not known whether this decrease in division is a stochastic event where all neuroblasts are inhibited from dividing or if specific populations of neuroblasts are targeted for inhibition. The technique of double staining of both *engrailed* expressing cells and dividing neuroblasts outlined in this study can be used to address this question. By identifying specific populations of dividing thoracic neuroblasts in non-mutant larvae and by comparing them to those that appear in *bnl;trol* and *hh;trol* double mutants, one can determine which neuroblast populations are responding to which growth factors, thus further characterizing the role that these growth factors play in cuing neuroblast division. Furthermore, by evaluating which neuroblast populations are dividing in different *trol* domain mutants, the description of *trol* can be enhanced. Through the use of this double staining technique, a greater understanding of stem cell biology and neural development will be achieved not only for *Drosophila melanogaster* but also *Homo sapiens*.

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