The overexpression of homeotic complex gene Ultrabithorax in the post-embryonic neuronal lineages of the ventral nervous system in Drosophila melanogaster

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THE OVEREXPRESSISON OF HOMEOTIC COMPLEX GENE
ULTRABITHORAX IN THE POST-EMBRYONIC NEURONAL LINEAGES OF
THE VENTRAL NERVOUS SYSTEM IN DROSOPHILA MELANOGASTER

by

Katie E. Dry

(A Thesis)

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ABSTRACT

Developing nervous systems face an immense challenge; neural precursor cells must differentiate into a variety of neuron types and then go on to establish functional synaptic connections at sometimes distant targets. Over a century of research has begun to elucidate the precise mechanisms responsible for this remarkable process. For most of that time, *Drosophila melanogaster*, the fruit fly, has proven to be an exceptional model organism for studying development processes. In addition to its short life cycle and ease of handling, *Drosophila* displays an array of genetic markers with which a number of sophisticated molecular tools have been generated. The genome of *Drosophila* can be manipulated with relative ease and the resulting phenotypes can be studied. Interestingly, hundreds of *Drosophila* genes have been conserved across species including humans, with their function providing insight to many normal biological and disease-related processes.

In *Drosophila*, most nervous system studies thus far have focused on the embryo or on particular neuronal classes in the adult brain. The postembryonic neurons of the ventral nervous system, however, are proving to be interesting and useful subjects for studying neurological mechanisms. Arising from neural precursor cells called neuroblasts, these adult-specific neurons appear later in larval life. The progeny of each neuroblast forms a morphologically distinct neuronal lineage that sends out characteristic axon projections that arrest prior to metamorphosis and later sprout to form the neural connections of the adult. Analysis of these arrested neurons in late larval stages has
revealed that their morphology depends on the segment in which they are present. This neuronal patterning in an apparent anteroposterior manner has implicated the Homeotic complex (Hox) genes as contributors to neural lineage specification.

Hox genes have long been studied and identified as important regulators of segment identity in *Drosophila*. In fact, manipulations of these genes can result in transformations in which one body segment looks like another. Previous loss of function experiments in the post-embryonic neurons of the ventral nervous system have resulted in more posterior lineages looking like more anterior lineages. Additional gain of function experiments are needed to confirm the role of the Hox genes in these transformation results.

This study uses a molecular technique called MARCM (Mosaic Analysis with a Repressible Cell Marker) to label neuronal lineages that overexpress the Hox gene *Ultrabithorax (Ubx)* in an unlabeled, wild type background. The results indicate that the overexpression of Ubx is sufficient to transform more anterior neuronal lineages to the morphology of their more posterior counterparts. The data presented here begin to elucidate the role that the Hox genes have in shaping segment-specific neural connections in the post-embryonic ventral nervous system.
INTRODUCTION

An intriguing and driving question of developmental biology is how a single cell develops into a complex organism with highly organized and diverse cell types. Somehow, the genome within that one cell initiates and directs a cascade of cellular division and differentiation. Subsequent cells continue the process, and amazingly, not only regulate their own genetic activity, but also influence and are influenced by those cells around them. Despite all of the cells of an organism possessing the same genotype, they end up performing very different, yet well-orchestrated tasks.

Neurons represent just one major type of cell that develops within an organism. Even within the nervous system, however, neurons diversify to take on a vast array of different forms and perform distinct functions. Early neural cells must establish an identity and then develop necessary projections so as to locate and form appropriate synaptic connections. Over a century of research has begun to elucidate the precise developmental mechanisms responsible for this remarkable process.

*Drosophila melanogaster*: A Model Organism for Studying Developmental Biology

The fruit fly (*Drosophila melanogaster*) represents one of the earliest established and most widely used model organisms for addressing the questions of developmental biology. In terms of husbandry, *Drosophila* is inexpensive and easy to maintain. Hundreds of flies can be housed in a relatively small space, and their entire life cycle takes a mere 10 to 14 days. Even more useful is the long history of study of the
Drosophila genome and the many tools developed to study the connections between genotype and phenotype.

The Drosophila melanogaster genome was fully sequenced in 2000 (Adams et al., 2000 and Myers et al., 2000), but the use of Drosophila in understanding genetics began nearly 100 years earlier with the work of Thomas H. Morgan and his students Sturtevant, Bridges, and Muller (reviewed in Arias, 2008). Their work provided the foundation on which all other fly research is based. Most notably, they performed controlled crosses, monitored adult mutant phenotypes, and related those phenotypes back to specific genes (reviewed in Rubin and Lewis, 2000). Today, many different mutant phenotypes affecting eye color, eye shape, wing shape, bristle shape, bristle color, etc. are used as genetic markers. These genetic markers aid in developing crosses and identifying fly lines that have desired genotypes for specific experiments (Greenspan, 2004).

Advances in the available molecular biological tools have increased the ease with which the fly genome can be manipulated and the resulting phenotypes can be studied. As a result, much insight has been gained as to the way genes function to control development in the fly. Drosophila, however, maintains its relevance as a model research organism because many of its genes and molecular processes have been conserved evolutionarily. It has been estimated that approximately 700 human disease genes have well-conserved homologues in Drosophila (reviewed in Bier, 2005). This means that Drosophila can be used to study the molecular mechanisms of human diseases like cancer, neurological disorders, developmental disorders, etc. Additionally,
Drosophila can be used to gain insight into the workings of fundamental biological processes common to many diverse organisms including humans.

**Development of the Ventral Nervous System in Drosophila melanogaster**

The development of the nervous system represents an incredible challenge for any organism. A diverse and intricate network of neurons must locate appropriate targets and establish functional connections. Interestingly, an insect like Drosophila that undergoes complete metamorphosis must generate two distinct nervous systems. The first innervates the larva, which is composed mainly of repeating parasegments. This nervous system specializes in abdominal mobility to facilitate crawling and feeding. The second innervates the adult fly, which is divided into the head, thorax, and abdomen, each of which contains its own set of morphologically unique segments. These neurons specialize in wing and leg mobility for flying and walking, as well as in sensory perception for feeding and mating. How are these two very different systems of connections established in such a short life cycle?

The ventral nerve cord originates from ventral neural ectoderm in the Drosophila embryo. At stage 9 of development, isolated neuronal precursor cells called neuroblasts delaminate from the neurogenic region of the ventral neural ectoderm and move internally in the embryo (Hartenstein and Campos-Ortega, 1984). After forming a monolayer of cells between the ectoderm and mesoderm, the neuroblasts begin a series of self-renewing asymmetric divisions. Each division produces another neuroblast and a ganglion mother cell (GMC). Each GMC then undergoes one mitotic division to give
rise to a pair of sibling cells that differentiate into neurons. Approximately 50 neuroblasts delaminate in each neuromere, the repeating segments within the central nervous system. This ultimately provides about 400 embryonic neurons per segment for larval life (reviewed in Campos-Ortega, 1993).

What is even more fascinating, however, is the means by which the adult-specific neurons later develop. It has been shown that the same embryonic neuroblasts that give rise to the larval neurons also generate the postembryonic neurons (Prokop and Technau, 1991). The neuroblasts enter quiescence or a state of dormancy after their initial set of divisions during embryogenesis. During the first instar of larval growth the neuroblasts reactivate and divide asymmetrically once again. The progeny of the GMCs at this stage, however, make up the adult specific-neurons of the ventral nervous system. In the thoracic neuromeres, segments where legs and wings will develop, approximately 47 of 50 embryonic neuroblasts reactivate in larval life. In the abdominal neuromeres, segments that are greatly reduced in the adult, only 6 of the embryonic neuroblasts persist. Remarkably, post-embryonic neuroblasts give rise to over 90% of the adult ventral nervous system (Truman and Bate, 1988).

The reactivated neuroblasts continue to divide as the larva grows, and each accumulates its own bundle of progeny. These immature neurons project their fasciculated neurites to an initial set of targets and then arrest neurite outgrowth. It is not until pupariation and metamorphosis that the post-embryonic neuron lineages intensely sprout and make their adult connections (Truman et al., 2004). In developing the post-embryonic lineages as a novel experimental system in which to study neural
development, Truman and colleagues utilized a powerful molecular genetic technique called Mosaic Analysis with a Repressible Cell Marker (MARCM) to identify and characterize these arrested adult-specific neurons during the 3rd instar larval phase just prior to metamorphosis (2004).

**Mosaic Analysis with a Repressible Cell Marker**

Mosaic Analysis with a Repressible Cell Marker (MARCM) provides an example of one of the powerful and sophisticated molecular tools that are utilized to study the development of *Drosophila*. By combining genetic recombination, directed gene expression, and cellular labeling techniques, MARCM allows for the visualization of experimentally manipulated cells against an unlabeled wild type background (Lee and Luo, 1999). Essentially, the effects of a gene that might otherwise be lethal if expressed or disrupted universally can be studied in a smaller subset of easily identifiable cells.

In general, a genetic mosaic refers to an organism with clones of cells that have different genotypes from the cells of the entire organism. Genetic mosaics can occur naturally, but mosaics can also be created by inducing mitotic recombination (Xu and Rubin, 1993). For a long time, scientists relied on X-rays to cause random chromosomal breaks that would lead to chromosomal exchange and eventually unpredictable mutant mosaic phenotypes. The FLP-FRT site-specific recombination system from yeast, however, was successfully introduced into the fly genome in 1989 and provides a means for site-directed homologous recombination (Golic and Lindquist, 1989).
Flip recombinase (FLP), also known as Flipase, is a protein that facilitates homologous recombination between FLP recombination target (FRT) sites on homologous chromosomes. When inserted in the *Drosophila* genome under control of the *hsp70* promoter, FLP will be actively transcribed following a heat shock treatment (Golic and Lindquist, 1989). Therefore, any cells that have replicated their genome at the time of a heat shock will be subject to mitotic recombination at the location of inserted FRT sites (Lee and Luo, 2001).

Along with the FLP/FRT system for site-directed genetic recombination, the MARCM technique also incorporates the GAL4/UAS system, another molecular tool from yeast that is utilized in *Drosophila* to drive expression of specific genes. GAL4 is a yeast transcriptional activator that can be expressed in the *Drosophila* genome. GAL4 will activate target genes whose promoters contain the GAL4 Upstream Activation Sequence (UAS) (Brand and Perrimon, 1993). To utilize the GAL4/UAS system for a particular experiment you need a GAL4 gene controlled by a nearby tissue-specific enhancer, and a UAS cloned in front of the gene of interest. With these two components in the genome, GAL4 will be expressed in a specific tissue and activate the gene of interest (Brand and Perrimon, 1993).

MARCM incorporates one more component in order to label only those cells with the genotype of interest after recombination occurs. GAL80, another yeast protein that transgenic flies can express, acts as an inhibitor of the transcriptional activator GAL4 (Lee and Luo, 1999). GAL80 binds to the activation domain of GAL4, blocking the
association of necessary transcription machinery with GAL4 and thus preventing transcription (Ma and Ptashne, 1987).

Combining each of these tools creates the powerful MARCM technique. Truman and colleagues utilized MARCM to identify and characterize the arrested adult-specific neurons of the ventral nervous system just prior to metamorphosis (2004). They created a wild type mosaic, which entailed labeling a few wild type neurons at a time against an unlabeled wild type background. In these experiments, the parent cell was heterozygous for the GAL80 repressor distal to the FRT recombination site. FLP and GAL4 were coded for elsewhere in the genome along with the membrane bound fusion protein mCD8-GFP (green fluorescent protein), a common cellular marker, under the control of UAS. Therefore, all parent cells were unlabeled since GAL4 could not activate the UAS for the cellular marker in the presence of the repressor GAL80. Upon heat shock, FLP induced mitotic recombination so that one daughter cell became homozygous for GAL80 while the other daughter cell lacked GAL80.

Heat shocking the embryo induces mitotic recombination in neuronal cells as well as non-neuronal cells. Since Truman and colleagues were only interested in labeling neuronal progeny, GAL4 was placed under the transcriptional control of the enhancer for the embryonic lethal abnormal visual system gene (elav). Elav is an RNA binding protein that is expressed in the majority of neurons throughout the Drosophila life cycle (Robinow and White, 1991). Therefore, along with elav, GAL4 will demonstrate pan-neuronal expression and drive expression of GFP only in those neuronal cells that lost
GAL80 during segregation. If GAL80 is not present in the daughter neuroblast, all subsequent neuronal progeny of that lineage will be labeled (i.e., express mCD8-GFP).

Identifying the Post-Embryonic Neuronal Lineages of the Ventral Nervous System

The neuroblasts in the embryo give rise to over 90% of the adult-specific post-embryonic neurons in the ventral nervous system. Therefore, initiating MARCM with a heat shock during embryogenesis results in the labeling of all of the post-embryonic progeny arising from the neuroblast(s) that lost GAL80 during segregation.

Neurotactin is a cell adhesion protein that is expressed by the cells of the larval central nervous system (Barthalay et al., 1990). Labeling this protein in a third instar larval nervous system reveals that the projections of the arrested post-embryonic neurons create a complex neurite scaffold in the thoracic and initial abdominal neuromeres. Double labeling the larval nervous system for Neurotactin and GFP provided a map of neurites from which to identify the MARCM clones (Truman et al., 2004).

Truman and colleagues found that 24 lineages (along with a 25th that was identified after publication) make up the neurite scaffolding of each thoracic hemineuromere. Numbered from 0 to 24, one of these lineages (lineage 0) only appears once per neuromere at the midline, while the others appear twice per neuromere, mirroring each other in each hemineuromere. Additionally, each lineage has a unique location and one or two neurite projection patterns to specific targets. For example, lineage 15’s cell bodies are lateral to the midline at the ventral surface of the neuromere, and its single projection travels dorsally and then laterally to reach the leg neuropil, a
region where other axonal projections will meet to form synaptic connections with target neurons within the leg. Meanwhile, lineage 13’s cell bodies are slightly more lateral and ventral than lineage 15’s. Also, lineage 13 has two neurite bundles, one contralateral that extends across the midline into the other hemineuromere and one ipsilateral that remains in the same hemineuromere as the cell bodies and innervates the leg neuropil (Truman et al., 2004).

**Daughter Cells of the GMCs Show Distinct Neuronal Phenotypes**

Recall that the postembryonic neurons arise from the mitotic daughters of a ganglion mother cell (GMC). At first, it appeared that these two daughter cells of the GMC, called A and B, either differentiated into two different neurons, as in the case of a lineage with two projections, or they differentiated into the same neuron type, as in the case of a lineage with one projection. Further analysis revealed that the two daughter cells are always fated to become different neurons, each forming a single distinct axonal projection or hemilineage. Both daughter cells survive in lineages with two projections, while programmed cell death kills one daughter cell in lineages displaying only one type of neuronal projection (Truman et al., 2010).

Interestingly, each hemilineage and thus each lineage demonstrates its own unique projection patterns. Additionally, although many lineages show consistent morphologies throughout each of the segments in which they appear, some other lineages show segment-specific morphologies and neurite projection patterns.
For example, lineage 1 has two projections that target the leg neuropil, which are present in the three thoracic segments T1, T2, and T3. Therefore, in T2, lineage 1 displays an ipsilateral branch that reaches the leg neuropil above in T1 as well as a contralateral branch that reaches the leg neuropil across the midline in T2. In T1, however, lineage 1 only displays its contralateral branch to the leg neuropil on the other side of the midline since there are no leg neuropils in the segment above. In contrast, in abdominal segment 1 (A1), lineage 1 only displays its ipsilateral branch that reaches the leg neuropil above in T3 since there are no leg neuropil in A1.

On the other hand, lineage 15, which was described earlier as having one projection that extends dorsally and then laterally, appears the same in all three thoracic segments. A majority of the lineages that are found in the thoracic neuromeres are not present in the abdominal neuromeres, and most of those lineages that are present in the abdominal neuromeres also display different morphologies from their thoracic counterparts (Truman et al., 2004). This poses an intriguing question, which is at the root of the work presented here: what is modulating neuron survival and hemilineage determination in an apparent anteroposterior segment-specific manner?

**Homeotic Complex (HOX) Genes Contribute to Segment Identity in Drosophila**

The *Drosophila* adult body plan is divided into the head, thorax, and abdomen, each of which contains its own set of morphologically unique segments. In the adult, the first of three thoracic segments (T1), for example, has a pair of legs, while T2 has a pair of legs and a pair of wings, and T3 has a pair of legs and a pair of halteres, (small
appendages required for balancing during flight). In 1978, E. B. Lewis identified a mutant fly that had two pairs of wings instead of the usual one pair of wings and one pair of halteres. He discovered that the mutated gene, Ultrabithorax (Ubx), was located within a cluster of genes called the Bithorax complex that control the unique patterning of the thoracic and abdominal segments of the fly (Lewis, 1978). Mutations yielding similar types of transformations in the head and more anterior thoracic segments led to the discovery of a second gene complex called the Antennapedia complex (Kaufman et al., 1990). Together, the Bithorax complex and the Antennapedia complex make up the Homeotic Complex (Hox) genes that play a major role in specifying the identity of each segment in the Drosophila body plan.

The expression pattern and interactions of the Hox genes have been studied extensively in the Drosophila embryo and larva. It is clear that the Hox genes exhibit segment-specific expression along the anteroposterior axis in the following order [beginning in the thorax]: Sex combs reduced (Scr), Antennapedia (Antp), Ultrabithorax (Ubx), abdominal-A (abd-A), and Abdominal-B (Abd-B) (reviewed in Hughes and Kaufman, 2002). It has also been shown that the protein products of the Hox genes display posterior dominance in their interactions with each other. This means that the more posterior Hox gene products inhibit the activity of the more anterior Hox gene products, thus establishing specific domains of Hox gene expression and action (Levine and Harding, 1987; reviewed in Hughes and Kaufman, 2002).

The concept of posterior dominance can explain the mutant phenotypes that Lewis observed. Antp is expressed in T2 where the normal pair of wings develops in the
adult fly. Ubx is predominantly expressed in T3 where the normal pair of halteres develops. When Ubx is missing in the mutant fly, Antp is no longer inhibited in T3, so T3 exhibits an anterior transformation into T2 and develops another pair of wings. Lewis also observed a posterior transformation when Ubx was misexpressed in T2. The expression of Ubx in T2 inhibits Antp in that segment, effectively transforming T2 into T3. This fly displayed two pairs of halteres instead of one pair of wings and one pair of halteres (Lewis, 1978). Similar transformations have been observed in the larvae, with focus on cuticle landmarks and parasegment transformations.

Just as the adult-specific post-embryonic neurons of the Drosophila ventral nervous system display segment-specific morphologies, the embryonic neuroblasts and embryonic-born larval neurons are also arranged in segment-specific patterns (reviewed Technau et al, 2006). Presumably, this organization provides the basis for establishing neural networks to support the segmentation pattern and function of the larger organism. As such, the roles of Hox genes have been studied in larval neurons, and it has been shown that the Hox genes contribute to anteroposterior patterning diversity in nervous system development (reviewed in Rogulja-Ortmann and Technau, 2008). Additionally, larval locomotion studies demonstrated that loss or gain of Hox function affects segment-specific movements, providing further evidence that the Hox genes are integral in establishing functional neural networks (Dixit et al, 2008).

Though they were first identified in Drosophila, the Hox genes have been highly conserved in many other organisms, and their function is universal. Mutations in these genes cause homeotic transformations from one body part to another. The Hox genes are
worthy of continued exploration because they play a major role in determining segmental identity and anteroposterior patterning across species, including in humans. For example, a mutation in the *HoxD13* gene in humans causes the disease synpolydactyly, in which segmental patterning during limb development is disrupted and patients present with extra and/or fused fingers or toes (Muragaki et al., 1996).

**HOX Gene Expression Patterns in Wild Type Post-Embryonic Neuronal Lineages**

In order to determine if the segment-specific morphologies of the post-embryonic neurons in the ventral nervous system can be attributed to Hox gene interactions as well, it was first important to determine whether the lineages express the Hox genes in a segment-specific manner. Previous studies had suggested that Hox genes are expressed in fairly broad, overlapping domains in the larval ventral nervous system (Cenci and Gould, 2005). Using the wildtype MARCM technique discussed previously and an antibody to counter stain for Ubx, Truman and colleagues characterized the expression pattern of Ubx in the lineages in the ventral nervous system (E.C. Marin, W. Moats and J.W. Truman, unpublished).

Interestingly, Ubx expression turns out to be segment-specific, lineage-specific, and sometimes even sibling-specific. For example, none of the lineages observed in T1 were positive for Ubx, confirming that Ubx expression is restricted to the more posterior regions of T2, along with T3 and A1, with the greatest expression appearing in T3 and A1. Additionally, some lineages, particularly those that appear in the thorax only (e.g. 4, 10, 12, 13, 14, 15, 16, 20, 21, 22), never exhibit Ubx expression. Other lineages,
however, express Ubx in a segment-specific manner. Lineage 7, for example is positive for Ubx in T3 and A1 but not in T1 or T2. Lastly, only half of the cells within lineages 3, 6, and 19 appear to be positive, which suggests sibling-specific Ubx expression by only one hemilineage.

**Hemilineage Rescue Reveals Additional Ubx Expression Patterns**

As described previously, one GMC daughter cell is killed in those post-embryonic neuron lineages that only display one projection (Truman et al., 2010). The removal of Dronc, a cell death initiator caspase, however, prevents cell death in these hemilineages, and promotes their survival. Marin and Truman generated MARCM clones with a *dronc* mutation and counterstained for Ubx in order to characterize the Ubx expression pattern in those hemilineages that do not normally survive. Remarkably, in several of the wild type lineages that were negative for Ubx, a sibling was rescued that was positive for Ubx. For example, the wild type lineage 1 only features the ipsilateral hemilineage in A1, which is negative for Ubx. The *dronc* mutation, however, rescues the contralateral hemilineage in A1, and the cells that generate this bundle are positive for Ubx. This suggests that Ubx may play a role in determining this hemilineage’s death (Marin and Truman, personal communication). In contrast, wild type lineage 0 normally expresses Ubx to varying degrees in T2, T3, and A1. The removal of *dronc* in this lineage rescued a second bundle that was negative for Ubx. Therefore, in the case of lineage 0, only the Ubx positive sibling survives.
The wild type Ubx expression data and the *dronc* mutant Ubx expression data suggested that Ubx might have a role in determining post-embryonic neuron segment-specific morphologies. It appeared that Ubx does not normally influence the survival or morphology of the lineages that are exclusive to the thorax since Ubx expression was not observed in these lineages. It did seem, however, that Ubx plays a critical role in the neurons that differ in their appearance from T3 to A1, as Ubx expression was observed in those lineages that survive in both the thorax and the abdomen. Since the exact nature of this regulation was not clear, additional loss and gain of function experiments with Ubx and the post-embryonic lineages of the ventral nervous system were performed.

**Anterior Transformation of Ubx Mutant Post-Embryonic Neuronal Lineages**

A *Ubx* homozygous mutant fly does not survive, so in order to analyze the loss of Ubx function in the lineages of the ventral nervous system, another application of the MARCM technique was used (Marin and Truman, personal communication). In this case, the parent cell contained all of the appropriate MARCM components in its genome, with the addition of a recessive *Ubx* mutation on the chromosome that did not carry GAL80. Therefore, all of the homozygous mutant cells were labeled in an unlabeled heterozygous background, making it easy to identify cells that would be displaying mutant phenotypes.

Ubx loss of function experiments resulted in some lineages demonstrating an anterior transformation. For example, lineage 1 in A1 only features an ipsilateral bundle projecting to the leg neuropil above in T3. Just as in the *dronc* mutants, in which cell death was directly prevented, the *Ubx* mutant clones in A1 displayed a contralateral
branch even though there are no leg neuropils to project to in A1. Essentially, the loss of Ubx allowed survival of the contralateral branch, and lineage 1 in A1 looked like lineage 1 in T2 or T3.

Interestingly, other lineages demonstrated an anterior transformation that went beyond simply rescuing a hemilineage. Wild type Lineage 12, for example, has a contralateral bundle (12c) and ipsilateral bundle (12id) in T1 as well as an additional small bundle (12im) protruding from the main ipsilateral bundle. In T2, often the 12im bundle is missing, and in T3 both the 12im and 12id bundles are missing. In lineage 12 drone mutants in T3, the 12id bundle is rescued, but in Ubx mutants in T3, both the 12id and the 12im bundles are rescued. This is clearly demonstrates that the two hemilineages of 12 in T3 are not just surviving but actually being transformed to the more anterior morphology of lineage 12 in T1.

Lineage 0 provides another excellent example of anteriorization occurring as a result of the loss of Ubx function. In T2 and T3, lineage 0 has one projection along the midline that travels dorsally. In T1, lineage 0 has the same type of projection; however, it stops sooner at the intermediate commissure, and sends out a lateral spray. Typically in the drone mutant, lineage 0 in T3 displays both projections because one sibling is rescued from programmed cell death. In the Ubx mutant, however, lineage 0 in T3 has the same morphology as lineage 0 in T1. There is only a projection to the intermediate commissure with accompanying lateral spray.
**Overexpression of Ubx in Post-Embryonic Neuronal Lineages**

Ubx expression data, *dronc* mutant data, and Ubx loss of function data have implicated Ubx in helping to define post-embryonic neuron segment-specific morphology, particularly in the third thoracic (T3) and first abdominal segments. Ubx expression is segment, lineage, and sibling-specific, and the loss of Ubx causes an anterior transformation of certain lineages in T3 and A1.

This thesis presents the final critical experiment in this series of Ubx manipulations in the post-embryonic neurons of the ventral nervous system. A gain of function MARCM technique was used to constitutively express Ubx in the clones. In order to use this method, a novel fly line was built that contained UAS-Ubx as well as the appropriate FRT (Appendix). Therefore, when the GAL4 repressor GAL80 segregated out of the daughter neuroblast, GAL4 could activate the expression of Ubx as well as the expression of the cellular marker GFP in all subsequent progeny.

This study uses gain of function MARCM to show that Ubx expression is sufficient to confer posterior identity, specifically T3 and A1, on post-embryonic neurons in more anterior thoracic segments. Experimental lineages observed in the thoracic neuromeres that normally appear in A1 and express Ubx now take on the morphologies of their more posterior counterparts, frequently resulting in the loss of one hemilineage. Meanwhile, those lineages that do not normally appear in A1 or express Ubx experience cell death in response to ectopic Ubx expression. These results confirm the importance of Ubx in defining segmental identity in the post-embryonic neurons of the ventral nervous system.
MATERIALS AND METHODS

Fly Stocks

For all experiments, the MARCM technique was used, in which the FLP/FRT system induced clones that lacked GAL80, a suppressor of GAL4, to make mCD8-GFP-labeled clones in an unlabeled background (Lee and Luo, 1999). The GAL4 driver used was GAL4C155, also known as Elav, a pan-neuronal driver. Parent stocks were generously shared by J. Parrish and Y-N. Jan (UCSF) or obtained from the Bloomington Drosophila Stock Center at Indiana University. The control genotype generated was GAL4C155, hsFLP, UAS-mCD8-GFP; FRT82B, tub-GAL80/FRT82B. For gain of function experiments, a novel fly line was constructed to make mCD8-GFP-labeled clones constitutively expressing Ubx (Appendix). The overexpression genotype generated was GAL4C155, hsFLP, UAS-mCD8-GFP; FRT82B, tub-GAL80/FRT82B, UAS-Ubx

Generation of MARCM Clones

The same heat shock regime was used to generate wild type and Ubx gain of function clones in order to control for the timing at which recombination was induced. Eggs were collected on grape juice plates for 12 hours and then held for 12 hours (both at 25°C). The 12-24 hour embryos were incubated at 37°C for 30 minutes, rested at room temperature for 30 minutes, then incubated once more at 37°C for 45 minutes. Larvae were raised on 4-24 instant fly media (Carolina Biological Supply) at 29°C to enhance GAL4 activity.
Immunocytochemistry and *in situ* Hybridization

As previously described (Truman et al., 2004), nervous systems were dissected from 3rd instar wandering larvae and fixed in 3.7% buffered formaldehyde for 1 hour at room temperature and then washed three times in PBS-TX [phosphate buffered saline (pH 7.2) with 1% Triton-X100]. Fixed samples were blocked in 2% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA USA) in PBS-TX for 30 minutes. To permit comparison of the mCD8-GFP-labeled clones to the neurotactin scaffold, the preparations were incubated in anti-neurotactin monoclonal antibody BP106 (Developmental Studies Hybridoma Bank) at 1:30 and rat anti-mCD8 monoclonal antibody (Caltag/Invitrogen) at 1:180 at 4°C overnight. Unbound primary antibodies were washed out and the nervous systems were incubated overnight at 4°C in a 1:300 dilution of FITC conjugated donkey anti-rat IgG and Texas Red conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories). The tissues were washed repeatedly in PBS-TX and mounted on poly-lysine coated coverslips, dehydrated, cleared through xylene and mounted in DPX (Sigma).

Experimental nervous systems that were examined for Ubx expression in the mCD8-GFP-labeled clones were treated with a similar protocol except they were fixed for 10 minutes after dissection and incubated in a 1:50 dilution of a mouse anti-Ubx::Abd-A monoclonal antibody and a 1:200 dilution of a rat anti-mCD8 monoclonal antibody before being washed and incubated in a 1:300 dilution of FITC conjugated donkey anti-rat IgG and Texas Red conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories).
Microscopy and Image Processing

Fluorescently stained nervous systems were imaged with a 40x oil immersion lens at 1.5 to 1.7 zoom using a Leica SP5 confocal microscope. Z-stacks were collected with optical sections at 0.5 µm intervals. The excitation wavelength was optimized for each fluorophore to avoid bleed-through and kept constant for each tissue.

Raw data stacks were imported into Image J (http://rsb.info.nih.gov/ij/) where the stacks were merged and movies were created. The lineages were identified using the neurotactin landmarks previously described (Truman et al., 2004). Lineage identity, segment, and phenotype were tallied for each identified lineage in wild type and experimental clones.

The data in this paper are presented as flattened z-projections or single slice snapshots, generally with neurotactin or Ubx expression in magenta and the clones in green. In some cases the clones appear in white on a black background. Necessary contrast and brightness adjustments were made to entire stacks in Photoshop (Adobe, San Jose, CA).
RESULTS

Novel fly line overexpresses Ubx in MARCM clones

It was first necessary to determine if the novel fly line containing the FRT and UAS-Ubx transgene overexpresses Ubx in the post-embryonic neurons when used in conjunction with the MARCM technique. Double staining for mCD8-GFP and Ubx revealed that 32 out of 33 observed clones were positive for Ubx. Of those clones, 16 were located anterior to the domain of normal Ubx expression, and of those, 15 clones were positive for Ubx (Fig. 1).

Since this Ubx gain of function MARCM technique had been shown to overexpress Ubx in the neuronal clones, approximately 80 UAS-Ubx and 75 wild type MARCM larval ventral nervous systems were dissected and double stained with antibodies against mCD8 and Neurotactin BP106 to label the clones against a neurotactin scaffold. Experimental nervous systems contained MARCM clones overexpressing Ubx and wild type nervous systems contained wild type MARCM clones. All clones were identified based on cell position, axon morphology, and position relative to the neurotactin scaffold (Truman et al., 2004), and a description of their morphology was recorded.

Morphological changes that were observed within the Ubx gain of function lineages can be summarized in three general categories. Some lineages underwent a posterior transformation and exhibited a typical posterior morphology in more anterior segments. Many lineages appeared to be dying or were observed very rarely in
comparison to matched wild type controls, which suggests that the overexpression of Ubx caused neuronal or neuroblast cell death. Lastly, a few lineages presented misguided axon projections and atypical morphologies in response to ectopic Ubx.

**Ubx gain of function causes posterior transformation in surviving lineages**

Five lineages, all of which normally express Ubx and survive in A1 as well as the thoracic and segments, exhibited a posterior transformation in response to the overexpression of Ubx. Lineage 0 is the only unpaired lineage in the ventral nervous system, and it displays one projection that travels anterodorsally along the midline. In the wild type T1 segment, the projection stops at the level of the posterior intermediate commissure (pI) and sends out very diffuse lateral processes ($n=4$) (Fig. 2A,B). In the wild type T2, T3, and A1 segments, the lineage 0 projection continues past the pI and does not terminate until the anterior intermediate commissure (aI) at the same level as the paired bundles of lineage 2 ($n=8$)(Fig. 2E,F). Of the 14 lineage 0 Ubx gain of function MARCM (UAS-Ubx) clones that were observed in T1, 13 demonstrated the phenotype typical of the more posterior segments T2 and T3 (Fig. 2C,D).

Thoracic wild type lineage 3 has two bundles, both of which remain ipsilateral (i) to the midline. One bundle extends dorsally (id), while the other branches off and extends laterally (il). In T1 and T2 these projections terminate with diffuse endings ($n=14$) (Fig. 3A), while in T3 these projections are less diffuse ($n=9$) (data not shown). In A1, wild type lineage 3 only displays the 3id bundle and lacks the 3il bundle ($n=6$) (Fig. 3C). In the UAS-Ubx larval nervous systems, 39 lineage 3 clones were identified in
the thorax. Of these clones, 24 exhibited only the id bundle, the same morphology of those in segment A1 in the wild type (Fig. 3B,C). Of the remaining 15 lineage 3 clones observed in the thorax, 6 presented an abnormal morphology that will be discussed later, and 9 displayed a morphology like that observed in the wild type.

Lineage 6 appears the same in all three thoracic neuromeres projecting two bundles contralaterally. The contralateral middle bundle (cm) crosses the midline at the pI commissure and extends anteriorly while the contralateral dorsal bundle (cd) crosses the midline at the posterior dorsal commissure (pD) (n=28) (Fig. 4A). In A1, the 6cm bundle is usually missing or very faint and does not project as far as the 6cm bundle in the thorax (n=6) (Fig. 4C). Of the 53 UAS-Ubx lineage 6 clones that were identified in the thorax, zero had the pronounced 6cm that is normally seen. Instead, 42 clones were missing the 6cm bundle all together (Fig. 4B), and 11 clones displayed a very faint 6cm bundle (data not shown).

In wild type ventral nervous systems, lineage 9 presents both a contralateral and ipsilateral hemilineage in the three thoracic neuromeres as well as in the abdominal neuromeres. In T1 through T3, the ipsilateral bundle is thick and pronounced and travels along the thin contralateral bundle (n=7). In some cases, the ipsilateral bundle curves away dorsally (Fig. 5A), while in others the ipsilateral bundle stays on track with the contralateral bundle (data not shown). In the abdominal neuromeres, the ipsilateral bundle is greatly reduced and does not curve away from the contralateral bundle (n=5) (Fig. 5C). Of the UAS-Ubx lineage 9 clones that were observed in the thorax (n=36), 30 clones projected reduced ipsilateral bundles that hugged the contralateral bundle in a
manner similar to that of wild type clones observed in the abdomen (Fig 5B). The other 6 clones appeared to have no ipsilateral bundle at all; however, they may have been greatly reduced and indistinguishable from the contralateral bundle (data not shown).

Wild type Lineage 19 in the thoracic neuromeres features a contralateral hemilineage as well as a short ipsilateral hemilineage that sprays out in close proximity to the cell bodies (n=10) (data not shown). Although zero wild type lineage 19s were observed in A1 in this study, Truman and colleagues (2004) reported that this ipsilateral spray is missing from those wild type lineage 19s found in A1. None of the UAS-Ubx lineage 19s that were observed in the thoracic neuromeres (n=37) (data not shown) presented an ipsilateral bundle; instead they projected only a contralateral bundle like those clones in wild type A1 neuromeres as previously reported.

**Lineages that do not normally express Ubx exhibit cell death in response to ectopic Ubx**

All of the lineages described in this category are normally found exclusively in the thorax and do not normally express Ubx. Specific criteria were used to conclude that these lineages experience cell death as a result of ectopic Ubx. First, the absence of lineages in the experimental nervous system compared to their presence in wild type nervous systems suggests that these lineages did not survive as a result of Ubx overexpression. Second, the neurotactin scaffold allowed for the identification of lineages that presented dying phenotypes (lack of cell bodies and faint and/or missing neural projections) that would have not been recognizable otherwise.
Lineage 1 is commonly observed among wild type MARCM clones. In fact, 46 were identified in the wild type nervous systems. Among the UAS-Ubx clones, however, only one was observed in the thorax, in T3, and it was missing cell bodies, sending out a very faint, incomplete contralateral projection, and missing its typical ipsilateral bundle (Fig. 6A,B). Lineage 1’s unique morphology allowed for its identification even its dying state.

Similar to lineage 1, lineage 13 has an easily identifiable morphology that allowed for identification. Within the thorax, all of the UAS-Ubx lineage 13 clones observed (n=8) were dying, while the lineage 13 clones observed in the wild type (n=17) were all normal (Fig. 7A,B). Additionally, lineages 15 (Fig. 8A,B) and 24 (data not shown) can be easily identified by their bright expression of mCD8-GFP and their direct projections to the leg neuropil in wild type nervous systems (n=17 and n=5). In the experimental nervous systems, all lineage 15s (n=15) and all lineage 24s (n=5) observed exhibited smaller cell clusters, suggesting some cell death.

Many faint cell clusters or “ghosts” that lacked any sort of projection were observed in the UAS-Ubx MARCM nervous systems. Most of these clones that were dying could not be identified without projections to analyze. Lineage 12, however, can be identified in the neurotactin scaffold as the medial point of a triangle with lineage 3 and lineage 6 as the other points (Fig. 9C). As a result, 20 UAS-Ubx lineage 12 clones were observed in the thoracic neuromeres and none demonstrated a phenotype like that of a wild type thoracic lineage 12 (Fig. 9A,B,C). In fact, all 20 were “ghosts” or dying
lineages without any projections. In the wild type nervous systems, however, all lineage 12 clones observed (n=15) exhibited normal projections.

Only 3 lineage 4 clones and 5 lineage 16 clones were observed in the UAS-Ubx MARCM nervous systems. Both lineages exhibited characteristic dying phenotypes. In wild type nervous systems, 15 and 16 normal clones respectively were observed for each lineage (data not shown). Additionally, in the experimental nervous systems zero clones were observed for lineage 11 and lineages 20 and 22. In the wild type, however, 6 clones were observed for lineage 11 and 16 total clones were observed for lineages 20 and/or 22 (very difficult to distinguish). This data suggests that each of these lineages were also being killed by ectopic expression of Ubx.

Lineage 8 provides an example of another lineage that normally appears only in the thorax, and does not express Ubx. A wild type lineage 8 includes two surviving hemilineages, one contralateral bundle and one ipsilateral bundle (n=12) (data not shown). Lineage 8 clones appeared relatively frequently in the UAS-Ubx MARCM nervous systems (n=20). These clones, however, showed variable phenotypes. Most clones (n=14) displayed a contralateral bundle only while others (n=2) displayed an ipsilateral bundle only. The other 4 clones showed dying phenotypes (data not shown).

Surviving lineages show misguided axon projections in response to ectopic Ubx

A few of the lineages in the experimental nervous systems had axon projections that appeared to be misguided. The most prevalent example of this was observed in lineage 7. Wild type lineage 7 appears the same in all segments from T1 to A1. It has
one projection that extends contralaterally through the aI commissure and then projects anteriorly in a very characteristic manner toward the next anterior segment (n=19) (Fig. 10A). In a majority of the UAS-Ubx lineage 7 clones that were identified (36 out of 50), the contralateral branch stopped after crossing the midline and did not project either anteriorly or posteriorly (Fig. 10D,E). Additionally, some of the UAS-Ubx lineage 7 clones (11 out of 50), all of which were observed in T2, had a contralateral bundle that projected toward the more posterior segment (Fig. 10B,C).

Recall that wild type lineage 9 clones present both a contralateral and ipsilateral hemilineage in the three thoracic neuromeres as well as in the abdominal neuromeres (Fig. 5A,C). Typically, the contralateral bundle ends without vertical deviation before reaching the lateral neuropil (Fig. 5A). Of the lineage 9 clones that were observed in the UAS-Ubx MARCM nervous systems (n=51), 32 clones had contralateral bundles that wandered either anteriorly or posteriorly after crossing the midline (Fig. 5B, yellow arrow).

Although most of the experimental lineage 3 clones, as described above (Fig. 3), presented a posterior transformation or a somewhat normal phenotype, a few UAS-Ubx lineage 3 clones (n=6) demonstrated a misguided projection. These clones featured only the 3id branch, but instead of projecting dorsally, the projections corkscrewed or wandered in an apparently nonspecific manner (data not shown).

Lineage 21 appears exclusively in the thoracic neuromeres in wild type nervous systems, and sends one ipsilateral projection anteriorly that ends quickly and sprays into the leg neuropil (n=19) (Fig 11A). In UAS-Ubx MARCM nervous systems, 9 lineage 21
clones were identified and each of these featured a projection that was longer and traveled farther than the wild type (Fig 11B).

**The effect of ectopic Ubx on infrequent and inconsistent lineages could not be characterized**

A few lineages appeared so infrequently in wild type nervous systems that no conclusions could be drawn from their appearance or lack of appearance in experimental nervous systems. Lineages 5, 10, and 18 were not confirmed to appear in any of the wild type nervous systems that were examined. In the UAS-Ubx MARCM nervous systems, zero lineage 18 clones were identified, and 1 or 2 lineage 5 and lineage 10 clones (difficult to distinguish) were identified to be dying.

Based on the neurotactin scaffold, lineage 23 is a lineage that appears in the thoracic and abdominal neuromeres. Its wild type phenotype, however, has not been well characterized and only 2 were observed in wild type nervous systems, neither of which was in the abdomen. Although 9 lineage 23 clones were identified in the UAS-Ubx MARCM nervous systems, it is difficult to say with certainty whether its phenotype was affected by the overexpression of Ubx.

Finally, lineage 2 appeared quite often in both the wild type and experimental nervous systems (n=24 and n=39), however, its phenotype varied in both types of systems. Some lineages presented an axonal projection that behaved normally as defined by Truman and colleagues (2004); the projection traveled dorsally and then laterally in the anterior dorsal commissure (aD). Other lineages, however, presented an axonal projection that traveled dorsally, but then bent back anteriorly with some never arriving at
the aD commissure. For this reasons, the effect of ectopic Ubx on lineage 2 could not be characterized.
Figure 1. Overexpression of Ubx in MARCM clones. Ventral view of a z-projection of Ubx expression (magenta) and HS induced neuronal clones (green) in a 3rd instar larval ventral nervous system. Yellow brackets indicate normal Ubx expression domain. Yellow arrows indicate neuronal clones expressing Ubx outside of the normal expression domain. Anterior is to the top of the page. Non-nuclear magenta spots outside of the normal expression domain represent unknown non-specific Ubx staining.
Figure 2. Posterior transformation of lineage 0 in response to ectopic Ubx. Ventral view z-projection of wild type lineage 0 clones in T1 (A) T2, and T3 (E) and of UAS-Ubx lineage 0 clone in T1 (C) (green) against a wild type neurotactin background (magenta) (A,C,E). (B,D,F) Single slice projections showing the relationship of the clone projection (green) to lineage 2s (two magenta dots) in the anterior intermediate (ai) commissure of the neurotactin scaffold (magenta). Wild type lineage 0 in T1 projecting to the posterior intermediate commissure (pl) and sending a lateral spray (B). UAS-Ubx lineage 0 in T1 projecting to the level of lineage 2 in the ai commissure (D) similar to wild type lineage 0 in T2 and T3 (F).
Figure 3. Posterior transformation of lineage 3 in response to ectopic Ubx. Ventral view z-projection of entire wild type lineage 3 clone in T2 (A) and A1 (C) and of entire UAS-Ubx lineage 3 clone in T2 (B). (A) Wild type lineage 3 in T2 showing both an ipsilateral dorsal (id) and ipsilateral lateral (il) bundle with diffuse projections. (B) UAS-Ubx lineage 3 in T2 showing a less diffuse id bundle only, which is similar to lineage 3 in A1 (C).
Figure 4. Posterior transformation of lineage 6 in response to ectopic Ubx. Ventral view z-projection of entire wild type lineage 6 clone in T2 (A) and A1 (C) and of entire UAS-Ubx lineage 6 clone in T2 (B). (A) Wild type lineage 6 in T2 showing both a contralateral dorsal (cd) bundle and a well-defined contralateral middle (cm) bundle. (B) UAS-Ubx lineage 6 in T2 showing a cd bundle only, which is similar to wild type lineage 6 in A1, which often lacks a cm bundle or shows a very faint and incomplete cm bundle (C).
Figure 5. Posterior transformation and misguided axon projections of lineage 9 in response to ectopic Ubx. Ventral view z-projection of entire wild type lineage 9 clone in T2 (A) and A1 (C) and of entire UAS-Ubx lineage 9 clone in T2 (B). (A) Wild type lineage 9 in T2 showing a contralateral (c) bundle and a thick, curving ipsilateral (i) bundle. (B) UAS-Ubx lineage 9 in T2 showing a thin c bundle and a reduced, straighter i bundle, which is similar to wild type lineage 9 in A1 (C). Note: the thicker projection at the right end of 9c (C) belongs to a different lineage. (B) Yellow arrow indicates anterior projection of 9c in UAS-Ubx lineage 9 that is absent in wild type lineage 9 (A).
Figure 6. Cell death of lineage 1 in response to ectopic Ubx. Ventral view z-projection of entire wild type (A) and UAS-Ubx (B) lineage 1 clones in T3. (A) Wild type lineage 1 clone displaying characteristic ipsilateral (i) and contralateral (c) bundles each with complete diffuse projections. (B) UAS-Ubx lineage 1 clone showing a dying phenotype with a reduced number of cell bodies, an incomplete c bundle, and a missing i bundle.
Figure 7. Cell death of lineage 13 in response to ectopic Ubx. Ventral view z-projection of entire wild type (A) and UAS-Ubx (B) lineage 13 clones in T3. (A) Wild type lineage 13 clone displaying an ipsilateral (i) and contralateral (c) bundle. (B) UAS-Ubx lineage 13 clone showing a dying phenotype with a reduced number of cell bodies, a thin c bundle, and a reduced i bundle.
Figure 8. Cell death of lineage 15 in response to ectopic Ubx. Ventral view z-projection of entire wild type (A) and UAS-Ubx (B) lineage 15 clones in T2. (B) UAS-Ubx lineage 15 clone characteristically showing bright GFP expression but consisting of fewer cells than wild type lineage 15 (A).
Figure 9. Cell death of lineage 12 in response to ectopic Ubx. (A) Ventral view z-projection of entire wild type lineage 12 clone in T1 showing normal projections. (B) Ventral view z-projection of a dying UAS-Ubx cell cluster in T1 lacking defined projections. (C) Single slice projection showing the relationship of the dying clone projection from (B) (green) to wild type lineage projections (magenta) in the neurotactin scaffold. The dying UAS-Ubx cell cluster is identified as lineage 12 for its location in the characteristic three-dot triangle that the axons of lineages 3, 6, and 12 project in each hemineuromere.
Figure 10. Misguided axon projections of lineage 7 in response to ectopic Ubx. Ventral view z-projection of entire wild type (A) and UAS-Ubx (B,D) lineage 7 clones in T2. (A) Wild type lineage 7 clone showing a contralateral projection extending anteriorly. (B) A pair of UAS-Ubx lineage 7 clones on either side of the midline, showing contralateral projections extending posteriorly. (C) Single slice projection showing UAS-Ubx lineage 7 clones from (B) (green) projecting posteriorly and meeting wild type lineage 7 clones projecting anteriorly (magenta) in the segment below. (D) UAS-Ubx lineage 7 clone showing a contralateral projection that stops and does not project anteriorly or posteriorly. (E) Single slice projection showing UAS-Ubx lineage 7 clone from (D) (green) stopping its projection in the anterior intermediate (al) commissure. The wild type projection of lineage 7 from the other hemineuromere is seen projecting anteriorly (magenta) while the UAS-Ubx lineage 7 projection does not project and cannot be seen traveling up.
Figure 11. Misguided axon projections of lineage 21 in response to ectopic Ubx. Ventral view z-projection of entire wild type (A) and UAS-Ubx (B) lineage 21 clone in T3. (A) Wild type lineage 21 sending one ipsilateral bundle that ends quickly and sends a curly spray into the leg neuropil. (B) UAS-Ubx lineage 21 sending a longer ipsilateral projection that travels farther and does not curl to spray in the leg neuropil.
DISCUSSION

The means by which an organism develops a diverse set of highly organized cell types from a single cell addresses the fundamental question of developmental biology research. Specifically, the necessary intricacies required of functioning nervous systems provide an intriguing example of the challenges of development. Many different types of neuronal cells must establish an identity relative to their neighbors and then extend their neurites to establish synaptic connections with appropriate targets. Although much progress had been made in recent decades, the mechanisms controlling these processes remain largely undefined.

The post-embryonic neurons of the ventral nervous system in Drosophila larvae have proven to be an excellent new model system for studying nervous system development (Truman, et al., 2004; Truman et al., 2010). These neurons arrest prior to metamorphosis, providing a snapshot of the neural processes that will eventually be elaborated to form the adult nervous system. Mapping of the post-embryonic neurons revealed that the ventral nervous system is composed of morphologically distinct cell lineages, and that the shape of these lineages often varies by segment in an anteroposterior fashion (Truman et al., 2004).

Preliminary experiments with the homeotic complex gene Ubx have suggested it plays a role in regulating neuron survival and morphology in the last thoracic (T3) and first abdominal (A1) segments of the ventral nervous system. Ubx is expressed primarily in segments T3 and A1, but in a lineage- and sometimes hemilineage-specific manner.
(E.C. Marin, W. Moats, and J.W. Truman, unpublished). Additionally, the loss of Ubx causes an anterior transformation of many of the post-embryonic neuron lineages in T3 and A1 (E.C. Marin and J.W. Truman, unpublished). Finally, the Ubx gain of function results presented here continue to support the role of Ubx in defining segment-specific lineage survival and morphology, particularly between T3 and A1 and the other thoracic neuromeres.

**Posterior transformation of lineages that normally both appear in A1 and express Ubx**

Lineages 0, 3, 6, 9, and 19 all appear in A1, and all express Ubx in wild type ventral nervous systems. Each of these lineages also displays a different phenotype in the more posterior segments within the Ubx expression domain than in the more anterior segments outside of the Ubx expression domain. Typically, these lineages project two hemilineages in the thoracic neuromeres but only one hemilineage in the abdomen, with the surviving hemilineage being Ubx positive. As expected, the overexpression of Ubx caused a posterior transformation in these lineages. The expression levels of Ubx in these lineages in T1 and T2 mimicked those of their T3 and A1 programs and thus they took on the morphology of their T3 and A1 counterparts.

The posterior transformations observed in this Ubx gain of function experiment suggest that Ubx is in some way dominant over Antp, the Hox gene that these lineages express in the more anterior segments. In fact, in the embryo, many negative regulatory Ubx binding sites have been characterized within the second of two Antp promoters.
This would allow Ubx to repress endogenous Antp expression when experimentally overexpressed in the Antp domain.

**Ubx overexpression triggers cell death in lineages that normally do not express Ubx**

Lineages 1, 4, 8, 12, 13, 15, 16, 20, 22, and 24 are only found in the thoracic segments and do not express Ubx in wild type ventral nervous systems. The phenotypes and infrequency or absence of these lineages in the UAS-Ubx MARCM ventral nervous systems compared to the wild type nervous systems strongly suggest that the misexpression of Ubx caused their cell death. Presumably, the expression of Ubx indicates to the lineages that they are located in the abdomen where they are not normally found, resulting in cell death. (Triple staining the nervous systems for a cell death marker could help confirm this conclusion.) Ubx, however, may not play a direct role in modulating lineage death and survival.

In the Ubx loss of function experiments, many of the lineages listed above were not rescued in the abdomen, which one would expect to see if Ubx normally acted to induce cell death. In fact, *abdominal-A (abd-A)*, the Hox gene whose expression begins posterior to that of Ubx, induces apoptosis in those neuroblasts fated to die in the abdomen (Bello et al., 2003). Since abd-A expression should not be affected by the UAS-Ubx MARCM technique, why wouldn’t more lineages survive in the thoracic neuromeres despite ectopic Ubx expression?

The most plausible explanation rests on the fact that Antp is normally required to prevent cell death of lineages in the thorax (Rogulja-Ortmann et al., 2008). Recall that
the Hox genes exhibit posterior dominance in which the more posterior Hox proteins inhibit those that are more anterior. Therefore, high levels of Ubx in the thoracic segments of the ventral nervous system may prevent Antp from promoting cell survival, instead of Ubx directly inducing cell death.

The respective roles of Antp and Ubx in segment-specific post-embryonic neuron fate determination

It is clear that Ubx plays an important role in modulating neuron survival and hemilineage determination in an anteroposterior manner. Specifically, Ubx seems to be integral in sculpting the nervous system at the interface between the thoracic and abdominal segments. What remains unclear, however, is the means by which Ubx acts to accomplish this segmental neuronal patterning.

The interactions between Ubx and Antp have been studied in the embryonic nervous system, which also demonstrates patterns of segment-specific cell survival. In some cases it appears that Ubx represses Antp expression directly (Appel and Sakonju, 1993), while in other cases it appears that Ubx might be out-competing Antp for shared target sites (Rogulja-Ortmann et al., 2008). Rogulja-Ortmann and colleagues, for example, have identified several binding sites for both Ubx and Antp within the enhancer of proapoptotic gene, reaper (2008).

In the future, several experiments need to be carried out to elucidate this interaction between Ubx and Antp further. For example, inducing UAS-Ubx MARCM clones and then staining for Antp will reveal whether ectopic Ubx is repressing Antp transcription or whether the two proteins are both present but competing in those
experimental clones. Conversely, staining for Antp in Ubx loss of function MARCM clones will show whether Antp expression increases in anteriorly transformed T3 and A1 lineages when Ubx is not present to repress it. Additionally, the generation of a novel UAS-Antp MARCM fly line will allow for the ectopic expression of Antp in MARCM clones. If Ubx normally only acts to repress Antp transcription, the overexpression of Antp via the MARCM system should generate anterior transformations like those in the Ubx loss of function experiments. If Ubx and Antp are competing, however, the phenotypes might vary across lineages depending on the levels of expression of each of the Hox genes.

A possible role for Ubx in axon guidance during development

The appearance of misguided axon projections in a few of the lineages was an unexpected result of this experiment, although perhaps not surprising, given the complexity of nervous system development. A major component of neuronal identity and function for the post-embryonic hemilineages is not simply survival of the neurons but the consistent trajectory of those neurite bundles within the scaffold of other neurons. As such, the neuronal cells not only need to respond to their own genetic program, but they need to be able to respond to the signals of other cells and target neurons.

Although the specific signals for appropriate axonal projections in the ventral nervous system are not yet known, it is likely that the overexpression of Ubx in post-embryonic neurons might affect their ability to respond to those signals. In fact, studies of the migration of larval sensory neurons in Drosophila offer some clues as to the
possible role of Hox genes in axon guidance. It has been shown that the Hox cofactor homothorax represses the activity of a well-known axon guidance molecule, Roundabout, during *Drosophila* development (Kraut and Zinn, 2004).

The findings above suggest that in the ventral nervous system, the overexpression of Ubx might alter the neuronal cells’ abilities to respond to axon guidance cues via Ubx forming abnormal levels of transcription complexes that go on to affect cell-signaling mechanisms. Analyzing the clones for levels of ectopic Ubx expression might provide some insight to these misguided phenotypes. The level of Ubx expression in each experimental clone depends on the strength of the Elav-GAL4 expression in that clone. Lineage 15, for example, expresses extremely high levels of GFP compared to other clones. Presumably, ectopic Ubx expression levels are also high in this lineage. Further knowledge about the interaction of Hox genes with axon guidance machinery will be needed, however, to understand how the overexpression of Ubx might be affecting the processes of neural development.

**Conclusion**

In conclusion, this gain of function study has shown that Ubx is sufficient to cause posterior transformation in the post-embryonic neurons of the ventral nervous system in *Drosophila melanogaster*. This implicates Ubx as playing a major role in establishing the segment-specific identities of neuronal lineages in an anteroposterior manner. Specifically, it appears that Ubx acts in the regions of T3 and A1 to define the boundary between thoracic and abdominal neuromeres. Future experiments with Ubx
and Antp should elucidate the nature of their interactions in regulating segmental patterning of neural networks during development.
REFERENCES


APPENDIX

Genetic crossing scheme used for the construction of a novel fly line containing UAS-Ubx and the appropriate FRT

yw; __UASUbx♀ ⊗ w; neoFRT82B♂
TM6BTb,Hu neoFRT82B

w; neoFRT82B♀ ⊗ yw; __CxDMale
yw UASUbx TM3Sb,Ser
G418 neomycin antibiotic selective food

(1) neoFRT82B,UASUbx♂ ⊗ __SbFemale
CxDMale
TM6BTb,Hu

Multiple crosses each with a different isoline

neoFRT82B,UASUbx♀ ⊗ neoFRT82B,UASUbx♂
TM6BTb,Hu TM6BTb,Hu

neoFRT82B,UASUbx
TM6BTb,Hu