AN ABSTRACT OF THE THESIS OF

Ho-Juhn Song for the degree of Doctor of Philosophy in Genetics presented on August 16, 2001. Title: The Genetic Dissection of the *fruitless* Gene's Functions During Embryogenesis in *Drosophila melanogaster*.

Abstract approved: Redacted for Privacy

Barbara J. Taylor

The *fruitless* (*fru*) gene in *Drosophila melanogaster* is a multifunctional gene having sex-specific functions in the regulation of male sexual behavior and sex-nonspecific functions affecting adult viability and external morphology. While much attention has focused on *fru* 's sex-specific roles, little is known about its sex-nonspecific functions. The embryonic central nervous system (CNS) is a prime model system in which to study the genetic control of axonal outgrowth and proper CNS formation. I have examined *fru*'s sex-nonspecific role in embryonic neural development. *fru* transcripts and FRU proteins from sex-nonspecific promoters are expressed beginning at the earliest stages of neurogenesis and subsequently in both neurons and glia. In embryos that lack most or all *fru* function, Fasciclin II- and BP102-positive axons appeared to defasciculate from their normal pathway and fasciculate along aberrant neuronal pathways, suggesting that one of *fru*'s sex-nonspecific roles is to regulate axonal differentiation. I next examined whether the loss of *fru* function in FRU-expressing neuronal precursors causes neuronal fate change. Analysis of *fru* mutant embryos revealed a lack of Even-skipped (Eve) staining in Eve-expressing neurons, ectopic Eve staining in non-Eve-expressing neurons and mispositioned dorsal Eve-expressing neurons, which suggests that *fru* functions to maintain neuronal identity rather than to specify neuronal fate. In *fru* mutants these defects in axonal projections and in Eve staining were rescued by the expression of specific *fru* transgenes.

To better understand *fru*'s function in the formation of the embryonic CNS, I dissected out *fru*'s function in neuron and glia through a genetic interaction study.

fru genetically interacts in neurons with *longitudinal lacking* to make proper axonal projections. In addition, *fru* might be in the same genetic pathway as *roundabout* (*robo*), a repulsive guidance receptor, and *commissureless*, a downregulator of Robo, to ensure proper axonal pathfinding. Surprisingly, *fru* interacts with *tramtrack* and *glial cells missing* to repress neuronal differentiation in the lateral glia and with *single-minded* for the development of midline glia. Taken together, *fru* function is required for proper axonal pathfinding in neurons and for proper development of lateral and midline glia.

The Genetic Dissection of the *fruitless* Gene's Functions During Embryogenesis in *Drosophila melanogaster*.

By

Ho-Juhn Song

A THESIS

Submitted to

Oregon State University

In partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented August 16, 2001

Commencement June 2002

Doctor of Philosophy thesis of Ho-Juhn Song presented on August 16, 2001

APPROVED:

Redacted for Privacy

Major Professor, representing Genetics

Redacted for Privacy

Chair of Genetics Program

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for Privacy

Ho-Juhn Song, Author

ACKNOWLEDGEMENTS

I would like to thank my advisor Barbara J. Taylor for giving me a chance to study in the field of neurosciences and for encouraging me to understand "genetics" in fruitfly, complex behavior and neuronal system. Without her guidance and knowledge, I could be less of a neurogenetist. I also want to thank Dr. Bruce S. Baker, Dr. Jeffrey C. Hall, and Dr. Chris Q. Doe. They have provided me with much of my professional knowledge and research in this study. I would like to thank my colleagues involved in this study and in *fruitless* projects; Margit Foss, Stephan Goodwin, Troy Carlos, Enrique Reynaud, Jean-Christophe Billeter, Lisa Ryner, Eric P. Spana. Without their assistant, my studies could be still in long dark tunnel. I do have to thank Dr. Nipam Petal. His support of various antibodies used in my study was crucial. I appreciated that Dr. Ed Giniger kindly shared his unpublished data with me. The member of Taylor's lab; Ginger Carney, Margit Foss, Kristin Latham, Laura Wilson, DeLane Larsen and Bryan Arnold have always helped me out with taking care of some troubles in my works and with providing me with emotional support whenever needed. My fellow ex-graduate students, John Melville and the member of Mason's lab including him, Mike DeMaster and Ignacio Moore also helped my graduate life. My parents and family in Korea have always been behind me to financially and spiritually support. And without support of my wife I can not make it this far.

CONTRIBUTION OF AUTHORS

Dr. Barbara J. Taylor was involved in the analysis and writing of each manuscript. Dr. Stephen F. Goodwin and Jean-Christophe Billeter made *fruitless* transgenes. Dr. Enrique Reynaud and Dr. Troy Carlos made FRU antibodies; Dr. Carlos was also involved in chromosome mapping. Dr. Eric P. Spana and Dr. Norbert Perrimon made new deficiency variant that uncovers *fruitless* locus.

TABLE OF CONTENTS

Page

CHAPTER 1: INTRODUCTION 1

General Overview of Thesis 1

The Specification of Neurons (Neurogenesis) in the CNS of *Drosophila* 4

The Specification of Glia (Gliogenesis) in the CNS of *Drosophila* 9

Axonal Pathfinding by CNS Neurons 12

Mechanisms for Axonal Fasciculation and Defasciculation 14

Mechanisms for Axonal Guidance by Attractive and Repulsive Environmental Cues 16

The Molecular Characteristics of the *fruitless* Gene 20

The Genes Containing a BTB/POZ Motif in *Drosophila* and Known BTB/POZ Functions 22

CHAPTER 2: THE *fruitless* GENE IS REQUIRED FOR THE PROPER FORMATION OF AXONAL TRACTS IN THE EMBRYONIC CNS OF *DROSOPHILA*. 25

Abstract 26

Introduction 27

Materials and Methods 30

Results 36

Discussion 73

Page

CHAPTER 3: THE *fruitless* GENE IS REQUIRED FOR REPULSIVE SIGNALING IN AXONAL PATHFINDING AND DEVELOPMENT OF GLIA IN THE EMBRYONIC CNS OF *DROSOPHILa*. 80

Abstract 81

Introduction 82

Materials and Methods 87

Results 88

Discussion 114

CHAPTER 4: THE *fruitless* GENE IS REQUIRED FOR MAINTAINING NEURONAL NEURONAL IDENTITY IN *EVENSKIPPED*-EXPRESSING NEURONS IN THE CNS DURING *DROSOPHILA* EMBRYOGENESIS. 122

Abstract 123

Introduction 124

Materials and Methods 128

Results 130

Discussion 150

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS 155

Putative Functions of Sex-Specific and Sex-Nonspecific *fru* in Adult CNS 158

BIBLIOGRAPHY 161

LIST OF FIGURES

Figure		Page
2.1.	The molecular map of the *fru* locus.	38
2.2.	The temporal and spatial distribution of *fru* mRNA and FRU protein during embryogenesis.	46
2.3.	FRU proteins are expressed in all neurons and lateral midline glia.	51
2.4.	β-galactosidase expression pattern in *fru* P-element mutant embryos.	55
2.5.	Axonal defects in the CNS of *fru* mutant embryos.	61
2.6.	The expression of specific *fru* transgenes rescues the axonal defects in FasII and BP102-positive axons in *fru* mutants but the expression of male-specific *fru* transgenes disrupt axonal tracts in the CNS.	67
3.1.	*fru* genetically interacts with *lola* in axonal pathfinding.	89
3.2.	*fru* genetically interacts with *robo* and *comm* in axonal pathfinding.	97
3.3.	*fru* genetically interacts with *ttk*, *gcm* and *sim*.	102
3.4.	A model of *fru*'s role and genetic hierarchy in guiding contralateral axonal projections with genes expressed in neurons.	108
3.5.	A model of *fru*'s role and genetic hierarchy in guiding longitudinal axonal projections with genes expressed in neurons and midline glia.	110
3.6.	A model of *fru*'s role and genetic hierarchy in guiding longitudinal axonal projections with genes expressed in lateral glia.	112
4.1.	Eve expression pattern in GMCs and neurons in wildtype and *fru* mutant embryos.	131
4.2.	Eve-positive neurons in wildtype and *fru* mutant stage 16 embryos.	137
4.3.	Wildtype and *fru* mutant embryos doubly labeled for axonal tracts and Eve.	141
4.4.	The expression of specific *fru* transgenes rescues the Eve phenotype in *fru* mutant embryos.	145

LIST OF TABLES

Table		Page
2.1.	Mutant phenotypes of *fru*^{AJ96u3} homozygotes and heterozygotes with other *fru* alleles.	40
2.2.	The temporal and spatial distribution of *fru* mRNAs and FRU proteins in embryos.	43
2.3.	Analysis of abnormal axonal projections in the CNS of *fru* mutant embryos.	63
2.4.	Pattern of FasII and BP102 axonal tracts in *fru* mutants expressing *UAS-fru* transgenes in the CNS.	69
3.1	Phenotypic analysis of FasII- and BP102-positive axonal tracts in double heterozygous embryos with *fru* and genes expressed in neurons, *lola* or *robo*.	91
3.2.	Phenotype analysis of FasII- and BP102-positive axonal tracts in double heterozygous embryos with *fru* and genes expressed in neurons, *comm,* and in midline glia, *sim*, *slit*.	99
3.3.	Phenotypic analysis of FasII- and BP102-positive axonal tracts in double heterozygous embryos with *fru* and genes expressed in lateral glia, *ttk*, *gcm* or *pnt*.	104
4.1.	Genetic analysis of Eve-expressing cells with the CNS.	134
4.2.	Genetic analysis of Eve-positive neurons in *fru* mutant embryos expressing *fru* transgenes.	147

The Genetic Dissection of the *fruitless* Gene's Functions During Embryogenesis in *Drosophila melanogaster.*

CHAPTER 1

INTRODUCTION

General Overview of Thesis

One of the fundamental questions of developmental neurobiology is how neuronal circuits are established in the nervous system of an animal to subserve particular behaviors. It is a reasonable assumption that an animal's ability to display a particular behavior derives from the presence of a specific organization of neurons, each identified by their individual pattern of connectivity and physiology, as part of a neuronal circuit. One productive avenue of research to elucidate the mechanisms underlying specific behavioral patterns, rather than those regulating an animal's general activity level, has been focused on the investigation of the cellular basis of neuronal differentiation and the formation of distinct neuronal connections.

In most animals, neuronal identity is determined through a series of discrete steps that restrict a cell's differentiative potential during development (Doe and Skeath, 1996; Doe et al., 1998; Kornack, 2000). Since neurons derive exclusively from ectodermal primordia in the embryo, the first differentiative decision is the choice of a neuronal fate rather than an ectodermal fate. Subsequent decisions restrict the potential fate of cells to the type of neuron generated. Neuroectodermal cells first segregate as a population of proliferating neuronal precursor cells, that ultimately produce the neurons that compose the nervous system. Neurons are postmitotic cells that undergo terminal differentiation and adopt a characteristic morphology, physiology and biochemistry.

One of the most important and distinctive features of neurons is their ability to make synaptic connections, that is, to form a network of synaptically interconnected cells, with specific cellular partners. This precise connectivity is due to the process of axonal guidance (axogenesis) and synapse formation (synaptogenesis). Axonal processes are tipped by growth cones, the active extending part of the axon, and these growth cones sample their environment and

make choices as to which direction they will next grow. A variety of cellular and molecular mechanisms have been shown to operate in this process of axonal pathfinding in both vertebrates and invertebrates (Tessier-Lavigne and Goodman, 1996; Mueller, 1999; Van Vactor and Flanagan, 1999; Featherstone and Broadie, 2000). Upon reaching the target cells, the growth cones stop and establish stable synaptic contacts with the target cells. The synaptic contact between one neuron and another neuron or non-neuronal cell forms the anatomical basis of a neuronal circuit. Finally, the strength of the synaptic contacts between neurons within a neuronal circuit has to be actively maintained through synaptic activity and trophic interactions with other cells, including glia. The formation of synaptic connections between neurons in a neuronal circuit, by itself, is often not sufficient for a behavior to be expressed by an organism. The actual display of a particular behavior often relies on other factors, such as cues in the abiotic environment or the presence of another animal.

While neuroscientists have analyzed a wide variety of behaviors, there are no examples in which the complete neuronal circuit has been established for a given behavior. Therefore, understanding the molecular, genetic and cellular processes required to establish individual neuronal circuits is a daunting task, and much more so in the case of behaviorally-related neuronal circuitry. Recent advances in the neural and behavioral sciences have contributed many pieces of the jigsaw-puzzle to allow us to understand how neuronal circuits are established to subserve a particular behavioral trait.

One of the most attractive behavioral systems to study is sexual behavior. Sexual behaviors have been studied in various species from humans to simpler animals, such as worms. To achieve proper reproductive behaviors in male and female animals, some regions of the brain in both sexes are somewhat specialized with respect to the other. Behavior also has a temporal component with a short time scale in which physical actions are rapidly strung together as the result of temporal sequences of activity in the underlying nervous system. This requirement, the construction of permanent specialized brain structures, which occurs on a long

time scale, as well as temporal usage of the nervous system underlying sex-specific behavior, lead one to consider that sexual behaviors are under genetic control.

In the fruit fly *Drosophila melanogaster*, *fruitless* (*fru*) is responsible for the expression of male sexual behavior and support the idea that the potential to generate a specific sexual behavior is genetically controlled in *Drosophila* (Ryner et al., 1996; Ito et al., 1996). Male flies that are mutant for the *fru* gene have a number of abnormal sexual behaviors (Hall, 1994; Villella et al., 1997; Anand et al., 2001; Lee et al., 2001). In those genotypes in which *fru* males show courtship behaviors, the males indiscriminately perform their courtship behaviors to both male and female potential mates; wild-type males, in contrast, overwhelmingly choose females as their courtship partners (Ryner et al., 1996; Ito et al., 1996; Villella et al., 1997). Mutations in other genes, such as *dissatisfaction*, *Voila*, *courtless* and *quick-to-court*, also, have been shown to affect the performance of male and female sexual behaviors in the fruit fly (reviewed in Yamamoto et al., 1998 and Greenspan and Ferveur, 2000; Finley et al., 1997; Balakireva et al., 1998; Orgad et al., 2000; Gaines et al., 2000). Thus, the presence of mutations that eliminate some or all expression of sexual behavior without affecting the ability of animals to perform other behaviors is one of several lines of evidence leading us to postulate that these genes may function to establish the neuronal circuitry subserving sexual behavior. However, remarkably little is known about the molecular mechanisms that establish any of these neuronal circuits in the CNS.

The *fru* gene has been studied in some detail, in part, because of its role in the control of all aspects of male courtship behaviors (reviewed in Yamamoto et al., 1998; Greenspan and Ferveur, 2000; Baker et al., 2001). The *fru* gene produces a complicated array of transcripts through alternative splicing of 5' and 3' end sequences, resulting in male- and female-specific as well as sex-nonspecific transcripts (Ryner et al., 1996; Ito et al., 1996; Goodwin et al., 2000). Much attention has focused on *fru*'s sex-specific role in order to understand its role in establishing the neuronal circuit for adult male sexual behavior. *fru* mutants also have sex-nonspecific phenotypes but little research has been done on how *fru* is involved in these functions.

My doctoral thesis has examined the role of *fru* in the differentiation of embryonic neurons in CNS. I have described the pattern of transcript and protein expression of different *fru* isoforms during embryonic development. To analyze *fru*'s function, I examined axonal phenotypes and the loss of neuronal identity in *fru* mutant embryos. Through the controlled expression of *fru* transgenes, I was able to rescue the neuronal defects in *fru* mutant embryos. Finally, to better understand *fru*'s function in proper formation of axonal projections in the embryonic CNS, I have identified other genes, through a genetic interaction study, that are likely to function along with *fru* in axonal pathfinding in the embryonic CNS. From these results, I have been able to place *fru* within the cascade of genes that have been identified as necessary for the establishment of wild-type neuronal connections in the embryonic CNS. My studies provide the first evidence that *fru* actually functions in establishing neuronal connections. My expectation is that my research in *fru*'s sex-nonspecific function in the embryonic CNS will contribute to the understanding of how genes, such as the *fru* gene, control the differentiation of neurons and create neuronal circuits to subserve complex behaviors, such as sexual behaviors. In this introduction to my dissertation, I have reviewed the formation of neurons and glia and the molecules involved in axonal guidance, mostly in *Drosophila*, as these pertain most closely to my research.

The Specification of Neurons (Neurogenesis) in the CNS of *Drosophila*

Neurogenesis is best understood at the cellular and molecular level in the *Drosophila* embryo (Bhat, 1999; Skeath, 1999; Doe et al., 1998; Doe, and Skeath, 1996). The formation of neurons begins with the production of the neuroblast (NB), which is equivalent to a vertebrate neural stem cell, from the ventrolateral ectoderm, termed the neuroectoderm. In the neuroectoderm, proneural gene activity by the genes of the *achaete-scute* complex (*AS-C*) promotes neuroblast formation. The action of the proneural genes is opposed by the activity of the neurogenic genes acting through the Delta ligand and the Notch membrane-bound receptor. These proteins mediate a cell-cell signaling cascade that antagonizes proneural gene function and limits NB formation to a single cell. The *AS-C* proneural genes,

achaete (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*), encode basic helix-loop-helix transcription factors whose expression is required in neuroectodermal cells for them to commit to the NB fate. Expression of specific combinations of *AS-C* genes in a small number of equivalent cells confers the potential to become a NB on all of the cells within a proneural cluster. The cell that becomes the NB expresses the *AS-C* genes at the highest level and retains this level of *AS-C* gene expression. The *AS-C* genes activate transcription factors that turn on the expression of genes required for the NB fate (Bhat, 1999; Skeath, 1999; Doe et al., 1998). Within each proneural cluster of cells, the level of Delta signaling through the Notch receptors on neighboring cells appears to help single out which cell will become the NB. Then, the presumptive NB, again acting through the Notch signaling pathway, inhibits the neural potential in the remaining cells of the cluster by extinguishing their expression of the *AS-C* genes (Bhat, 1999; Skeath, 1999; Doe et al., 1998). In embryos lacking proneural gene function, there is a decrease in the number of NBs formed (Skeath and Carroll, 1994). Embryos with mutations in genes involved in Notch signaling pathway, which disrupt the "lateral inhibition" within a proneural cluster, have the opposite phenotype to the loss of proneural genes, and all cells of the cluster form NBs (Skeath and Carroll, 1992; Martin-Bermudo et al., 1995).

The pattern of *AC-S* gene expression in the proneural clusters determines the NB pattern in an orthogonal array of seven rows and three columns, from medial to lateral (Bhat, 1999; Skeath, 1999; Doe and Skeath, 1996). Within each hemisegment, the first 10 proneural clusters, named SI proneural clusters because they produce the first wave of NBs, are born. Each SI NB delaminates into the interior of the embryo directly, from within its proneural cluster (Bhat, 1999; Skeath, 1999; Doe and Skeath, 1996; Campos-Ortega and Hartenstein, 1997). Over the next hour, five SII proneural clusters arise at specific locations within this pattern, and SII NBs interdigitate between SI NBs. Subsequently, 16 SIII-SV proneural clusters and their NBs arise at invariant positions within this pattern.

The identity of individual NBs is determined by the particular subsets of transcription factors that are expressed in each one (Bhat, 1999; Skeath, 1999; Doe and Skeath, 1996). Individual NBs produce a defined set of embryonic progeny in

a specific birth order (Doe et al., 1998; Campos-Ortega and Hartenstein, 1997). The process by which NBs divide to create their progeny is now well understood in *Drosophila*. The majority of neuroblasts within the CNS are stem cells that divide asymmetrically to produce one daughter that retains the NB fate and a daughter that becomes a ganglion mother cell (GMC), a secondary precursor cell (Doe, 1992; Doe et al., 1998). These cells are vastly different in their characteristics; the larger NB can divide over 100 times throughout the embryonic and postembryonic phases of neurogenesis. By contrast, the smaller GMC divides just once. The more limited developmental potential of GMCs means that they divide to produce a pair of neurons, a pair of glia, or one of each type of cell (Doe et al., 1998). During the asymmetric division, the fate of the NB and GMC hinges on the unequal distribution of a number of proteins and RNAs acting as intrinsic determinants for GMC and NB fate (Spana et al., 1995; Shen et al., 1997; Li et al., 1997; Broadus et al., 1998). The delineation of one of the daughters as a GMC depends on the localization of the Prospero protein. Prospero is a divergent homeodomain transcription factor which is synthesized in the NB but required in GMCs to activate GMC-specific gene expression and to repress neuroblast-specific gene expression (Doe et al., 1991; Spana and Doe, 1995). The segregation of *prospero* mRNA requires the RNA-binding protein, Staufen, to help tether it to the cytoplasmic actin cortex along the basal side of the NB. The prospero protein localization requires Miranda, a coiled-coil domain protein (Shen et al., 1997), for proper function. In embryos lacking Miranda or Staufen, Prospero fails to become localized and is equally distributed to both daughter cells after the division of the neuroblast; thus both daughter cells adopt a GMC fate. Numb, a signaling protein with a zinc finger (ZnF) domain, has been found to interact with Miranda, which contributes to its localization to one daughter cell (Shen et al., 1997). Numb is an important regulator of signaling between Delta and Notch proteins and contributes to making the two daughter cells take disparate fates (see below).

Another protein, Inscuteable (Insc), which is a cytoskeletal adapter protein, acts as a key regulator of asymmetric cell division (Kraut and Campos-Ortega, 1996). In neuroblasts, Insc protein is distributed as an apical crescent associated

with the actin cortex close to the plasma membrane of the NB. The localization of Insc begins during late interphase and continues through early mitosis; Insc is localized to the side opposite the one from which the GMC will segregate. During mitosis, Insc is required for the correct apical-basal orientation of the mitotic spindle and for the asymmetric segregation of the proteins Numb, Prospero and Miranda into the basal daughter cell (Kraut et al., 1996). Insc remains asymmetrically distributed after mitosis and remains in the daughter cell that will retain the NB fate, whereas Numb and Prospero are distributed to the daughter cell that will become the GMC. In embryos homozygous for a null allele of *insc, miranda, prospero* and *staufen* are unable to localize preferentially to the basal cortex, but crescents of these proteins and mRNAs are randomly distributed along the cell membrane (Li et al., 1997). The combined action of these intrinsic determinants contributes to the specification of GMC.

The mechanism described above for the differential distribution of determinants is common to all divisions that generate a NB and a GMC. In order for a GMC to have a separate identity from other GMCs produced by the same NB, all NBs, in addition, must express individual combinations of determinants as they produce early-, mid- and late-born GMCs. The production of different determinants in the NB results in each GMC inheriting a different combination of gene products (Broadus et al., 1995; Cui and Doe, 1992; Kambadur et al., 1998). Two genes with the potential to distinguish GMC fates are *klumpfuss* and *castor* (also called *ming*), which encode different putative ZnF transcription factors. These transcription factors are produced in neuroblasts following the production of the first GMCs, so they are detected in late-born but not early-born GMCs (Yang et al., 1997; Cui and Doe, 1992; Kambadur et al., 1998). By their pattern of expression, these genes appear necessary for distinguishing late-born GMC fates from early-born GMC fates.

The last step of neurogenesis is the division of each GMC to produce a pair of terminally differentiated cells, which often take on different cell fates with different morphologies, physiologies and synaptic targets (Doe and Skeath, 1996; Campos-Ortega and Hartenstein, 1997; Spana and Doe, 1996). The mechanism by

which GMCs divide to produce sibling neurons with different identities has many parallels to the ways that NBs and GMCs are generated. The main features are the asymmetric distribution of a determinant that subsequently regulates the cell-cell communication between the two sibling cells. GMCs possess the same apical-basal polarity that is exhibited by the NB (Buescher et al., 1998). As a result, GMCs distribute determinants in an asymmetric manner to generate pairs of sibling neurons that are intrinsically distinct. The molecular mechanism that drives the asymmetrical distribution of determinants in the daughters of the GMC division is essentially the same as the mechanism by which determinants were distributed between the NB and the GMC. Insc again plays an important role since it is responsible for the asymmetric distribution of Numb protein into one of the sibling neurons. The loss of *insc* function randomizes this distribution and results in the generation of equivalent sibling neurons. The correct asymmetric distribution of Numb has been shown to be crucial for the specification of distinct fates of sibling neurons in several studies (Guo et al., 1996; Spana et al., 1995; Buescher et al., 1998; Skeath and Doe, 1998). Numb acts to downregulate Notch signaling between the two daughter neurons, so that in one cell Notch signals are high and in the other cell Notch signals are low. This difference in Notch signaling leads to differences in the expression of transcription factors and, thus, the fate taken by the two siblings (Buescher et al., 1998). A direct link between intrinsic (Numb) and extrinsic (Notch) mechanisms for the specification of distinct fates of sibling neurons has been shown by various overexpression and mutant studies (Buescher et al., 1998; Spana and Doe, 1996).

At the end of neurogenesis, each segment of the embryonic CNS of *Drosophila melanogaster* contains around 350 neurons (Bossing et al., 1996; Schmid et al., 1999; Campos-Ortega and Hartenstein, 1997) and 36 glia derived from around 30 stem cell-like progenitor cells, NB and glioblasts, respectively (Ito et al., 1995; Granderath and Klämbt, 1999). These neurons are now ready to develop their characteristic dendritic and axonal projections to their targets to form their neural connections. Since many genes are expressed in most or all neurons of the CNS, the identity of each neuronal cell type is probably acquired progressively

and involves a large number of genes, each regulating specific aspects of neuronal development and function. The proper differentiation of neurons requires not only that neurons be specified through the intrinsic mechanisms that have just been described, but also the formation of support cells of the CNS, the glia.

The Specification of Glia (Gliogenesis) in the CNS of *Drosophila*

In the *Drosophila* embryonic CNS about 10% of the cells are glia (Ito et al., 1995). Two types of glia, lateral and midline glia, are formed; they are specified by different molecular mechanisms. Both types of glial cells play a crucial role during early nervous system development. In addition to roles in the regulation of neuronal apoptosis and in providing electrical insulation to neurons, they often act as substrates for migrating neurons and growth cones and secrete factors that are capable of guiding growth cones over long distances (Granderath and Klämbt, 1999; Booth et al., 2000; Hidalgo and Booth, 2000; Hidalgo et al., 1995; Kinrade et al., 2001; Lemke, 2001).

There are two sources of glial cells; the ventral neuroectoderm that is the primordium for the lateral glial cells and the mesectoderm which come to lie at the ventral midline of the embryo after gastrulation and is the primordium for a small but critical set of midline glial cells. For the neuroectodermally-derived lateral glia, there are two types of neuroblast-type precursors, the glioblasts (GBs) and the neuroglioblasts (NGBs), which are subdivided into type I and type II NGBs (Udolph et al., 2001; Bernardoni et al., 1999). GBs possess only gliogenic properties, giving rise exclusively to glial cells. At the first NB division, the type I NGB divides into a GB and NB, thus creating progenitors with a more restricted developmental potential that give rise either to neurons or to glial cells. The type II NGBs create intermediate progenitor cells that have the potential to generate neurons as well as glia via an asymmetric cell division.

The development of these neuroectodermally-derived lateral glia depends on the gene *glial cells missing* (*gcm*, also known as *glial cell deficient* [*glide*]). *gcm/glide* encodes a novel type of transcription factor that serves as a binary switch between neuronal and glial cell fates because this transcription factor promotes

gliogenesis at the expense of neurogenesis (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). In *gcm* mutants, all lateral glial cells are transformed into neuronal cell types. In the reverse situation, ectopic expression of *gcm* in neurons causes neurons to adopt the glial cell fate (Hosoya et al., 1995; Jones et al., 1995). Recently, it has been demonstrated that an early bifurcation of the glial versus neuronal sublineages takes place during the first division of the parental NGB. This process involves the asymmetric expression of *gcm* in the glial sublineage (Akiyama-Oda et al., 1999; Bernardoni et al., 1999). Furthermore, it has been suggested that in type II NGBs, Notch is used for specifying the glial part of the lineage (Udolph et al., 2001). The Gcm transcription factor in daughter cells stimulates the expression of other transcription factors required for normal gliogenesis. G*cm* regulates two pathways in glia development. One pathway leads to the activation of glial-specific genes, and the other pathway suppresses the expression of neuronal-specific genes. Three target genes of *gcm* have been found: two genes, *reversed-polarity* (*repo*), and *pointed* (*pnt*), are required for glial development, and a third gene, *tramtrack* (*ttk*), is involved in suppressing genes controlling neuronal differentiation. The *repo* gene encodes a homeodomain transcription factor, and the *pnt* gene encodes two transcription factors that share a conserved ETS DNA-binding domain (Klämbt, 1993; Klaes et al., 1994). Only one of these proteins, PointedP1, is expressed in lateral glial cells; the other protein, PointedP2, is expressed only in the midline glia (Klämbt, 1993; Klaes et al., 1994). The *ttk* gene encodes a BTB/POZ family transcription factor. One of the two Ttk isoforms, Ttk69, is expressed by lateral and midline glia cells (Giesen et al., 1997). At the end of gliogenesis, about 35 lateral glial cells are formed and are classified according to their position, morphology and expression of molecular markers. The perineural glial cells form a continuous glial sheath around the CNS and provide a hemolymph-brain barrier. The longitudinal (interface) glial cells ensheath the axonal processes of neurons that project into the longitudinal axonal tracts (Granderath and Klämbt, 1999).

The failure of lateral glial cell differentiation has severe consequences for neurogenesis. In *repo* mutant embryos, axonal fibers that extend in longitudinal

tracts through the CNS are unable to maintain the wild-type level of adhesion between the axons and instead travel through the nervous system as individual fibers or as small groups of fibers (Halter et al., 1995). There are also defects in how well the nervous system condenses after germ band retraction (Halter et al., 1995). Glial cells provide trophic support for neurons, so that in mutants in which glial cells do not form, as in mutants of *gcm*, more neurons die by apoptosis (Xiong and Montell, 1995). In *pnt* mutant embryos, the lateral glial cells fail to extend cellular processes to surrounding bundles of axons. These glial cell processes shield neuronal axons from exposure to the high concentration of potassium ions found in the hemolymph; these ions affect the ability of axons to conduct action potentials and lead to a failure of nerve transmission to target cells, such as muscles (Klaes et al., 1994). *pnt* expression in glial cells is necessary and sufficient for the expression of a neuronal-specific antigen recognized by the 22C10 antibody in a small subset of motorneurons and interneurons (Klaes et al., 1994).

The formation of mesectodermally derived glia, the so-called midline glia (MG), does not require *gcm* but rather involves a much more complicated cascade of genes. The regulation and determination of MG start with the specification of mesectodermal cells (MECs) between the ventral mesodermal and lateral neuroectoderm cells in the blastoderm stage. The Dorsal transcription factor is transported to the nucleus of ventral cells in the blastoderm embryo and turns on the expression of the *twist* and *snail* genes, which encode transcription factors that specify mesodermal fates. The MECs are specified at the lateral margin of the mesodermal region by the activity of the Twist and Snail proteins. The boundaries of Snail and Twist expression are established by the level of Dorsal expression in the lateral nuclei (reviewed in Jacobs, 2000; Morisato and Anderson, 1995). MECs are first identified by the expression of the bHLH transcription factor Single minded (*sim*) and the cell surface protein Rhomboid (*rho*) (Nambu et al., 1991), which is regulated by the transcription factors, Snail and Twist (Kosman et al., 1991). Sim expression also requires maternal Notch function (Morel and Schweisguth, 2000). Sim proteins form heterodimers with other proteins, such as Tango, to activate the transcription of genes that specify MG identity (Sonnenfeld

et al., 1997; Crews, 1998; Jacobs, 2000). The MECs produce a small subset of MG and midline neurons (Jacobs, 2000). At the end of midline gliogenesis, 3-4 midline glial cells form in each neuromere (Bossing and Technau, 1994). These cells ensheath axonal processes within the commissures from neurons whose processes cross the midline and extend to the side of the CNS contralateral to the neuronal cell body (Granderath and Klämbt, 1999).

The MGs play a special role in the formation of proper axonal tracts in the embryonic CNS. Their survival and differentiation is crucial for correct axonal tract. The differentiation of MG is controlled by the *Drosophila* epidermal growth factor (EGF) receptor homologue (DER), whose expression in the CNS is found specifically in the midline glia (Zak et al., 1990; Scholz et al., 1997). In response to DER activation, PointedP2 becomes phosphorylated by mitogen-activated protein kinase (Brunner et al., 1994; O'Neill et al., 1994). In the CNS, PointedP2 is required for MG's differentiation (Klämbt, 1993).

Axonal Pathfinding by CNS Neurons

In *Drosophila*, the embryonic ventral nerve cord (VNC), which is the rough equivalent of the vertebrate spinal cord, consists of longitudinal connectives with two commissures across the midline that link the connectives in each segment (Goodman and Doe, 1993). In the developing VNC, neurons are classified into two types based on their axonal projection to the midline-ipsilateral and contralateral neurons. The axons of about 20% of the 350 or so embryonic interneurons in a hemisegment grow longitudinally on the same side of the midline where their cell bodies lie; these ipsilateral neurons never cross the midline of CNS (Bossing et al., 1996; Schmid et al., 1999; Campos-Ortega and Hartenstein, 1997). About 70 % of the interneurons called contralateral neurons cross the midline once as part of a commissural tract and then bundle together (fasciculate) with other axons to grow along the longitudinal pathways up to the brain (Bossing et al., 1996; Schmid et al., 1999; Campos-Ortega and Hartenstein, 1997). Many motorneurons also send their axons across the midline before exiting the CNS to innervate their muscle target

cells (Bossing et al., 1996; Schmid et al., 1999; Campos-Ortega and Hartenstein, 1997).

In general, the precise wiring of neuronal connections is thought to be achieved by two types of mechanisms. In early stages of axonogenesis, axonal pathfinding depends on mechanisms that are independent of neural activity but require long- and short-range signals. The formation of axonal tracts in the CNS starts with the growth of axons from a special subset of early pioneer neurons whose processes set up the basic axonal scaffold in the CNS (Bate, 1976). The axon has a specialized region at its tip at which point growth of the axon takes place by the addition of new plasma membrane and by the reorganization and elongation of the actin- and microtubule-based cytoskeletons. Neurons frequently extend processes over very long distances to reach their synaptic partners. In order to reach their targets, they are guided first by long-range secreted signals and later, as they come closer to the target, by short-range signals that direct the axon's growth. Later stages of axonal pathfinding occur when the neurons are close to, or at, their target and require activity-based refinement mechanisms to prune exuberant projections and adjust synaptic strengths (Tessier-Lavigne and Goodman, 1996).

Many of the long- and short-range signals have been identified as secreted diffusible molecules (Tessier-Lavigne and Goodman, 1996; Mueller, 1999). For some secreted short-range cues, the diffusion of the molecule may be restricted, since the secreted protein may bind to molecules in the extracellular matrix. Other guidance cues are thought to be mediated by the direct contact between the axonal growth cone and the extracellular matrix or the glial cells. In these cases, the glial cells at specific locations in the pathway have been proposed to behave as guideposts or intermediate attachment points for growing axons (Tessier-Lavigne and Goodman, 1996; Mueller, 1999;Auld, 1999; Hidalgo and Booth, 2000). Located in one of the most important regions for controlling axonal pathfinding by commissural axons, midline glial cells secrete both attractive and repulsive signals (see below; Dickson, 1998; Tessier-Lavigne and Goodman, 1996; Thomas, 1998; Tear, 1999). In addition to midline glia, lateral glia are also involved in guiding

growth cones and mediating fasciculation and defasciculation of axons at choice points (Auld, 1999; Hidalgo and Booth, 2000; Booth et al., 2000; Hidalgo et al., 1995).

While cues may exist along the entire path of the axon, it is at the growth cone on the tip of each axon where the decision as to the direction of axonal growth is made. Each growth cone thus makes multiple decisions at a series of choice points to change direction or fasciculation partners. At these choice points, the growth cones typically cease forward growth and sample the environment by filipodial extensions. The choices that growth cones make reflect the integration of information provided by local cues, long-range signals and neighboring support cells. Once these primary axonal trajectories are formed, later-born neurons project their growth cones along the same pathway. Axons that are going to the same target cells adhere to each other to fasciculate together. When only a part of a pathway is shared by neurons that ultimately take different trajectories, follower axons must decide which route to take and at a specific choice point, defasciculate from the other axons in the fascicle, turn, and fasciculate with a new group of axons. Thus, neurons have to decipher cues in their environment with precision in order to execute multiple fasciculation and defasciculation decisions in an environment packed with inappropriate partners and to reach their target cells and make correct synapses (Goodman and Shatz, 1993; Tessier-Lavigne and Goodman, 1996; Auld, 1999).

Here I focus on the early events of axonal pathfinding that are independent of neural activity. I will discuss the major molecules expressed in neurons and midline glia that have been identified molecularly and genetically to play major roles in axon-axon interactions, such as fasciculation into tracts, and in axonal guidance events for finding their correct synaptic partners.

Mechanisms for Axonal Fasciculation and Defasciculation

When axons fasciculate they are selecting one of several pre-existing axonal pathways and they make this choice through their response to attractive and repulsive cues that may be either secreted or on the surface of other axons and glia

(Tear, 1999; Tessier-Lavigne and Goodman, 1996; Goodman, 1996). Since axons do not remain fasciculated to the same neurons throughout their pathfinding, they undergo cycles of fasciculation, defasciculation and refasciculation. This means that there must be ways to modulate the strength of axonal adhesion to various partners during axonal pathfinding. The best examples of the decision to defasciculate can be found in motoneuronal axons (Tear, 1999). For example, motoneuronal axons bundle together into discrete fascicles as they leave the CNS on their way to the periphery. Individual axons must then choose to defasciculate from the common bundle to reach their target muscle (Tear, 1999; Goodman, 1996). A number of different molecules control the choice of axonal fasciculation partners. These proteins belong to one of several families of membrane-bound receptors that homo- and hetero-dimerize.

One important member of a family of cell adhesion molecule genes that mediate axonal fasciculation is the *Drosophila fasciclin II* (*fas II*) gene. This gene encodes a protein that is a homologue of the vertebrate neural cell adhesion molecule (NCAM), which is a member of the immunoglobulin superfamily. NCAMs and FasII proteins are expressed on the membranes of many axons that fasciculate together, including motoneurons (Goodman, 1996). These proteins appear to promote fasciculation because they have adhesive properties and function in a concentration-dependent fashion. In *Drosophila*, there are between 7 and 9 fascicles of FasII-positive axons; in whole-mount preparations of the embryonic CNS, these appear as three major longitudinal tracts. The pattern of these fascicles has been used as a structural landmark of the organization of neuronal tracts in the embryonic CNS. In *fas II* mutants, the axons in these FasII-positive fascicles are no longer closely apposed, so that the axons do not bundle tightly together and now grow along independent pathways through the neuropil (Grenningloh et al., 1991; Lin et al., 1994). If there is an increase in the expression of FasII, either by increasing the number of *fasII* genes in an animal or through molecular means to increase expression, axons show increased fasciculation and failure to defasciculate properly (Lin and Goodman, 1994). The results of the mutant and overexpression studies suggest that the control of axonal fasciculation is a process that is relative,

in that the dosage of different molecules alters the strength of the interaction between neighboring axons.

How do axons defasciculate? Four receptor linked protein tyrosine phosphatases (RPTPs), DLAR, DPTP10D, DPTP69D and DPTP99A, have been shown to play an important role in the ability of axons to defasciculate in motoneurons that are growing through the CNS and to the periphery to their muscle target cells (Desai et al., 1997;
Krueger et al., 1996; Sun et al., 2000; Sun et al., 2001). These RPTPs are also selectively expressed on axons in the *Drosophila* embryonic CNS. In an embryo mutant for both DPTP10D and DPTP69D, more axons cross the midline than in single mutant embryos, showing that there is a synergistic effect from the loss of both genes (Sun et al., 2000). In addition, heterozygous embryos that have one wild-type copy of either the DPTP10D or DPTP69D gene and one wild-type copy of either the *commissureless* (*comm*), *roundabout*(*robo*) or *slit* genes showed a midline-crossing phenotype that is not seen when any single gene is heterozygous (Sun et al., 2000). Heterozygous embryos with one wild-type copy of either the DPTP10D or DPTP69D gene and one wild-type copy of the D-Netrin gene did not show any mutant phenotype (Sun et al., 2000). All of the genes that showed an interaction in the trans-heterozygous embryos are involved in the production or reception of the Slit repulsive signal from the midline. The one gene, which showed no interaction is a secreted ligand that mediates attractive signals. Double mutant combinations with mutations in other RPTPs also show defects in the CNS, suggesting that RPTPs are partially redundant, compete with one another, and that there are dosage-sensitive for repulsive cues but not for attractive cues mediating axonal guidance (Sun et al., 2000; Sun et al., 2001).

Mechanisms for Axonal Guidance by Attractive and Repulsive Environmental Cues

The choice of axonal pathway is primarily orchestrated by molecules that bind to receptors on the growth cone. These signals may produce the positive effects of enhanced axonal elongation and chemoattraction to intermediate or terminal target

tissues. Alternatively, negative cues lead to growth inhibition and repulsion from a particular region of the CNS. Molecules implicated as guidance cues or as receptors for these cues in vertebrates and *Drosophila* are well reviewed elsewhere (Tessier-Lavigne and Goodman, 1996; Mueller, 1999; Tear, 1999; Goodman, 1996).

The *slit* gene encodes an extracellular EGF-like leucine-rich repeat motif which functions as a secreted ligand for a repulsive cue for the growth cones of axons and muscle cells (Rothberg et al., 1988; Kidd et al., 1999; Battye et al., 1999; Battye et al., 2001). Embryos lacking *slit* function show a medial collapse of lateral axon tracts. The loss of *slit* function affects other tissues because ventral muscles migrate across the midline in these mutants (Kramer et al., 2001; Kidd et al., 1999). A transgene that induces *slit* expression in the midline restores axon patterning (Kidd et al., 1999). By contrast, ectopic *slit* expression outside of the midline inhibits formation of axon tracts at locations of high Slit production and misdirects axon tracts towards the midline (Kidd et al., 1999). In double heterozygous embryos, *slit* interacts genetically with *robo* which encodes a putative receptor for growth cone repulsion (Kidd et al., 1998a; Battye et al., 1999; Kidd et al., 1999).

The receptors of the Slit repulsive factor are members of the *robo* gene family. These proteins encode membrane spanning proteins with a novel subfamily of immunoglobulin domains followed by three fibronectin (FN) type-III repeats in their extracellular domain. These domains are similar in all three Robo proteins but Robo2 and Robo3 lack a cytoplasmic domain called CC2 (CC stands for conserved cytoplasmic), a binding site for the protein, Enabled, and CC3, a site that interacts with the Abelson tyrosine kinase; both domains are found in Robo1 (Kidd et al., 1998a; Rajagopalan et al., 2000a, 2000b; Simpson et al., 2000a, 2000b; Bashaw et al., 2000). In *robo1* mutants, axons cross and re-cross the midline. This aberrant growth of axons across the midline is thought to be due to axonal growth cones that lack repellent activity and ignore repulsive Slit signals from the midline. The severity of the mutant phenotypes among the three different *robo* mutants in terms of the number of FasII axons crossing the midline is *robo1* > *robo2* > *robo3* (Rajagopalan et al., 2000a, 2000b; Simpson et al., 2000a, 2000b). Double *robo1*; *robo2* mutants reveal that all axons turn toward the midline and stay there unable

to leave, a phenotype identical to that found in *slit* mutants. The three Robo proteins are selectively expressed in neurons in the CNS, and their expression pattern is confined to the FasII-positive longitudinal fascicles (Rajagopalan et al., 2000a, 2000b; Simpson et al., 2000a, 2000b). In the *robo3* mutant, the intermediate FasII axon bundle disappears, and its axons are now found in the medial fascicle. If *robo2* is expressed in all neurons, the axons from these neurons converge in a single fascicle in a lateral position. The Abelson (Abl) tyrosine kinase and its substrate, Enabled (Ena), play direct and opposing roles in Robo signal transduction; Abl functions to antagonize Robo1 signaling, while Ena is required in part for Robo1's repulsive output. Both Abl and Ena can directly bind to Robo1's cytoplasmic domain. A mutant form of Robo that interferes with Ena binding is partially impaired in Robo function, while a mutation in a conserved cytoplasmic tyrosine that can be phosphorylated by Abl generates a hyperactive Robo receptor (Bashaw et al., 2000).

The *commissureless* (*comm*) gene encodes a novel membrane protein with a single membrane-spanning domain and a short Adaptin (AP-2) recognition sequence commonly found among molecules that are endocytosed (Tear et al., 1996) and is expressed in neurons and midline glia (M. Seeger, unpublished data). Embryos with mutations in *comm* lack all embryonic CNS commissures, suggesting that Comm has a role in how axons cross the midline. Normally, Robo is expressed at very low levels on commissural axons and at high levels on longitudinal axons. In *comm* mutant embryos, Robo expression in the longitudinal tracts appears as if it might be higher than normal. Interestingly, in *comm* hypomorphic alleles, the occasional commissures are thin. These axons express Robo protein at levels that are higher than normally seen in the commissures and closer to what is typically seen in the longitudinal tract. Comm protein might function by suppressing Robo expression on commissural axons, allowing axons to cross the midline (Kidd et al., 1998b). The double mutant for *robo* and *comm* is indistinguishable from *robo* (Kidd et al., 1998b). Recently it is found that Comm and Robo form a complex that leads to clearance of Robo from the cell surface of axons, a process facilitated by a specific region of the Comm cytoplasmic domain

(M. Seeger, unpublished observation). In summary, Slit, a secreted protein from midline glia, binds to the Robo receptor on neurons, and the response of neurons to Slit is mediated by the ability of Comm protein to remove Robo proteins.

The attractive midline signal is a protein, Netrin, secreted by midline glia. *Drosophila* has two genes, *netrin-A* (*netA*) and *-B (netB)*, which are structurally similar. The Netrin N-terminal contains three EGF-like repeats similar to the subunit structure of laminin B2. The Netrin protein forms a large heterotrimeric protein in the extracellular matrix found in other species from the worm *Caenorhabditis elegans* to humans (Harris et al., 1996; Mitchell et al., 1996). Embryos mutant for both *netrin* genes have thinner or absent commissures as well as occasional breaks in the longitudinal tracts. These findings suggest that this molecule functions as an attractant to commissural axons (Harris et al., 1996; Mitchell et al., 1996).

There are several receptors for the Netrins; these fall into two broad groups; those related to the DCC (human gene Deleted in Colorectal Cancer) protein and those similar to the *Caenorhabditis elegans* protein unc-5 (Keino-Masu et al., 1996; Leonardo et al., 1997). In *Drosophila*, the netrin receptor is Frazzled (fra), a DCC-like immunoglobulin-C2-type domains with FN III repeats (Kolodziej et al., 1996). As expected for a receptor controlling axonal pathfinding, Frazzled is expressed at high levels on commissural and longitudinal axons in the developing CNS (Kolodziej et al., 1996; Hiramoto et al., 2000). In *fra* mutants, the commissures do not develop properly, but these defects are only partially penetrant. The commissures are sometimes thin or absent, with the posterior commissure being more severely affected than the anterior (Kolodziej et al., 1996). Recently it has been shown that Fra can capture Netrin and 'present' it for recognition by other receptors; Frazzled itself is actively localized within the axon through its cytoplasmic domain, and thereby rearranges Netrin protein into a spatial pattern completely different from the pattern of Netrin gene expression. Frazzled-dependent guidance of one pioneer neuron in the CNS can be accounted for solely on the basis of this ability of Frazzled to control Netrin distribution, and not by Frazzled signaling. A model for the mechanisms of patterning has been proposed in

which a receptor rearranges secreted ligand molecules, thereby creating positional information for other receptors (Hiramoto et al., 2000).

To determine if attraction or repulsion is the primary modular function encoded in the cytoplasmic domain of these receptors, chimeras were created carrying the extracellular domain of one receptor and the cytoplasmic domain of the other, and their function in transgenic *Drosophila* was tested. Fra-Robo (Fra's ectodomain and Robo's cytoplasmic domain) functions as a repulsive Netrin receptor; neurons expressing Fra-Robo avoid the Netrin-expressing midline and muscles. Robo-Fra (Robo's ectodomain and Fra's cytoplasmic domain) is an attractive Slit receptor; neurons and muscle precursors expressing Robo-Fra are attracted to the Slit-expressing midline (Bashaw and Goodman, 1999). These results suggest that a key factor controlling attraction versus repulsion is the receptor's cytoplasmic domain (Bashaw and Goodman, 1999).

It has recently been suggested that lateral glial cells dictate pioneer axon projections in the *Drosophila* embryonic CNS (Hidalgo and Booth, 2000) and that the interaction between glia and neurons is required for proper axonal projection (Hidalgo et al., 1995; Booth et al., 2000). Midline glial cells express important molecules for axonal attraction and repulsion involved in midline guidance. Both ligands, Netrin and Slit, are expressed at the midline. Robo proteins are temporarily expressed in lateral glia, at a stage early in the process of axonal pathfinding, which may indicate that trophic factors are likely released from lateral glia (Kinrade et al., 2001). Compared to what is known about the role of midline glia, little is known about guidance molecules, or trophic factors, and how they interact from lateral glia cell. Lateral glia also require contact with axons to maintain their survival (Kinrade et al., 2001).

The Molecular Characteristics of the *fruitless* Gene

The *fru* gene is multifunctional and encodes a family of BTB/POZ (Broad complex, Tramtrack, and Bric-a-brac /Poxvirus and Zinc finger) transcription factors (Ryner et al., 1996; Ito et al., 1996; Anand et al., 2001). *fru* is functionally complex, producing both sex-specific and sex-nonspecific transcripts from four

promoters (P1, P2, P3 and P4), and alternative splicing at both the 5' and 3' ends of the primary transcripts (refer to Fig. 2.1; Ryner et al., 1996; Ito et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000). Transcripts from the distal *fru* promoter (P1) are spliced in females by the Transformer and Transformer-2 proteins, whereas a default splice occurs in males (Ryner et al., 1996; Heinrichs et al., 1998; Usui-Aoki et al., 2000). In females, the splicing of the P1 derived transcripts gives rise to mRNAs with the potential to encode proteins with a BTB/POZ domain at their amino-termini and one of three alternative C_2H_2 Zinc finger pairs at their carboxy-termini. In males, the default splicing of the P1 transcripts generates male-specific mRNAs encoding proteins that differ from the proteins that could be translated from the female specific mRNA by the addition of 101 amino acids N-terminal to the BTB domain (Ryner et al., 1996; Ito et al., 1996). Surprisingly, immunostaining with FRU antibody shows that the mRNAs produced from the P1 *fru* promoter are translated in males but not in females (Lee et al., 2000; Usui-Aoki et al., 2000). FRU protein-expressing cells are found beginning in the late third instar larval through adult stage males in a small subset of neurons in the CNS. These neurons are thought to be responsible for male sexual behaviors.

The transcripts from the three more proximal promoters (P2, P3 and P4) are found in both sexes and encode a BTB/POZ domain followed by one of three Zn-finger pairs although it has yet been determined whether each promoter actually produces all three Zn finger isoforms as shown in female specific transcripts (Ryner et al., 1996; Anand et al., 2001; Ryner et al., in preparation). P2, P3 or P4 derived transcripts encode sex-nonspecific proteins that differ from the male-specific proteins by having just short stretches of amino acids preceding the BTB/POZ domain (Ryner et al., in preparation). Furthermore, they are expressed widely in the nervous system as well as in other non-neuronal tissues in both sexes (Ryner et al., 1996; Lee et al., 2000). It has been suggested that one or more of these sex-nonspecific proteins are responsible for *fru*'s vital function (Ryner et al., 1996; Anand et al., 2001). *fru* mutants that do not make transcripts from P3 and P4 have defects in the adult derivatives of the imaginal discs and a reduction in the

terminal arborization of motor axons on adult abdominal muscles, suggesting that sex-nonspecific *fru* plays a role in the differentiation of the adult epidermis and CNS (Anand et al., 2001). While male-specific expression is found from the late third instar larvae onward, sex-nonspecific expression derived from one of P2, P3, or/and P4 is detected from embryos to adults (S.Goodwin, unpublished data). Taken together, *fru* functions sex-specifically in the sex-determination hierarchy to control male behavior and sex-nonspecifically to control the development of imaginal discs and motoneuronal synapses during adult development. However, it is not clear what differences exist between the functions of the male-specific and sex-nonspecific FRU proteins in neurons.

The Genes Containing a BTB/POZ Motif in *Drosophila* and Known BTB/POZ Functions.

The BTB/POZ domain is an evolutionally-conserved protein-protein interaction motif at the N-terminus of many proteins in species from yeast to human (Reviewed in Collins et al. 2001 and Read et al., 2000). Various genome projects have revealed that the fruitfly *D. melanogaster* has 64, the human *H. sapiens* has 98, the worm *C. elegans* has 91, the yeast *S. cerevisiae* has 2 and the plant *A. thaliana* has 31 BTB domain-containing protein sequences (Rubin et al., 2000; Venter et al., 2001). The BTB/POZ domain is found in a variety of proteins including actin-binding proteins, pox virus proteins, and many transcriptional regulators that usually contain zinc-finger DNA-binding motifs.

This variety in the ways that BTB/POZ proteins can interact with other proteins suggests that they are involved in a wide variety of regulatory events throughout development. For example, in *Drosophila*, the *abrupt* gene, a BTB/POZ ZnF protein, is required for the embryonic formation of a subset of neuromuscular connections and muscle attachments (Hu et al., 1995). The *kelch* gene is necessary for the construction of ring canals that connect oocyte and nurse cells in the ovary (Xue and Cooley, 1993). The *pipsqueak* gene functions in the dorsal-ventral patterning of the eggshell and embryo (Horowitz and Berg, 1996). The *Broad complex* (*BR-C*) is especially interesting, since it has a very similar structure to the

fru gene. *BR-C* is a steroid hormone-regulated locus required for metamorphosis of the epidermis and multiple internal tissues including the CNS (Restifo and White, 1991; Restifo and Merrill, 1994). Three additional BTB/POZ ZnF proteins are involved in different developmental functions: *bric-a-brac* is involved in homeotic function (Kopp et al., 2000), *longitudinal lacking* (*lola*) is involved in axonal pathfinding during embryogenesis (Giniger et al., 1994), and *tramtrack* (*ttk*) plays an important role in specifying cell fate in the developing eye and for development of embryonic glial cells (Lai and Li, 1999; Wen et al., 2000; Giesen et al., 1997). In mammals, several BTB/POZ-encoding genes are implicated in cancer development (Collins et al., 2001). For example, the B cell lymphoma 6 (Bcl-6) gene is implicated in pathogenesis of non-Hodgkin lymphomas (Ye et al., 1993). A fusion protein between the human promyelocytic leukemia zinc-finger protein (PLZF) BTB and the retinoic acid receptor (RARalpha) is strongly associated with acute promyelocytic leukemia (Dong et al., 1996).

A general property of the BTB/POZ domain is that this protein domain mediates homomeric or heteromeric dimerization (Bardwell and Treisman, 1994). The crystal structure of the PLZF BTB domain revealed that BTB monomers are tightly intertwined when they dimerize (Ahmad et al., 1998). Mutations that disrupt the interface and block dimerization result in BTB/POZ proteins that do not function, suggesting that dimerization of BTB/POZ domains is essential for the proper folding of the entire zinc finger protein (Melnick et al., 2000). However, it has also been shown that the BTB/POZ domains facilitate heterodimer formation or oligomerization with a number of proteins. In one model for transcriptional repression of Bcl-6 and PLZF, the BTB/POZ domain directly interacts with Sin3A, SMRT (silencing mediator for retinoid and thyroid hormone receptors) corepressor, and N-CoR (nuclear receptor corepressor) (David et al., 1998; Grignani et al., 1998; Hong et al., 1997; Lin et al., 1998) to form a transcriptional repressor complex. These corepressors, in turn, recruit the HDAC1 histone deacetylase to the promoter of the target gene, resulting in local changes in chromatin structure that diminish transcription (Grignani et al., 1998; Lin et al., 1998).

Another case of oligomerization can be found in *D. melanogaster*, in the GAGA transcription factor of the BTB/POZ family, which is encoded by the essential *Trithorax-like* (*trl*) gene (Espinas et al., 1999). The GAGA transcription factor, named for its pentamer consensus binding site (multiples of GAGAG/CTCTC), is required for normal expression of several developmental regulatory genes. Based on biochemical studies, GAGA activates transcription by counteracting chromatin repression, e.g. heterochromatin silencing (Farkas et al., 1994). In addition, it has been shown that the mutation of conserved residues in the BTB/POZ domain of GAGA encoding *trl* disrupts the formation of higher-order complexes and the BTB/POZ domain in *mod* (*mdg4*) protein can substitute for that of GAGA (Read et al., 2000). The BTB/POZ domain in GAGA is responsible for oligomerization of transcription factors into higher-order complexes and strong cooperative binding of GAGA to multiple DNA sites. It was suggested that oligomerization of GAGA mediated by the BTB/POZ domain bends the target gene promoter, probably resulting in disruption of nucleosome barriers (Espinas et al., 1999; Katsani et al., 1999). It was also reported that the BTB/POZ domain is capable of mediating transcriptional activation and repression (Kaplan and Calame, 1997). Therefore, it is postulated that with respect to the transcriptional regulation by the BTB/POZ family, the versatile BTB/POZ domain mediates oligomerization and interaction with cofactors, ultimately leading to chromatin remodeling and changes in gene expression (Collins et al., 2001).

A comprehensive survey of *fru*'s functions during embryogenesis will be presented in the following chapters; first the expression pattern of *fru* in the embryo will be described and then the implications of this expression pattern will be explored. I (and we) expect that the following findings will contribute to a better understanding of *fru*'s function in the adult CNS.

CHAPTER 2

THE *fruitless* GENE IS REQUIRED FOR THE PROPER FORMATION OF AXONAL TRACTS IN THE EMBRYONIC CNS OF *DROSOPHILA*.

Ho-Juhn Song, Stephen F. Goodwin, Enrique Reynaud, Troy Carlo, Jean-Christophe Billeter, Eric P. Spana, Norbert Perrimon, Bruce S. Baker, and Barbara J. Taylor.

To be submitted to Development

Abstract

The *fruitless* (*fru*) gene in *Drosophila melanogaster* is a multifunctional gene, which has sex-specific functions in the regulation of male sexual behavior and sex-nonspecific functions affecting adult viability and external morphology. While much attention has focused on *fru* 's sex-specific roles, much little is known about its sex-nonspecific functions. We have examined *fru*'s sex-nonspecific role in embryonic neural development. *fru* transcripts from sex-nonspecific promoters are expressed beginning at the earliest stages of neurogenesis, and FRU proteins are present in both neurons and glia. By examining the pattern of FasII- and BP102-positive axons in *fru* mutants, we found defects in the formation of both longitudinal and commissural tracts in the CNS. In embryos that lack most or all *fru* function, FasII- and BP102-positive axons appeared to defasciculate from their normal pathway and fasciculate along other aberrant neuronal pathways. These defects in axonal projections in *fru* mutants were rescued by the expression of specific *UAS-fru* transgenes under the control of a pan-neuronal *scabrous-GAL4* driver. These results suggest that one of *fru*'s sex nonspecific roles is to regulate axonal differentiation during embryogenesis.

Introduction

The *fruitless* (*fru*) gene has a prominent role in both the expression of male sexual behavior as well as adult viability and external morphology (Ryner et al., 1996; Ito et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000 Anand et al. 2001). *fru*'s functional complexity is reflected in the structural complexity of the locus. The *fru* gene encodes a large set of sex-specific and sex-nonspecific transcripts generated by differential promoter usage and alternative splicing at the 5' and 3' ends (Ryner et al, 1996; Ito et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000). *fru* transcripts are generated from at least four different promoters, P1, P2, P3 and P4 and have been shown to have one of at least three different 3' ends (Ryner et al, 1996; Ito et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000; Anand et al., 2001). *fru* transcripts encode closely related BTB/POZ-Zn finger (ZnF) proteins, which likely act as transcription factors (Ryner et al, 1996; Ito et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000).

The male-specific behavioral functions of *fru* depend on transcripts produced from the P1 promoter (Ryner et al, 1996; Ito et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000). P1 transcripts are spliced in a sex-specific manner; in females, the Transformer (TRA) and Transformer-2 (TRA-2) proteins lead to the use of a second 5' acceptor splice site, whereas in males a default splice site is used (Ryner et al, 1996; Heinrichs et al., 1998; Usui-Aoki et al., 2000). The sex-specific splicing of P1 primary transcripts generates a set of P1 transcripts in females that are not translated, and a set of P1 transcripts in males that upon translation have an amino-terminal extension before the BTB/POZ domain (Ryner et al, 1996; Usui-Aoki et al., 2000; Lee et al., 2000). In addition, in both sexes alternative splicing at the 3' end leads to the production of three types of P1 transcripts, each with a different pair of ZnF domains (Goodwin et al., 2000; Usui-Aoki et al., 2000). The male-specific FRU proteins are expressed in nearly 2000 neurons in the pupal and adult CNS and play an important role in male sexual behavior and in the formation of a male-specific abdominal muscle known as the Muscle of Lawrence (MOL) (Ryner et al., 1996; Ito et al., 1996; Goodwin et al., 2000; Lee et al., 2000; Usui-

Aoki et al., 2000). Recently, it has been shown that the FRU^M protein is required for acquisition of the serotonin transmitter in a set of abdominal neurons that innervate the masculine internal genitalia, which are likely involved in aspects of male fertility (Lee and Hall, 2001; Lee et al., 2001).

fru's role in adult viability and morphology has been revealed by analyzing *fru* mutants that also affect the activity of the other three promoters, P2, P3, and P4 (Ryner et al., 1996; Goodwin et al., 2000; Anand et al., 2001). *fru* transcripts from these promoters are expressed in both sexes and encode similar BTB/POZ ZnF proteins. However, these transcripts are not targets for the activity of the TRA and TRA-2 proteins, and thus they are not directly downstream of the sex-determination pathway that governs P1 transcript processing (Ryner et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000). *In situ* hybridization using a probe from the common coding region of *fru*, which detects all or most of the known *fru* transcripts, shows that *fru* transcripts are ubiquitously expressed in the adult CNS and in particular subsets of non-neuronal tissues during larval and pupal development of both adult male and female flies (Ryner et al., 1996; Goodwin et al., 2000; Lee et al., 2000). In addition, genetic analysis of various *fru* mutants shows that mutants lacking the P3 and/or P4 transcript classes die at the pupal stage, suggesting that the expression of a subset of *fru* transcripts is essential for adult viability (Ryner et al., 1996; Anand et al., 2001). In some *fru* mutant genotypes, adult escapers show a variety of defective external phenotypes indicating that these transcripts likely have some function in the development of these adult structures (Anand et al., 2001). To gain a better understanding of *fru*'s role in development, we examined the expression pattern of *fru* RNAs and proteins in the embryonic CNS.

The embryonic CNS has been an important model system for defining how specific genes govern neuronal identity and the process of axonal pathfinding necessary for the formation of proper neuronal connections (reviewed in Goodman and Doe, 1993; Tear, 1999). The main axonal tracts in the ventral nerve cord (VNC) of the fruitfly embryonic CNS consist of two bilaterally symmetrical longitudinal connectives with a pair of commissures, anterior and posterior, that

cross the midline in each segment (Goodman and Doe, 1992). These axonal tracts are created by the growth of axonal processes of interneurons and motoneurons that form specific fascicles (e.g. reviewed in Tear, 1999). The proper formation of this longitudinal and commissural axon scaffold is complex. Among the steps involved are the attraction and repulsion of axons mediated by families of diffusible molecules released from the midline, which produce specific responses in the growing axons (reviewed in Tear, 1999; Guthrie, 1999; Brose and Tessier-Lavigne, 2000). Other steps in axonal pathfinding involve the selective fasciculation and defasciculation of axons as they grow through different regions of the CNS (Tear, 1999; Rusch and Van Vactor, 2000).

In this paper, we show that *fru* RNAs and proteins are expressed in a dynamic pattern during embryogenesis and are widely expressed in the developing embryonic CNS. By co-labeling with the appropriate cell-specific markers, FRU proteins are shown to be present in lateral and midline glia and neurons in the CNS. Parallel analyses of *fru* mutants, in which transcripts from the P1, P2 or P3 promoters are disrupted or in which no *fru* transcripts are made, revealed defects in the formation of proper FasII-positive tracts as well as other longitudinal and commissural tracts in the CNS. These findings suggest that transcripts from the P3 and/or P4 promoters are needed in the embryonic CNS for the formation of wild-type axonal tracts. We also show that the defects in the FasII-positive and BP102-positive axonal tracts found in *fru* mutants are rescued by the expression of specific *fru* isoforms under the control of the pan-neuronal *sca-GAL4* driver, which has a pattern of expression similar to that of FRU proteins, but not by the *elav-GAL4* driver, which is expressed exclusively in post-mitotic neurons.

Materials and Methods

Fly stocks and crosses

Canton-S flies were used as the wild-type genotype. The following *fru* mutant alleles were used: fru^{3}; fru^{4}; fru^{sat}; $In(3R)fru^{1}$ (fru^{1}); $Df(3R)Cha^{M5}$(Cha^{M5}); $Df(3R)fru^{w24}$(fru^{w24}); $In\ (3R)\ fru^{w27}$(fru^{w27}); $Df(3R)fru^{sat15}$(fru^{sat15}); $Df(3R)fru^{440}$ (fru^{440}): $In(3R)fru^{w12}$(fru^{w12}); *Df(3R)P14* (*P14*) (Castrillon, et al., 1993; Ryner et al. 1996; Ito et al. 1996; Anand et al., 2001). A deficiency, *Df(3L)XDI98* (65A02-65E1), was used to examine the other inversion break point of the fru^{w12} chromosome, which is located at 65C-D on the left arm of the third chromosome. fru^{w12}/*Df(3L)XD198* mutants were fully viable and fertile adults (n=10 male; n=10 female) and did not have *fru* mutant external phenotypes, such as defects in the morphology of the wings or legs or duplicated thoracic bristles (n=10 male; n=10 female), nor do they show typical *fru* mutant behavior, such as courtship chaining (n=20, 10 males each group).

For embryonic immunohistochemical analysis, all *fru* mutations were maintained over the "Blue Balancer" chromosome *TM3, Sb, P[ftz-lacZ]*. For adult phenotypic analysis, *fru* mutations were maintained over *TM6B, Tb, Hu*. For further phenotypic mapping of *fru* deficiencies, we used a mutation in the *spread* gene, $sprd^{05284}$ (Spradling et al, 1999; Bloomington Indiana Stock Center). To label midline glial cells in *fru* mutants (see below), the P-element in the enhancer-trap line *AA142* (Scholz et al., 1997; Bloomington Stock center) was recombined onto the fru^{sat15} and fru^{AJ96u3} chromosomes.

Fly stocks were maintained at room temperature on an agar, sucrose, cornmeal and yeast medium supplemented with 0.1% nipagin (p-hydroxybenzoic acid methyl ester, Sigma) for mold inhibition.

Generation and molecular characterization of the fru^{AJ96u3} deficiency

To determine the location of the $AJ96w^{+}$ P-element (Spana and Doe, 1996), flanking genomic sequences around the P-element were isolated by Inverse PCR and sequenced (Yeo et al., 1995). These sequences were Blast-searched against the

Drosophila genome and mapped to the genomic sequences that include the *fru* locus (ACC# AE003722; FlyBase, 1999). To create new *fru* mutations, excisions of the $AJ96w^{+}$ P-element were induced by standard techniques (Robertson et al., 1988). One lethal excision out of 56 w^{-} revertant lines was recovered. The molecular limits of the fru^{AJ96u3} deficiency were determined by a combination of Southern blot analysis and genomic PCR using oligo primers obtained from plasmid subclones from across the *fru* region and flanking sequences (data not shown). The exact location of the deficiency breakpoints was determined by using oligo primers flanking these sequences to PCR amplify fragments that contain sequences around the breakpoints. These PCR fragments were sequenced and compared to the published sequences for this region.

Lethal phase, phenotypic, fertility and behavioral analysis of fru^{AJ96u3} mutant animals

To characterize the fru^{AJ96u3} mutation, transheterozygotes for fru^{AJ96u3} and other *fru* alleles were examined. In those crosses in which adult *fru* transheterozygotes were not present, the pupal stage at which the *fru* mutant died was determined from aged collections of white prepupae and by gauging the final developmental stage based on the staging guide published by Bainbridge and Bownes (1981). For genotypes that reached late stages of pupal development, the pupal case was opened and the animals allowed to emerge or dissected out and their adult phenotype examined.

Males were tested for sterility by collecting males a few hours after eclosion, grouping 8-10 of them in food vials. After four days, individual males were mated with 2-3 Canton-S virgin females and the vials were examined after 7 days for the presence of larvae and/or pupae. To determine whether males would form courtship chains (Villella et al., 1997), males of the same genotype were collected after eclosion and aged for 3-4 days. Eight males were then put together in a food vial and observed in the late afternoon or early evening for the presence of chains of greater than three males courting during a one-hour period over 3-4 days.

Embryonic immunohistochemistry

Timed embryo collections were staged by morphological criteria (Campos-Ortega and Hartenstein, 1997) and prepared for immunohistochemistry according to Patel (1994). To label neurons or glia in the CNS, the following primary antibodies were used (except where noted, these antibodies were a gift from N. Patel): anti-Fasciclin II (1D4, 1:5, Grenningloh *et al.*, 1991), mab22C10, (1:200, Fugita *et al.*, 1982), anti-Elav (9F8, 1:30, O'Neill et al., 1994), mabBP102 (1:20, Seeger *et al.*, 1993), anti-Repo, (1:100, a gift of Dr. A. Travers; Halter *et al.*, 1995) and anti-Odd-skipped (1:200; a gift from Dr. J. Skeath; Spana et al., 1995). To label *fru*-positive cells, rat anti-FRU^{COM} (1:500; Lee et al., 2000), rat anti-FRU^{BTB} (1:500; this study, see below), anti-rat FRU^{A} (1:500, this study) and rat anti-FRU^{C} (1:500, this study) were used. Anti-ß-galactosidase (1:10,000, Cappel, NC) labeling permitted *fru* mutant embryos to be distinguished from control siblings.

For detection of primary antibodies, we used secondary antibodies, which were either directly conjugated with horseradish peroxidase (Jackson Laboratory, PA), with AP, or with the fluorochromes Alexa-594, Alexa-488, or Alexa-395 (Molecular probes, OR). For anti-*Repo* labeling, a biotinylated secondary antibody was used followed by incubation with the ABC-reagent (Vector Lab, CA). For some double-label experiments, colorimetric visualization of diaminobenzidine (DAB, Sigma) was nickel-enhanced (Patel, 1994). Enzymatic processing of alkaline phosphatase used NBT/ X-phosphatase (Boehringer Mannheim) in AP reaction buffer (0.1M NaCl, 0.1 M Tris-HCl , pH 9.5, 0.05 M $MgCl_2$, 0.1% Tween 20, Patel, 1994). To improve the signal obtained from the anti-FRU antibodies, we used the tyramide-based signal amplification kit (NEN, MA) prior to staining for alkaline phosphatase (AP, Jackson Laboratory, PA) reaction.

Labeled whole-mount and filleted embryos were viewed and photographed with a Sony DKC-5000 digital camera under DIC optics, using an Olympus Vanox-TX microscope. For certain double- or triple-labeling experiments, fluorescently labeled embryos were viewed on a TCS-Leica confocal microscope. Composite images were assembled in Adobe Photoshop 5.0.

In situ hybridization of embryos

Single-strand antisense and sense riboprobes for *in situ* hybridization were prepared as previously described (Ryner et al., 1996; Goodwin et al., 2000). The following antisense riboprobes were used: the common coding region probe (antisense-Com, nts 2785-3612, Genbank ACC# U72492), the BTB domain region probe (nts 2075-2422, Genbank ACC# U72492), the P1-promoter-derived sex-specific probe (Probe P1.S, nts 160,236-159,918, ACC# AE003722), the P2 5' end probe (nts 121168-121101 plus 120967-120936; AE0037222.2), the P3 5' end probe (nts 95,033-94,958, ACC# AE003722), the P4 5' end probes (nts 61,666-61,268, ACC# AE003722), and the Zn finger motif probe A (nts1743-2294 ACC# D84437), B (nts 37,721-37,479 ACC# AE003722) and C (nts 3872-4114, Genbank ACC#U72492).

The fixation of whole-mount embryos and the *in situ* hybridization protocols were carried out according to Broadus and Doe (1995) except RNase treatment (1mg/ml) was included after hybridization to remove non-specific binding of riboprobes. To visualize the signal, embryos were incubated in dilute anti-digoxigenin-alkaline phosphatase (1:2000) and reacted with AP (Broadus and Doe, 1995). Embryos were mounted in 70% glycerol and viewed as either whole mount or dissected preparations.

Generation of transformants

Six different UAS-*fru* constructs were made from *fru* cDNAs subcloned in pBluescript KS (Ryner *et al.*, 1996; Ryner *et al.*, in preparation). These constructs all have a minimal 5'UTR and were subcloned into a pUAST vector (Sullivan *et al.*, 2000; Brand and Perrimon, 1993; LC Ryner personal communication). The *UAS-fruA, UAS-fruB* and *UAS-fruC* constructs all started at the same 5' EcoRI site (128,354 on genomic ACC# ACC#AE003722.2) and included identical 5' UTR, BTB and common *fru* coding sequences but different 3' end sequences. The *UAS-fruA* construct contains a 3kb EcoRI fragment of *fru* cDNA #7 (LC Ryner, personal communication; the A 3' end terminates at nt 40563, ACC#AE003722.2). The *UAS-fruB* construct contains a 4.5 kb EcoR1-XbaI fragment from a *fru* cDNA#25

(LC Ryner, personal communication; the B 3' terminates at nt 36684, ACC#AE003722.2). The *UAS-fruC* construct contains a 3.6 Kb *Eco*RI-*Kpn*I fragment of the female cDNA #1 (Genbank ACC# U72492, nts 1763 to 5409). The *UAS-fruMA, UAS-fruMB* or *UAS-fruMC* constructs all have the same 5' sequences that encode the 101 male-specific amino terminus and the BTB domain derived from *fru* male cDNA#5-19 (L. C. Ryner, personal communication), which was truncated at the 3'end and subcloned into pMartini (a gift from Nick Brown) as a *Sac*I-*Pvu*I fragment. The final constructs were generated via a three-way ligation into the pUAST vector in which the 5' sequences (1.2kb EcoRI-PvuI fragment) were ligated to a 1.948 kb PvuI-EcoRI fragment (A 3' end; *fru* cDNA #7; L.C. Ryner, personal communication), a 1.98 kb PvuI-HindIII fragment (B' end; fru cDNA #25; L.C. Ryner, personal communication) or a 1.98 kb PvuI-KpnI (C 3' end; nts 2824-5409, Genbank ACC# U72492)

The pUAST vectors also contained a mini-*white* reporter gene and the final transgene constructs were introduced into *Df(1)yw* parental strain by germ line transformation as described in Rubin and Spradling (1982). Transgene constructs (300 μg/ml) were co-injected with the helper plasmid pUCHSΔ2-3 (100 μg/ml). The chromosomal location of 3 to 10 transformant lines was determined. If a transformant line contained two insertions on different chromosomes, the transgenes were segregated and treated as independent lines. Two transformant lines for each *UAS-fru* construct were crossed into a fru^{W12} or fru^{sat15} mutant background and balanced over the Blue Balancer.

To express these various *UAS-fru* constructs in the CNS, lines containing *scabrous-GAL4* (*sca-GAL4*) and *embryonic lethal abnormal vision-GAL4* C155 (*elav-GAL4*; Lin and Goodman, 1994, Bloomington Stock Center) transgenes were crossed into a fru^{W12} or fru^{sat15} mutant background. The *scabrous* sequences in the transgenic construct drive pan-neuronal expression of GAL4 from neuroblasts through neurons (Klaes et al., 1994; see below) and the *elav* sequences in this transgenic construct drive expression in post-mitotic neurons (Lin and Goodman, 1994). Crosses between a *GAL4* line and a *UAS-fru* line generated the *fru* mutant embryos in which one of the *fru* isoforms was expressed in the CNS and PNS.

Generation of FRU antibodies

For the GST fusion constructs, coding regions for the *fru* BTB domain and alternative ZnF A and C (Fig. 1) were generated by PCR. Oligonucleotide pairs containing sites for in-frame directional cloning in the pGEX-4T-1 vector (Pharmacia) were designed for BTB and each unique ZnF sequence. DNA was amplified from *fru* cDNA clones (Primer sequences for BTB domain: BTB-1-For: GGG GGA ATT CAT GGA CCA GCA ATT CTG CTT3', BTB-115-Rev: GGG GGC TCG AGC TAG TTG TTA TCT GTG AGA 3'; A form ZnF domain: A-For: 5' CCG GAA TTC CAG CAG CGC CCG CCA CC 3', A-Rev:5'GCC GCT CGA GCG GGA TGG GCT GCA CTT GGG C and those for C form ZnF domain: C-For: 5' CCG GAA TTC CGC GTC AAG TGT TTT AAC ATT AAG C 3', C-Rev: 5' CCG CTC GAG GTT TGC TTG ATT CTT GGT TAC TTA G 3'), digested with EcoRI and XhoI enzymes and cloned. Individual clones were validated by sequencing.

Fusion proteins were purified according to Smith (1993). These fusion proteins were SDS-PAGE purified and not proteolytically cleaved from the GST so that the whole GST-ZINC FINGER or GST–BTB peptides were used as the immunogen. These materials were injected into rats using a 77-day protocol. In brief, 750 μg of purified protein were injected to each animal with complete Freund's adjuvant, followed by three 750 μg immunizations (boosters) using incomplete Freund's adjuvant. Serum was collected by exsanguinations at the end of the protocol. Titer and specificity of the antibodies were assayed by Western blots of the recombinant protein (data not shown). Harvested polyclonal antisera against *fru* BTB domain and A and C form Zn finger domain, hereafter, are named as anti-FRU^{BTB}, anti-FRU^{A}, anti-FRU^{C}, respectively.

Statistics

The frequency of defective embryos in various *fru* mutant and wild-type embryos were analyzed statistically by one-way ANOVA (SAS program version 6.12; SAS Institute) and post-hoc analyzed by Tukey HSD comparisons or by two-sample t-test (SAS program version 6.12).

Results

Isolation and characterization of a new *fru* null mutation

The *AJ96w*$^+$ P-element, which labels a pair of midline neurons in each segment of the embryonic CNS (Spana and Doe, 1996), was mapped molecularly to approximately 3 kb downstream of the 3' end of the *fru* locus (Fig. 2.1; Materials and Methods). Although this P-element would not disrupt *fru* coding sequences, it was possible that it might still affect *fru* function. We examined homozygous *AJ96w*$^+$ males to determine whether they would be only partially male-fertile (Anand et al., 2001; Lee et al., 2001) or form chains of courting male flies (Villella et al., 1997; Anand et al, 2001). In single-pair tests for male fertility, 97% of the tested *AJ96w*$^+$ males were fertile (31/32 males, each paired with 3 virgin Canton-S females). In addition, *AJ96w*$^+$ males did not form male-male courtship chains (6 tests of 8 males each; total n=48). Therefore, homozygous *AJ96w*$^+$ males do not exhibit *fru* mutant phenotypes.

However, because of its genomic location, we expected that imprecise excisions of the *AJ96w*$^+$ P-element would lead to lesions in *fru* coding sequences and generate strong *fru* mutations. One line, *Df(3R)AJ96*^{U3} (*fru*^{AJ96u3}), out of 56 *w*$^-$ revertant lines, was shown by molecular mapping to remove all *fru* common coding sequences because sequences from within the *AJ96w*$^+$ P-element to sequences between the P3 and P4 promoters were deleted (Fig. 2.1; Materials and Methods). Only one putative ORF has been detected between the 3' end of the *fru* locus and the *AJ96w*$^+$ P-element (29144-27188 nts, ACC# AE003722; FlyBase, 1999).

Phenotypic analysis confirmed that *fru*^{AJ96u3} is a *fru* null allele. The phenotypes of transheterozygotes for *Df(3R)fru*^{AJ96u3} and other *fru* alleles were determined with respect to male and female viability, lethal phase and defects in external morphology (Table 2.1). *fru*^{AJ96u3} transheterozygotes with hypomorphic *fru* alleles, such as *fru*^{4} or *fru*^{440}, were fully viable (Table 2.1A). By comparison, *fru*^{AJ96u3} transheterozygotes and *fru* null alleles, such as *fru*^{sat15}, failed to emerge as adults but reached the pupal stage (Table 2.1C). Aged white prepupal collections were used to determine when various *fru* mutant animals died during

metamorphosis. These *fru* null transheterozygotes, such as fru^{AJ96u3}/fru^{sat15}, died around or just after pupal ecdysis, the same lethal phase as other known *fru* nulls (Table 2.1B, Anand et al. 2001). More than half of the *fru* transheterozygotes with null *fru* alleles, such as fru^{w27}/fru^{AJ96u3}, reached late pupal stages and would survive if dissected out of the pupal case. Adult male and female survivors of fru^{w27} with null *fru* allele, such as fru^{AJ96u3}/fru^{w27}, had visible external defects including the failure to inflate their wings or if inflated held in an outstretched position and malformed leg joints (Table 2.1D), as do other null *fru* mutant combinations (Anand et al., 2001). Male fru^{AJ96u3} transheterozygotes with viable *fru* mutant alleles were sterile (0/18 fru^{440}/fru^{AJ96u3} males; 0/18 fru^{4}/fru^{AJ96u3} males). Therefore, fru^{AJ96u3} behaves as a *fru* null allele and the molecular data indicates that it is the smallest deletion available of the *fru* locus (Fig. 2.1).

The outstretched wing phenotype and low viability of these *fru* mutant genotypes are similar to phenotypes caused by the loss of the *spread* gene (*sprd*, map location 91A5-6; Spradling et al., 1999). We tested whether the $sprd^{05284}$ allele, derived from a P-element insertion, would complement various *fru* alleles. There were no adult $sprd^{05284}/fru^{w24}$ survivors, indicating that large *fru* deficiencies uncover the *sprd* locus (0/36 control siblings). However, $sprd^{05284}$ transheterozygotes with other *fru* alleles whose lesions did not affect neighboring genes were fully viable and had a normal wing phenotype ($sprd^{05284}/fru^{440}$, n=61; $sprd^{05284}/fru^{w12}$, n=30; $sprd^{05284}/fru^{AJ96u3}$, n=24). In addition, when housed together as small groups of males in a food vial, none of these $sprd^{05284}/fru$ males formed male-male courtship chains ($sprd^{05284}/fru^{440}$, n=20; $sprd^{05284}/fru^{w12}$, n=8; $sprd^{05284}/fru^{AJ96u3}$, n=9). These phenotypic and viability analyses indicate that the phenotypes of *fru* escapers are not due to disruption of *sprd* function and support attribution of these mutant phenotypes to the loss of *fru* function (see also, Anand et al., 2001).

Figure 2.1. The molecular map of the *fru* locus

The insertion sites of the fru^2, fru^3, fru^4, fru^{sat} and $AJ96w^+$ P-elements are indicated by triangles (Ito et al., 1996; Ryner et al., 1996; Goodwin et al., 2000; Anand et al, 2001; this paper). The $AJ96w^+$ P-element is located 3 kb from the 3' end of the *fru* locus (this paper). The breakpoints of the inversion alleles fru^1 and fru^{w12} are located on the genomic map. The inversion allele fru^{w27} is more complex with the relevant break somewhere between P2 and P3, since no P2 transcripts are detected by RT-PCR (Anand et al., 2001). The limits of relevant *fru* deficiencies, including the $Df(3R)fru^{AJ96u3}$ described in this paper, are delineated with thick black lines to represent deleted sequences and dashed lines represent breakpoints mapped to the relevant restriction fragment (Anand et al, 2001; this paper). *fru* exons are positioned on the genomic map. Four promoters (P1-P4) are distributed throughout the locus and the alternative 3' ends (A, B, C). Each 3' end encodes a different pair of ZnF domains (Goodwin et al., 2000; Ryner et al., in preparation; see also Usui-Aoki et al., 2000). The P1 promoter produces primary transcripts that are spliced female-specifically by the TRA and TRA-2 proteins binding to three repeats located in the S exon. These transcripts also contain the BTB domain and other common exons (C1-5) and are spliced alternatively to one of the three different 3' ends (Ryner et al., 1996; Usui-Aoki et al., 2000; Goodwin et al., 2000). Transcripts derived from the other three promoters P2, P3, and P4 contain the BTB and common exons but it is not known whether these transcripts utilize all of the possible alternative 3' ends. Therefore, the full extent of transcript complexity from these promoters is not known (Ryner et al., 1996; Usui-Aoki et al., 2000; Anand et al., 2001; Ryner et al., in preparation).

Figure 2.1.

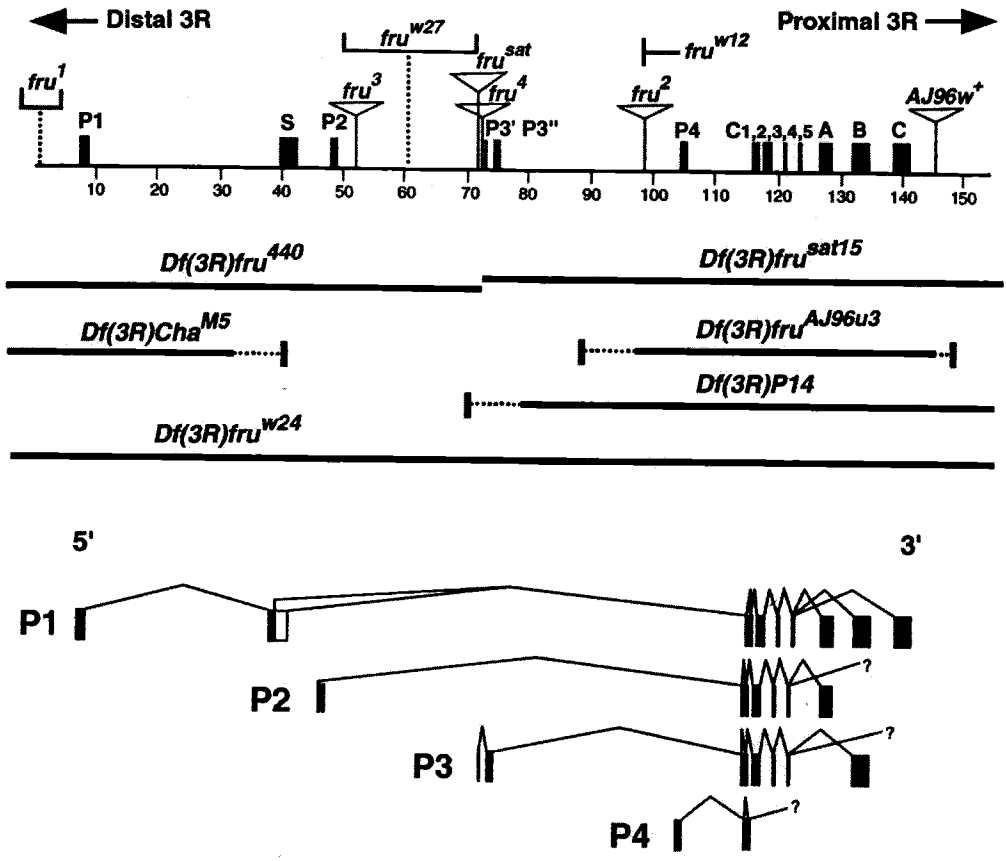

Distal 3R
Proximal 3R
fru1
fruw27
fru3
frusat
fru4
fruw12
fru2
AJ96w+
P1
S
P2
P3' P3"
P4
C1,2,3,4,5
A
B
C
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
Df(3R)fru440
Df(3R)frusat15
Df(3R)ChaM5
Df(3R)fruAJ96u3
Df(3R)P14
Df(3R)fruw24
5'
3'
P1
P2
P3
P4
?

Table 2.1. Mutant phenotypes of *fru*^{AJ96u3} homozygotes and heterozygotes with other *fru* alleles.

Standard crosses were made to determine the viability of various *fru*/*fru*^{AJ96u3} transheterozygotes; in three of the crosses the transheterozygotes were fully viable since their number was within the Mendelian expectations for the cross. In order to determine the lethal phase during pupal development, animals were collected as white prepupae and aged until they no longer appeared to develop. The stage at which they ceased development was assessed by reference to the metamorphic stages described in Bainbridge and Bownes (1981). To simplify presentation of this table, some stages were grouped together. In some genotypes, a small number of animals, in parentheses, emerged on their own. The phenotype of adult escapers was determined for *fru*^{w27}/*fru*^{AJ96u3} animals that were dissected out of the pupal case.

* The number of adult survivors that eclosed on their own.

@ This phenotype was scored as positive if one uneverted was found per animal.

Only wings that were inflated were scored for their position with respect to the body.

** Only the anterior scutellar and the sternopleural bristles in the thorax were obviously duplicated.

N; Total number of transheterozygotes scored.

Table 2.1. Mutant phenotypes of fru^{AJ96u3} homozygotes and heterozygotes with other *fru* alleles.

A. Adult viability of $Df(3R)AJ96^{U3}$ transheterozygotes with representative *fru* alleles.

Genotype	Number of adult transheterozygotes/total progeny	Genotype	Number of adult transheterozygotes/total progeny
fru^{4}/fru^{AJ96u3}	99 (305)	fru^{440}/fru^{AJ96u3}	161 (432)
Cha^{M5}/fru^{AJ96u3}	146 (379)	fru^{w12}/fru^{AJ96u3}	0 (644)

B. Lethal phase of fru^{AJ96u3} transheterozygotes with *fru* null alleles.

Genotype	N	%Brown Prepupa (St 2/P3)	% Bubble stage (St P4i/P4ii)	% Pupal Ecdysis (St P5)
$fru^{AJ96u3}/fru^{AJ96u3}$	72	4	96	
fru^{sat15}/fru^{AJ96u3}	50		100	
$P14/fru^{AJ96u3}$	113	40	60	
fru^{w24}/fru^{AJ96u3}	109		39	61

C. Lethal phase of fru^{AJ96u3} transheterozygotes with strong *fru* alleles.

Genotype	N	% Pre-pupal (St P2-P4)	% Mid-pupal (StP5-P13)	% Late pupal (St P14-P15ii)	% Adult with dissection
fru^{w12}/fru^{AJ96u3}	381	1	34	9	56
fru^{w27}/fru^{AJ96u3}	60	12	10	22	53 (3*)

Table 2.1. Continued

D. External phenotypes of fru^{AJ96u3} transheterozygote adult escapers.

Genotype	% Uneverted discs@		% Uninflated wing		%70-90^0 wing position#		% defective Leg joint		% Duplicated bristles**	
	M	F	M	F	M	F	M	F	M	F
fru^{w27}/fru^{AJ96u3} (M=18, F=11)	100	63	89	63	38	100	63	72	100	100

Table 2.2. The temporal and spatial distribution of *fru* mRNAs and FRU proteins in embryos.

A) Wild-type (CS) and *fru* mutant (Null, homozygous fru^{w24} or fru^{sat15}) embryos were hybridized with digoxigenin-labeled antisense riboprobes and visualized with alkaline phosphatase histochemistry. The presence or absence of in situ hybridization signal at different stages is presented. The intensity of the *in situ* hybridization signals is graded by the number of (+) marks in an arbitrary scale; the absence of a detectable signal is indicated by (-). The temporal and spatial patterns in embryos labeled with the antisense BTB and-Com riboprobes were identical and thus data from both probes are combined in the same column. More than 10 labeled embryos were examined for each stage.
B) Wildtype (CS) and *fru* mutant (Null, homozygous fru^{w24} or fru^{sat15}) embryos were labeled with FRU antibodies and the signal visualized with AP histochemistry. FRU antibody signals were intensified with the treatment of biotinylated tyramide. The presence and relative level of FRU protein immunohistochemical signal is indicated by (+) and the absence of a detectable signal by (-).

Table 2.2. The temporal and spatial distribution of *fru* mRNAs and FRU proteins in embryos.

A. *fru* mRNA distribution

	Antisense riboprobes								
	BTB/Com		*fru* 5' end probes				*fru* 3' end probes		
Labeled cell types	CS	Null	P1.S	P2	P3	P4	A	B	C
Preblastoderm stage (st 1-3)	++	++	-	-	+	+	+	+	+
Pole cells (st 4)	++	++	-	-	+	+	+	+	+
Ventral & cephalic furrow (st 5-6)	++	-	-	-	+	+	+	+	+
Mesectoderm (st 7-8)	++	-	-	-	+	+	+	+	+
Neuroectoderm (st 9)	++	-	-	-	+	+	+	+	+
Neuroblast (st 9-10)	++	-	-	-	+	+	+	+	+
Tracheal placodes (st 9-10)	++	-	-	-	+	+	-	-	+
Amnioserosa (st 9-10)	++	-	-	-	+	-	-	-	+
PNS (st 11-16)	-	-	-	-	-	-	-	-	-
CNS (st 11)	++	-	-	-	+	+	+	+	+
CNS (st 12-13)	++	-	-	-	+	+	+	+	+
CNS (st 14-16)	+/-	-	-	-	-	+	-	-	-

able 2.2. Continued

B. FRU protein distribution

	Antibodies							
	FRU^{BTB}		FRU^{COM}		FRU^{A}		FRU^{C}	
Labeled cell types	CS	Null	CS	Null	CS	Null	CS	Null
Preblastoderm stage (st 1-3)	+	+	+	+	+	+	+	+
Pole cells (st 4)	+	+	+	+	+	+	+	+
Ventral & cephalic furrow (st 5-6)	+	-	+	-	+	-	+	-
Mesectoderm (st 7-8)	+	-	+	-	+	-	+	-
Neuroectoderm (st 9)	-	-	-	-	-	-	-	-
Neuroblasts (st 9-10)	+	-	+	-	+	-	+	-
GMCs (st 10)	+	-	+	-	+	-	+	-
Tracheal placodes (st 10)	+	-	+	-	-	-	+	-
Amnioserosa (st 9-10)	+	-	+	-	-	-	+	-
Midline cells (st 9-11)	+	-	+	-	+	-	+	-
Neurons (st 11/12)	+/+	-	+/+	-	+/-	-	+/-	-
CNS neurons (st 13-16)	+	-	+	-	+	-	+	-
PNS neurons (st 13-16)	+	-	+	-	+	-	+	-
CNS Glia (st 13-16)	+	-	+	-	+	-	+	-
PNS Glia (st 13-16)	+	-	+	-	+	-	+	-
Posterior epidermal cell (st 13-16)	+	-	+	-	-	-	+	-
Muscle (st 14-16)	+	-	+	-	-	-	+	-

Figure 2.2. The temporal and spatial distribution of *fru* mRNA and FRU protein during embryogenesis.

In situ hybridization was carried out with digoxigenin-labeled antisense fru^{BTB} riboprobes and AP histochemistry (A-D). FRU protein distribution was determined by immunohistochemical localization of anti-FRU^{BTB} followed by AP histochemistry (E-I).
A) Stage 4 wildtype embryo. *fru* mRNA is found in pole cells (arrow).
B) Stage 8 wildtype embryo. Mesectodermal cells (arrow) are strongly labeled for *fru* mRNA.
C) Stage 11 *fru* null (fru^{w24}/fru^{w24}) embryo. No *fru* mRNA was detected in any cell types at this stage.
D) Stage 11 wildtype embryo. Medial neuroblasts, but not more lateral ones, express *fru* mRNAs. Cells surrounding the tracheal pits are weakly labeled (arrow).
E) Stage 9 wildtype embryo. Cephalic regions (white asterisk) and two rows of mesectodermal cells (arrow, see Fig 2B) express FRU proteins.
F) Stage 14 *fru* null embryo (fru^{w24}/fru^{w24}). No FRU protein was detected in any cell types at this stage.
G) Late stage 9 wildtype embryo. FRU proteins are found in three columns of neuroblasts: S1 neuroblasts (l, lateral column and m, medial column) and S2 neuroblasts (I, intermediate column).
H) Stage 10 wildtype embryo. FRU protein is detected throughout in the CNS and appears to be in most or all GMCs (white arrow) and is also more strongly expressed in anterior half of the segment.
I) Stage 11 wildtype embryo. FRU protein is expressed in a large number of cells in the nervous system. There is slightly stronger expression in many medial neurons, anterior neurons and cells along the midline cells. The size of cells (white arrow) and their relative positions indicate that they are likely to be neurons.
A-C, E are whole mount preparations, F-L are filleted embryo preparations. Anterior to the left (A-E) or to the top (F-I). White bar (G-I) indicates the ventral midline.
Magnification bars 20μm (panels G-I, same magnification).

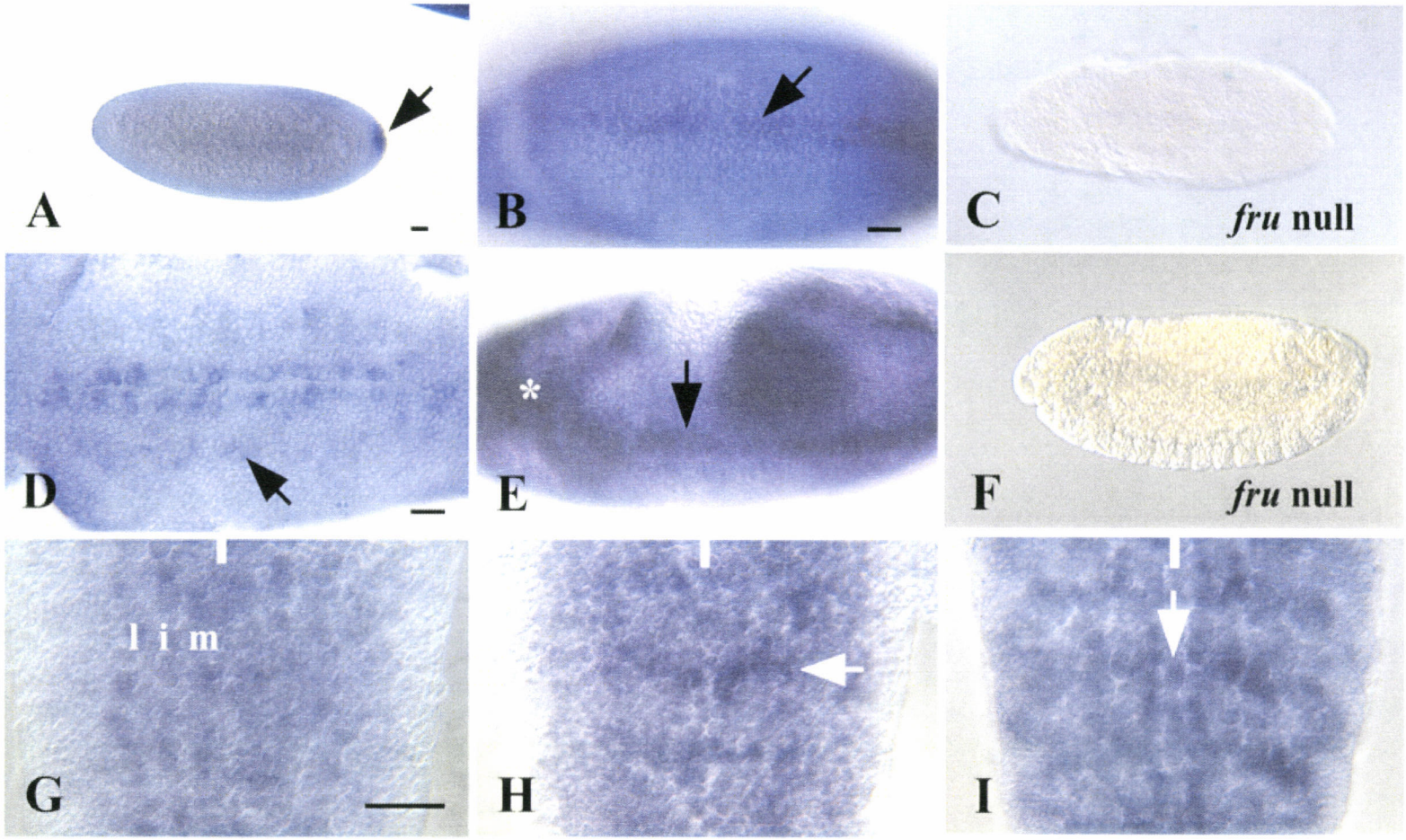


Figure 2.2.

Temporal and spatial distribution of *fru* mRNA during embryogenesis.

To determine the spatial and temporal distribution of *fru* transcripts in embryos, we performed *in situ* hybridizations with antisense-BTB and antisense-Com riboprobes, which detect most or all *fru* transcripts (Ryner et al., 1996; Goodwin et al., 2000; Lee et al., 2000). We found that *fru* mRNAs are expressed in a dynamic temporal and spatial pattern from the beginning of embryogenesis until stage 16 (Fig. 2.2; Table 2.2A). *fru* transcripts are uniformly distributed in very early embryos and become incorporated into the pole cells (st 1-5; Fig 2.2A). At the start of gastrulation (st 6), the cells forming the ventral and cephalic furrows were the most heavily labeled cells in the embryo. In slightly older embryos (st 7-8; Fig. 2.2B), the most prominent distribution of *fru* transcripts is found in the developing CNS and is initially seen as labeled cells in the mesectoderm and ventral neuroectoderm. *fru* transcripts are present in the delaminating neuroblasts, and continue to be expressed in medial neuroblasts (st 9-11) but not in lateral neuroblasts, since *fru* signal in these neuroblasts declines around stage 10 (Fig. 2.2D). After stage 12, the level of *fru* expression in these tissues declined until stage 16 when it became undetectable (Table 2.2A). The cells of the amnioserosa and the tracheal placodes also expressed *fru* transcripts (st 9-11; Table 2.2A), but no *in situ* hybridization signal was detected in other tissues, such as the PNS or body wall muscles. Thus, *fru* transcripts are expressed throughout the development and early differentiation of the CNS but become undetectable at later stages.

The *fru* locus encodes multiple transcripts that are derived from four promoters and have alternatively spliced 3' ends. To better define the embryonic *fru* transcription pattern, we carried out *in situ* hybridization using riboprobes derived from *fru* 5' end, which detect sequences from transcripts made from the P1, P2, P3 or P4 promoters, and riboprobes to *fru* 3' end, which detect transcripts containing the A, B, or C ends (Materials and Methods; Ryner et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000).

When riboprobes specific to P1 and P2 transcripts were used, neither P1 nor P2 transcripts were detected by *in situ* hybridization in embryos (Table 2.2A). The P1 expression result is consistent with the findings of Lee et al. (2000). The

temporal and spatial distribution of P3 transcripts on using riboprobes specific to P3 transcripts was very similar to the distribution of *fru* transcripts labeled by antisense-BTB or -Com riboprobes. The expression pattern in the developing CNS began with labeling of the mesectoderm and neuroectoderm followed by labeling of neuroblasts (Table 2.2A). At slightly later times (st 9-11), medial neuroblasts continued to express P3 transcripts, but lateral neuroblasts no longer had detectable transcripts. Overall, P3 transcript levels declined and became undetectable in all tissues after stage 12. When riboprobes specific to P4 transcripts were used, we found that P4 transcripts were present in the developing CNS in a pattern similar to that found for P3 transcripts, but the signal appeared to be less intense suggesting that the level of P4 transcripts was lower than P3 transcripts for stages 7-12. However, P4 transcripts were still detectable at stage 16, a time when P3 transcripts were undetectable. In summary, the *in situ* hybridization data suggest that both P3 and P4 *fru* transcripts are expressed likely within the same cells in the developing CNS. Furthermore, one explanation for the higher level of *fru* transcripts detected with riboprobes to *fru* common sequences at earlier stages in the developing CNS (st 9-12) is that both P3 and P4 transcripts are present while the lower level of transcripts detected at later embryonic stages (st 12-16) reflects the presence of only P4 transcripts.

Additional complexity in *fru* transcripts is due to alternative splicing at the 3' ends to generate transcripts that contain one of three different pairs of Zn finger domains (Ryner et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000). While it is known that P1 transcript isoforms are spliced to each of the alternative 3' ends (Goodwin et al., 2000), the full complexity of the 3' alternative splicing of transcripts produced from the P2, P3 and P4 promoters is not known. We examined embryos by *in situ* hybridization with three different riboprobes to detect transcripts having the A, B or C 3' ends. Overall, the temporal and spatial expression pattern of transcripts containing the A, B and C 3' ends was consistent with the pattern found for *fru* transcripts labeled with antisense- BTB and – antisense Com riboprobes (Table 2.2A). In the developing CNS, transcripts containing the C 3' end appeared to be more abundant than those containing the A

or B 3' ends (data not shown). In addition, some tissues, such as the tracheal placodes and amnioserosal cells, were labeled with only the C 3' end containing transcripts (Table 2.2A).

In order to confirm that we were detecting authentic *fru* transcripts, *fru* mutant embryos, fru^{w24}/fru^{w24} and fru^{sat15}/fru^{sat15}, in which the coding regions of the *fru* gene are deleted, were labeled with the antisense-BTB, antisense -Com and -A 3' end riboprobes. At the beginning of embryogenesis, we only detected transcripts in these null mutant embryos up to stage 5; later stage embryos were not labeled (Fig. 2.2C). By contrast, *fru* transcripts in wild-type embryos are present up to stage 16 (Table 2.2A). From these results, we infer that there are two sources of *fru* transcripts. The presence of the transcripts in very early mutant and wildtype embryos suggest that these may have a maternal source, whereas the transcripts found only in older wildtype embryos suggest that these have an embryonic source. The presence of maternal and zygotic *fru* transcripts was consistent with the results of FRU protein immunohistochemistry (see below).

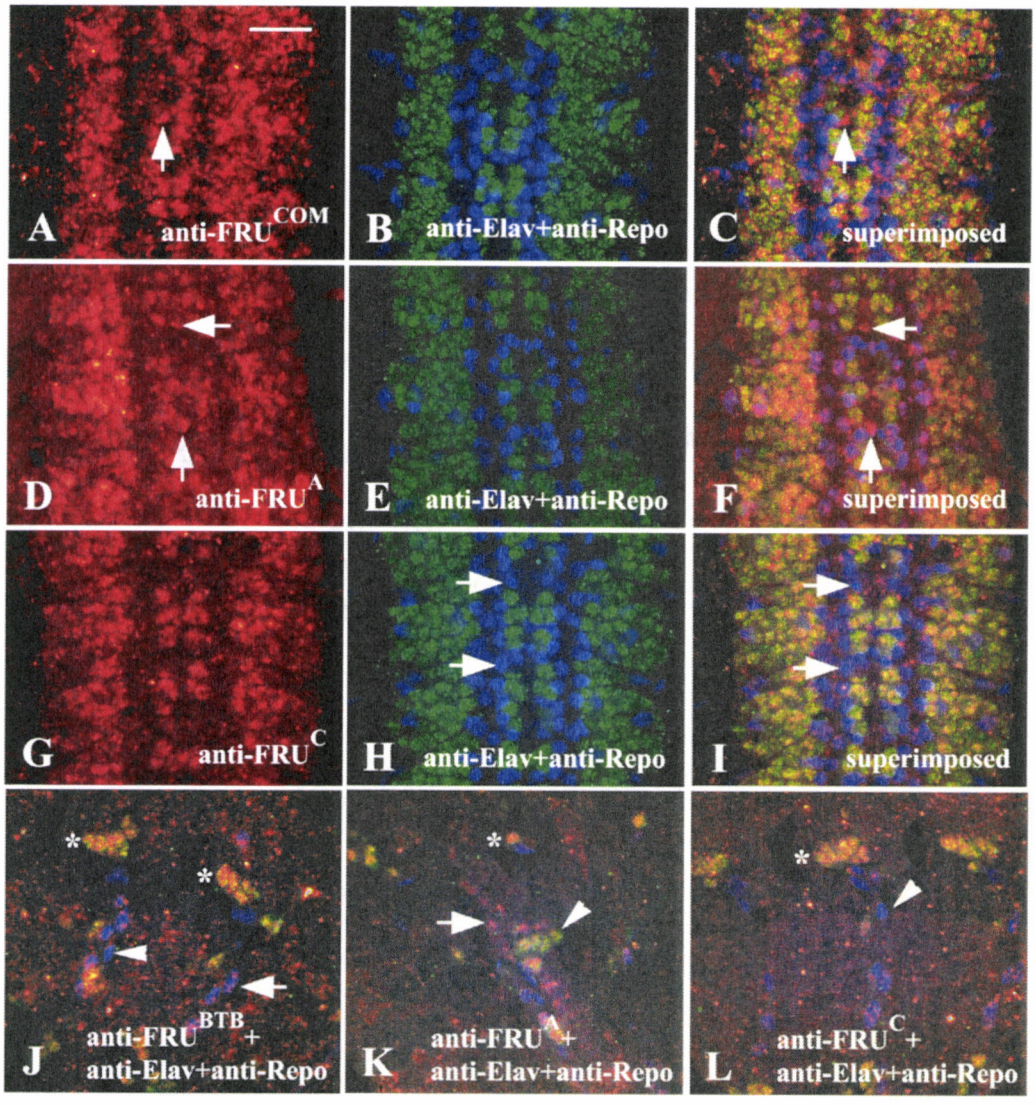
<u>The temporal and spatial expression of FRU protein during embryogenesis</u>

To determine whether the *fru* transcripts present during embryogenesis are translated, embryos were labeled with antibodies directed against the common coding regions of FRU proteins (anti-FRU^{COM} and anti-FRU^{BTB}) and against the class A 3' end (anti-FRU^{A}) and the class C 3' end (anti-FRU^{C}). Our expectation was that FRU proteins would be present in multiple cell types in the developing CNS, and we find that the localization of FRU proteins in the embryo is consistent with the spatial and temporal RNA distribution described above (Table 2.2). Before neuroblast delamination, mesectodermal cells (st 9), but not neuroectodermal cells, were labeled with both anti-FRU^{COM} and anti-FRU^{BTB} (Fig. 2.2E; Table 2B). All neuroblasts, as they delaminate, and their progeny express FRU proteins (st 9-10; Fig. 2.2G). GMCs located in the anterior regions of each segment label more strongly for FRU proteins than those in the posterior regions (st 10; Fig. 2.3H). At later stages (st 11-16), many cells in the CNS were strongly labeled (Fig. 2.3I).

Figure 2.3. FRU proteins are expressed in all neurons and lateral and midline glia.

All panels are from filleted wildtype embryos (st 16), which were triple labeled for FRU (red), the neuronal marker ELAV (green) and the glia marker REPO (blue). Panels A-C, D-F and G-I are single confocal images in a dorsal CNS focal plane merged in Adobe Photoshop. Panels J-L are composite merged images of the lateral body wall from embryos stained as above.
A) CNS cells are labeled with anti-FRU^{COM} antibody. The arrow points to a FRU-positive midline cell.
B) CNS cells labeled with anti-ELAV and anti-REPO antibodies.
C) In this superimposed image of 3A and 3B, all neurons (yellow) and lateral glia (purple) co-label with FRU protein. The arrow points to a FRU-positive midline cell.
D) CNS cells labeled with anti-FRU^{A} antibody.
E) CNS cells labeled with anti-ELAV and anti-REPO antibodies.
F) Most neurons (yellow) and glia (purple) co-label for FRU^{A} isoforms. The arrows point to midline cells that are only FRU positive.
G) CNS cells labeled with anti- FRU^{C} antibody.
H) CNS cells labeled with anti-ELAV and anti-REPO antibodies.
I) Most neurons (yellow) but few glia (purple, arrows in H, I) co-label for FRU^{C} isoforms.
J) All peripheral sensory neurons (*, yellow) are colabeled with anti-ELAV and anti-FRU^{BTB} but some glia cells are FRU positive (arrow, purple) and others are FRU-negative (arrowhead, blue). The level of FRU expression is low in some sensory neurons and glial cells.
K) A few peripheral sensory neurons (arrowhead, yellow) but many glia cells (arrow, purple) co-label for FRU^{A} isoforms.
L) Many peripheral sensory neurons (asterisk, yellow) but only a few glia cells (arrowhead, purple) express FRU^{C} isoforms.
Anterior is up for A-I and to the right for J-L. Magnification bar is 20μm.

Figure 2.3.


A
anti-FRU COM
B
anti-Elav+anti-Repo
C
superimposed
D
anti-FRU A
E
anti-Elav+anti-Repo
F
superimposed
G
anti-FRU C
H
anti-Elav+anti-Repo
I
superimposed
J
anti-FRU BTB +
anti-Elav+anti-Repo
K
anti-FRU A +
anti-Elav+anti-Repo
L
anti-FRU C +
anti-Elav+anti-Repo

To identify the FRU-positive cell types in stage 13-16 CNSs, we carried out double- and triple-labeling experiments to co-localize neuronal and glial markers. FRU-positive neurons were identified by co-expression of ELAV protein and FRU-positive lateral glia by co-expression of REPO protein (Figs 2.3A, 2.3B). From a detailed comparison of double (n= 5) and triple labeled (n=3) CNSs, it appears that all ELAV-positive cells co-express FRU proteins, even though the level of FRU protein expression appears variable in individual cells (Fig. 2.3C). All REPO-positive cells were also observed to be FRU-positive (n=5, Fig. 2.3C). However, some cells, predominantly along the midline, were only FRU-positive (arrow, Fig. 2.3C). By their location, it is likely that these cells are midline glia, which do not express REPO protein (Halter et al., 1995). In summary, all neurons and glia appear to express FRU proteins.

We used antibodies specific to individual carboxy termini to determine whether FRU proteins in embryos have either the A or the C ZnF terminus or whether FRU proteins with both types of ZnF domains co-exist in the same cell types (Fig. 2.3D, 3G, 3K, 3L). The pattern of cells labeled with anti-FRU^{A} is very similar to the spatial and temporal pattern of cells detected with anti-FRU^{COM} and anti-FRU^{BTB} (Table 2.2B). In contrast, in young embryos (st 1-11) the anti-FRU^{C} labeling pattern is similar to the pattern of cells stained with anti-FRU^{COM} and anti-FRU^{BTB}, whereas at later stages (st 12-16), fewer cells were labeled with anti-FRU^{C} than with anti-FRU^{COM} and anti-FRU^{BTB}. By double- and triple-labeling experiments in stage 16 embryos, all ELAV-positive neurons, REPO-positive lateral glia and midline cells were FRU^{A}-positive (n=4, Fig 2.3D, 3E, 3F). However, all neurons, but only some REPO-positive glia, were FRU^{C}-positive (n=5, Fig 2.3G, 3H, 3I). These results suggest that all neurons and some lateral glia contain FRU isoforms having at least two different 3' ends, but that some lateral glia and midline cells contain FRU proteins that may have only one type of 3' end. Currently, there is not an antibody specific to the B 3' end.

In contrast to the findings by the *in situ* hybridization experiments, FRU proteins were also detected in cells within the PNS by anti-FRU^{BTB}, anti-FRU^{COM}, anti-FRU^{A} and anti-FRU^{C} immunohistochemistry (Fig. 2.3J, 3K, 3L). PNS cells

were identified by use of anti-ELAV and whether these expressed FRU proteins was ascertained by double- and triple-labeling experiments with anti-FRU^{BTB} and anti-FRU^{COM} antibodies. By their location, FRU expressing PNS cells are external sense organs, neurons of the mechanosensory and chordontonal organs, or are multidendritic neurons. Co-localization with anti-REPO identified other FRU-positive cells as peripheral glial cells (Fig. 2.3J; Table 2B). However, in double- and triple- labeling experiments, only a subset of the FRU-positive sensory neurons and peripheral glia were also labeled with anti-FRU^{A} and anti-FRU^{C} (Fig. 2.3K, 3L, Table 2.2B).

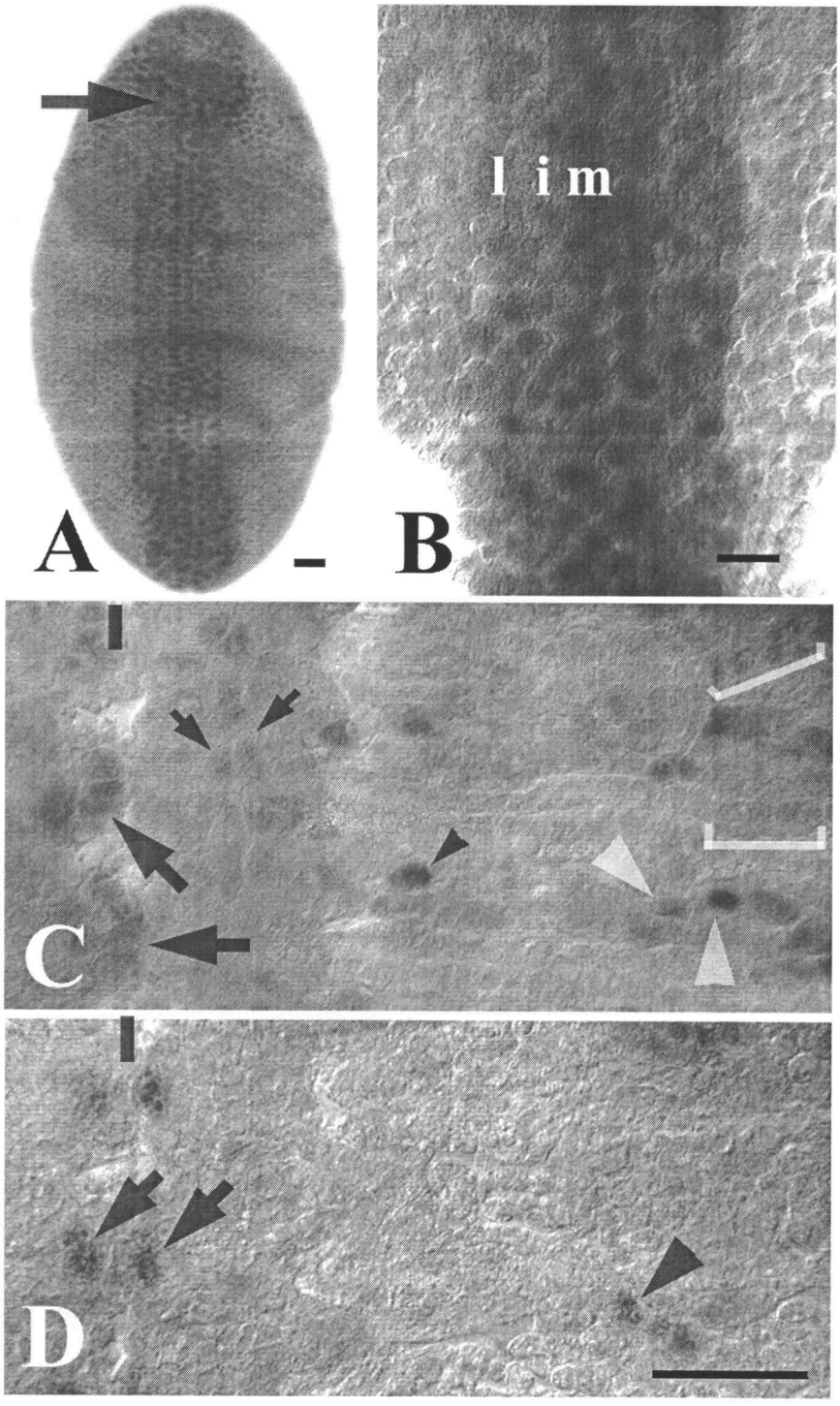
FRU proteins are also expressed in other embryonic tissues. The posterior epidermis (st 13-16) and most body wall muscles (st 14-16, Table 2.2B) were FRU-positive by anti-FRU^{BTB} and anti-FRU^{COM} labeling. These tissues were labeled with anti-FRU^{C} but not with anti-FRU^{A} antibodies (Table 2.2B). These findings suggest that FRU protein isoforms with specific ZnF domains have a tissue-specific pattern in non-CNS tissues. The presence of FRU proteins in cell types that were not labeled in the *in situ* hybridization experiment, such as, the PNS may be due to differences in sensitivity of the molecular probes used and/or the relative levels of *fru* transcripts and proteins in these cell types.

To be certain that authentic FRU proteins were being labeled in wild-type embryos, *fru* mutant embryos, fru^{w24}/fru^{w24} and fru^{sat15}/fru^{sat15}, which are deleted for the *fru* gene were labeled with anti-FRU^{BTB} and anti-FRU^{COM} antibody. At early embryonic stages (st 1-5), the pattern of FRU protein expression in null mutant embryos was comparable to wild-type embryos. At mid to later stages (st 6-16), no FRU protein was detected in null mutant embryos, even though there is still robust labeling in wild-type embryos (Fig. 2.2F). These results indicate that the earliest transcripts present, which we infer to be maternal in origin, are translated in embryos and that FRU proteins in stage 6-16 embryos are derived from zygotic *fru* transcripts.

Figure 2.4. β-galactosidase expression pattern in *fru* P-element mutant embryos.

A) Whole mount *fru*^{4} embryo (stage 9, ventral view). Cephalic (arrow) and ventral neuroblasts strongly express β-galactosidase.
B) Filleted *fru*^{4} embryo (early stage10, dorsal view). All ventral neuroblasts, including S1 neuroblasts (l, lateral column and m, medial column) and SII neuroblasts (i, intermediate column), are uniformly labeled.
C) Filleted *fru*^{4} embryo (stage 13, composite of dorsal to ventral views to show ventral nerve cord and lateral tissues). At later stages, the expression pattern in the ventral nerve cord shifts to a small number of midline cells (large arrows) and lateral cells, which are likely to be both, glia (small arrows) and neurons based on the focal plane. Sensory neurons (white arrowheads), peripheral glia (small black arrowhead), and epidermis (white brackets) also strongly express β-galactosidase.
D) Filleted *AJ96w*$^+$embryo (stage 13). Two midline neurons, dMP2 and vMP2, are strongly labeled (arrows) as are sensory neurons (arrowhead).
Black bar (C, D) indicates the ventral midline of embryo. Anterior is up.
Magnification bars 20 μm (panels C-D same magnification).

Figure 2.4



Neurons in the embryonic CNS and PNS are labeled in *fru* P-element lines.

To determine whether β-galactosidase expression pattern of *P*-element inserts into or near *fru* locus may reflect expression pattern of *fru* gene, we examined fru^3, fru^4 and fru^{sat} mutant and $AJ96w^+$ embryos (Fig. 2.1; Castrillon et al, 1993; Ito et al, 1996; Spana and Doe, 1996). In three *fru* mutant alleles, fru^3, fru^4 and fru^{sat}, a P-element is inserted into the genomic region downstream of the P1 and P2 promoters and upstream of the P3 promoter (Fig. 2.1; Castrillon et al, 1993; Ryner et al 1996, Ito et al., 1996, Goodwin et al., 2000) and consequently lead to alteration of the pattern of sex-specific and sex-nonspecific *fru* transcripts in pharate adult animals (Goodwin et al., 2000). Since the P-elements in these *fru* mutants were located in close proximity to the P2 and P3 promoters, we expected that β-galactosidase expression from these P-elements might assess the *fru* expression patterns in embryos.

In both fru^3, fru^4 and fru^{sat} mutant and control sibling embryos, cells in the CNS and PNS were labeled by antibody β-galactosidase. Similar temporal and spatial labeling patterns were observed in embryos from the fru^3 and fru^4 lines. In embryos from these lines, anti-β-galactosidase expression was first detected in the mesectoderm of early post-gastrula embryos (st 8). At later stages, delaminating neuroblasts were uniformly labeled (st 9; Fig. 2.4A, 4B), followed by labeling in a variety of smaller cells, which appear by their location to be GMCs (st 10), neurons (st 11), and lateral glia and midline cells (st12; Fig. 2.4C). β-galactosidase labeling persisted in these CNS cells until stage 16. In the PNS of embryos from the fru^3 and fru^4 lines, sensory organ precursors (st 10) and later their progeny, the external and chordotonal sensory neurons (st 12 - 16), were β-galactosidase positive (Fig. 2C). In addition, tracheal cells (st 11) and epidermal cells in the posterior part of each hemisegment (st 13-16) were labeled (Fig. 2.4C). By contrast, in embryos from the fru^{sat} line, no β-galactosidase-positive cells were found in the embryonic CNS, and only the chordotonal neurons in the PNS were labeled (data not shown). In summary, these results indicate that β-galactosidase expression from these P-elements somewhat reflects *fru*'s pattern expressed widely in the developing CNS and PNS during embryogenesis.

We also examined the expression pattern of the enhancer trap line *AJ96w*$^+$. Previous studies have shown that the MP2 neuroblast and its progeny, the dorsal and ventral midline precursor neurons (dMP2 and vMP2), express β-galactosidase in the *AJ96w*$^+$ line (Spana and Doe, 1996). The ventral CNS also showed very weak generalized neuronal expression from stage 14 to 16 and expression in a few lateral sensory neurons (Fig. 2.4D). The generalized ventral nerve cord and sensory neuron labeling in embryos of the *AJ96w*$^+$ line is similar to the pattern of expression in the *fru*^{3} and *fru*^{4} lines, but these two *fru* lines did not show prominent MP2 or dMP2/vMP2 neuronal staining.

fru function is required for formation of longitudinal tracts within the CNS.

The finding that the *fru* gene was expressed in the embryonic CNS led us to examine *fru* mutant animals to determine whether *fru* plays a role in CNS development. We used two antibodies, anti-FasII and mAb BP102, to assay specific and general axonal projections within the CNS of selected *fru* mutant genotypes. FasII is a neural adhesion molecule expressed on the cell surface of axons forming specific longitudinal fascicles or tracts running throughout the entire ventral nerve cord and into the brain (Fig. 2.5A; Grenningloh et al., 1991; Lin et al., 1994; Goodman and Doe, 1993; Hidalgo and Brand, 1997). In whole-mount preparations of stage 15-17 wild-type embryos, three tracts are visible. These tracts have been named for the neurons that pioneer each tract: the medial tract is identified as the pCC/MP2 pathway; the middle tract is pioneered by the MP1 neurons; and the lateral tract for which the pioneering neuron is unknown (Hidalgo and Brand, 1997; Hidalgo and Booth, 2000).

In *fru* null mutant embryos (*fru*^{sat15}/*fru*^{w24}, *fru*^{sat15}/ *fru*^{AJ96u3}, and *fru*^{AJ96u3}/*P14*, and *P14*/*fru*^{w24}) a significant number of stage 15/16 embryos, ranging from 12-25% depending on the *fru* genotype, had disruptions in the normal orderly pattern of FasII-expressing tracts (Fig. 2.5B, 5C; Table 2.3D). By comparison, wild-type embryos with disrupted FasII tracts were rare (<3%, Table 2.3A). In the most common disruption of the axonal pattern, the FasII tracts were no longer distinct, suggesting that FasII-positive axons have defasciculated, but this

defasciculation was usually confined to one or a few adjacent hemisegments. In some cases, axons that had defasciculated crossed and joined an adjacent fascicle or approached and crossed the midline (Fig. 2.5C). In other cases, the bilaterally symmetric medial tracts appeared to merge along the midline (data not shown). The frequency and severity of the defects in *fru* mutant embryos was the same in large and small deficiencies (e.g., $P14/fru^{w24}$ compared to fru^{sat15}/fru^{w24} and fru^{sat15}/fru^{AJ96u3}; Table 2.3D, see deficiency map in Fig. 2.1). Since fru^{sat15}/fru^{AJ96u3} mutants lack *fru* and potentially one other ORF, these results suggest that either fru^{+} or the other ORF functions in the proper differentiation of FasII-expressing neurons.

To determine whether these FasII axonal defects depended on the loss of *fru* function, we used the fru^{w12} allele, which has a chromosomal break within the *fru* locus, in combination with *fru* deletion mutations (Fig. 2.1, Table 2.3C). We found that 15% to 23% of these mutant embryos had hemisegments with defects in FasII-labeled fascicles similar to those of the *fru* null embryos (Figure 2.5B, 2.5C). We examined fru^{w12}/DfXD198 mutants, in which the other inversion breakpoint of fru^{w12} is uncovered and found that the pattern of FasII fascicles was wildtype (n=10; data not shown). These results show that it is the loss of *fru* function that causes the axonal defects in FasII fascicles (Table 2.3C, 3D). Since the *fru* locus chromosomal break in the fru^{w12} allele separates the P1, P2 and P3 promoters from the *fru* coding region but allows P4 *fru* transcripts to be made, we infer that the defective FasII axonal pattern is most likely due to the loss of P1, P2 or P3 *fru* transcripts (Anand et al., 2001).

To further define which *fru* transcripts are required for the formation of FasII tracts, we examined *fru* mutants lacking subsets of P1, P2 and P3 *fru* transcripts. The FasII pattern was wild-type in mutants in which only the P1 transcripts are affected, fru^{1}/fru^{w24} and Cha^{M5}/fru^{sat15} (Table 2.3B; Anand et al., 2001; LC Ryner, unpublished observation). Likewise, the FasII pattern was wildtype in mutants in which P1 and P2 transcripts are eliminated, fru^{440}/fru^{sat15} and $fru^{440}/P14$ (Table 2.3B; Anand et al., 2001). Thus, embryos that express P3 and P4 or just P3 *fru* transcripts have wild-type FasII fascicles. In summary, only embryos

in which P3 and/or P4 transcripts are eliminated, such as fru^{w12}/fru^{AJ96u3}, are FasII fascicles defective, suggesting that P3 and/or P4 transcripts are important for axonal differentiation. The lack of embryonic phenotype in mutants lacking P1 and P2 transcripts is consistent with the absence of these transcripts as determined by *in situ* hybridization (Table 2.2A).

To determine whether many or most axonal tracts were disrupted in *fru* mutants, we labeled the longitudinal connectives and commissures with BP102 antibody which can label all longitudinal and commissural tracts of the wildtype embryo CNS (Fig 2.5D; Seeger et al., 1993). Almost 20% of the *fru* null mutant embryos had defects in both the pattern and distribution of BP102-positive axons in the longitudinal connectives and commissures compared to only 1% of wild-type embryos (Table 2.3A, 3D). Most commonly, the longitudinal connectives and commissures were not uniform but were either thicker, as though more axons were present, or thinner, as though fewer axons were present (Fig. 2.5E, 5F; Table 2.3C, 3D). The similarity in the severity and frequency of defects in the BP102 axonal pattern among the *fru* null genotypes (fru^{sat15}/fru^{w24}, $fru^{AJ96u3}/P14$ and fru^{sat15}/fru^{AJ96u3}) and in fru^{w12}/fru^{w24} and fru^{w12}/fru^{sat15} mutant embryos indicates that this phenotype is also due to the loss of *fru* function (Table 2.3D).

In order to determine which *fru* transcripts were needed for the formation of normal BP102 tracts, we examined *fru* mutants that expressed subsets of *fru* transcripts. *fru* mutant embryos that do not express P1 and/or P2 but do express P3 and P4 transcripts (fru^{440}/fru^{sat15}, $fru^{440}/P14$, fru^{1}/fru^{w24} and Cha^{M5}/fru^{sat15}) have wild-type BP102-positive tracts (Table 2.3B, 3C). In summary, the mutant analysis shows that both FasII and BP102-positive axons require P3 and/or P4 *fru* transcripts to form the wild-type pattern of axonal tracts. However, the fact that the frequency of abnormality in both FasII and BP102-positive axons when P3 and P4 transcripts are absent is the same as when just P3 transcripts are absent suggests that *fru* transcripts from P3 are much important than those from P4 to form the wildtype axonal tracts.

Figure 2.5. Axonal defects in the CNS of *fru* mutant embryos.

Filleted wildtype and *fru* mutant embryos were stained for anti-Fas II (A-C) and BP102 (D-F) and visualized using HRP histochemistry, or mAb 22C10 (G-I) using AP histochemistry. Very late stage 16 (A-F) and early stage 13 (G-I) embryos.
A) In a wildtype embryo, three bilaterally symmetric FasII longitudinal fascicles are visible.
B) In a fru^{w12}/fru^{sat15} embryo, all fascicles are disrupted within one segment. Brackets indicate area shown at higher magnification in C.
C) Axons in all three fascicles have defasciculated. Axons in the medial MP1 fascicle appear to extend toward the midline (arrowhead and arrow).
D) In a wildtype embryo, BP102 positive axonal processes form a bilaterally symmetric pair of longitudinal connectives and pairs of anterior and posterior commissures in each segment.
E) In a fru^{w12}/fru^{sat15} embryo, both the longitudinal connectives and commissures are not uniform in size (arrow) suggesting that unequal numbers of axons are present. In some segments, the commissures are missing (bracket).
F) A higher power view of Fig 5E (bracketed area), in which no axons (arrowhead) appear to be crossing the midline.
G) In a wildtype embryo, vMP2 neurons (big arrow) and dMP2 neurons (small arrow) label with mAb 22C10. The vMP2 axon initially projects anteriorly (big arrowhead) and the dMP2 axon projects posteriorly (small arrowhead).).
H) In a fru^{w12}/fru^{sat15} embryo, vMP2 and dMP2 neurons do not express mab22C10 as strongly as wildtype. In some segments, mab22C10 positive neurons were not in their typical anterior-posterior location (*). The white arrowhead indicates two cell bodies with no clear axonal projection. The white arrow points a cell with a posteriorly projecting axonal growth cone.
I) A fru^{AJ96u3}/fru^{sat15} embryo, in which some segments have a nearly normal pattern of dMP2 and vMP2 label (arrows and arrowheads, compare to Fig 5G). However, in other segments, very little mab22C10 (white arrow) staining in these neurons is apparent
Anterior is to the top. Magnification bars 20 μm (A, B, D, E, G, H and I are at the same magnification; C and F are at the same magnification) .

Figure 2.5.

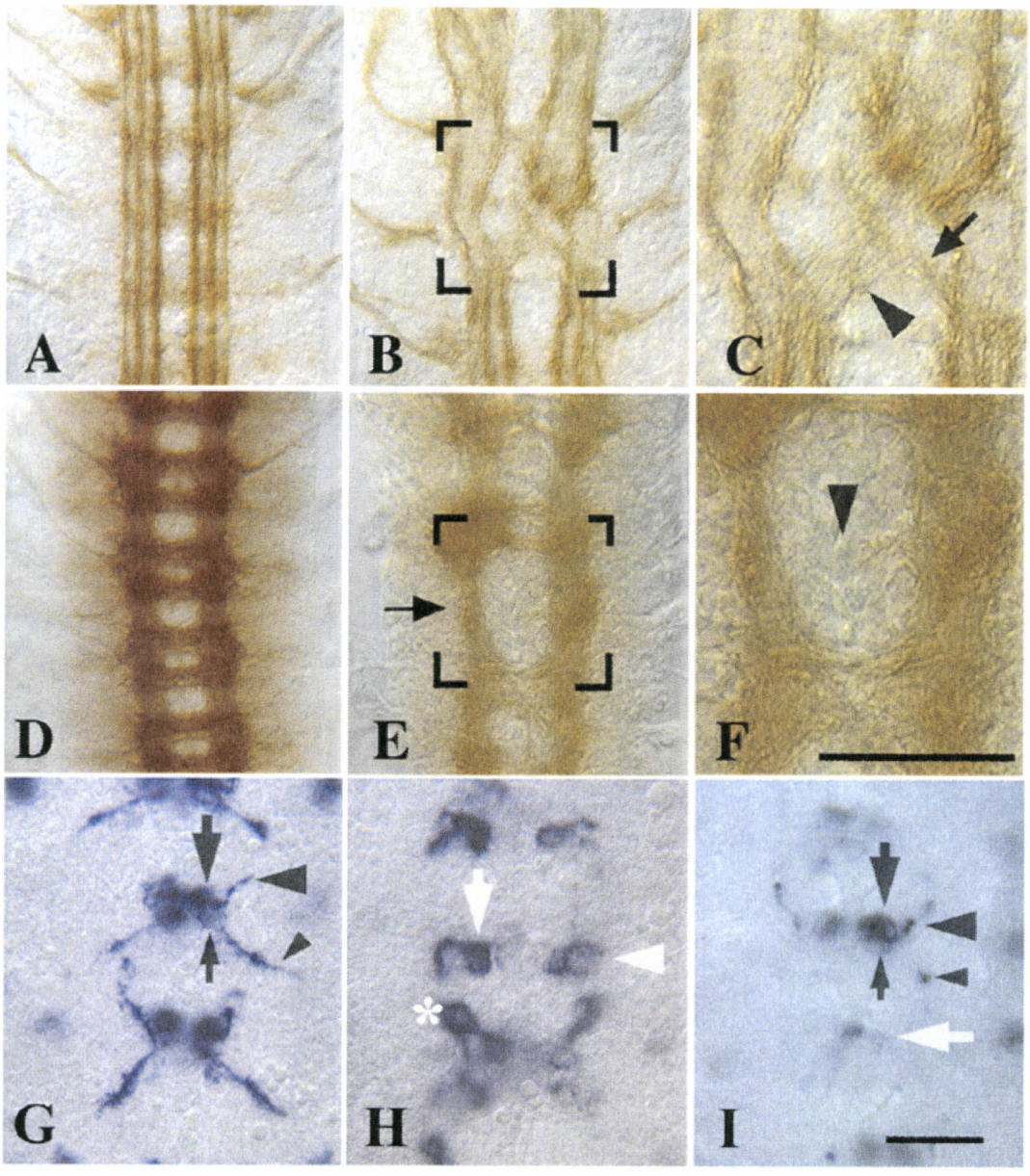


Table 2.3. Analysis of abnormal axonal projections in the CNS of *fru* mutant embryos.

A. fru^{+} genotype

Genotypes	Percent embryos with abnormal Fas II (+) fascicles	Percent embryos with abnormal BP102 label
Wildtype (Canton-S)	3.2 (n=558)	0.7 (n=153)

B. *fru* genotypes with reduced P1or lacking P1 and P2 transcripts but producing P3 and P4 transcripts.

fru^{1}/fru^{w24}	5.2 (n=135)	3.2 (n=124)
Cha^{M5}/fru^{sat15}	2.4 (n=167)	0 (n=72)
fru^{440}/fru^{sat15}	1.6 (n=316)	3.3 (n=159)
$fru^{440}/P14$	2.1 (n =437)	3.6 (n=111)

C. *fru* genotypes lacking P1, P2 and P3 transcripts.

fru^{w12}/fru^{sat15}	15.0 (n=176)*	16.3 (n=151)*
fru^{w12}/fru^{w24}	23.4 (n=259)*	23.9 (n=251)*
$fru^{w12}/P14$	20.5 (n=251)*	ND
fru^{w12}/fru^{AJ96u3}	14.4 (n=227)*	ND

D. *fru* genotypes producing no *fru* transcripts.

fru^{sat15}/fru^{AJ96u3}	15.8 (n=133)*	17.4 (n=190)*
fru^{sat15}/fru^{w24}	12.4 (n=239)*	18.4 (n=137)*
$fru^{AJ96u3}/P14$	24.5 (n=316)*	17.0 (n=106)*
$P14/fru^{w24}$	25.5 (n=263)*	ND

For each genotype, the percentage of stage 15/16 embryos with at least one hemisegment having a defective pattern of FasII or BP102 axonal projections from the total number of embryos examined (n). The percentage represents the average value of two independent labeling experiments. For statistical analysis, one-way ANOVA with genotypes as the main effect revealed significant differences with genotypes labeled with FasII [$F_{12, 1706} = 13.09$, $P < 0.0001$] and within those labeled for BP102 [$F_{9, 1205} = 20.84$, $P < 0.0001$], respectively. Subsequent comparisons (Tukey's HSD) revealed that both FasII staining and BP102 staining in the CNS of *fru* genotypes (identified by *) were significantly different (all Ps < 0.05) compared to wildtype and the viable *fru* genotypes, fru^{1}/fru^{w24}, Cha^{M5}/fru^{sat15}, fru^{440}/fru^{sat15}, and $fru^{440}/P14$. ND is data not determined.

fru function is required for early *futsch* expression in dMP2 and vMP2 pioneer neurons.

Upon finding that FasII tracts in the CNS were abnormal in *fru* mutants, we examined the development of neurons that pioneer these fascicles in *fru* mutants. In the wild-type CNS, the outgrowth of the pCC, vMP2, dMP2, and MP1 axons initiate the FasII longitudinal fascicles (Hidalgo and Booth, 2000). The axonal process of the pioneer neuron vMP2 grows anteriorly toward the descending pCC axon, and the process of the dMP2 axon grows posteriorly along with MP1 axon. These axons initially produce one fascicle at stage 13, which then splits into two fascicles, the pCC/vMP2 (medial) and dMP2/MP1 (intermediate) fascicles. We therefore examined the differentiation of vMP2 and dMP2 neurons in fru^{sat15}/fru^{AJ96u3} and fru^{w12}/fru^{AJ96u3} embryos with mab22C10, which recognizes the *futsch* protein that is required for normal neuronal process outgrowth (Hummel et al., 2000). The vMP2 and dMP2 neurons in only 20-33 % of the hemisegments from late stage 12 fru^{w12}/fru^{AJ96u3} and fru^{sat15}/fru^{AJ96u3} embryos were strongly labeled (Fig. 2.5; 10/50 hemisegments of fru^{w12}/fru^{AJ96u3} embryos; 28/84 hemisegments of fru^{sat15}/fru^{AJ96u3} embryos). All *fru* mutant embryos had hemisegments in which dMP2 and/or vMP2 were not detectable (Fig. 2.5H, 5I; fru^{w12}/fru^{AJ96u3} n=16; fru^{sat15}/fru^{AJ96u3} n=20 embryos) whereas 92% of the wild-type embryos had heavily labeled neurons (Fig. 2.5G; n= 100 hemisegments, 13 embryos). In both fru^{w12}/fru^{AJ96u3} and fru^{sat15}/fru^{AJ96u3} embryos, the cell bodies of dMP2, vMP2 or both neurons were not mab22C10-positive in most hemisegments, even when processed for immunohistochemistry using a more sensitive chromagen, alkaline phosphatase (Fig. 2.5). In order to assess whether fewer dMP2 neurons were present in *fru* mutants, embryos were labeled with anti-*Oddskipped* antibody normally enable to stain cell body of dMP2 neuron, and all segments were shown to have dMP2 neurons in fru^{w12}/fru^{AJ96u3} and fru^{sat15}/fru^{AJ96u3} (n=35 segments, n=5 embryos each genotype; data not shown). The lack of mab22C10 staining in these dMP2/vMP2 pioneer neurons meant that their initial axonal projections could not be assessed. The finding that these pioneer neurons are present in *fru* mutant

embryos suggests that it is not their survival but some aspects of their differentiation that depends on *fru* function.

fru function is not required for lateral and midline glial cell survival.

One of the potential causes for the axonal phenotypes in *fru* mutant embryos could be the loss of normal function in both midline and lateral glial cells which have been shown to be necessary for the formation of normal axonal tracts (Halter et al., 1995; Giesen et al., 1997; Scholz et al., 1997). Since FRU proteins are clearly expressed in both types of glial cells, we examined *fru* mutant embryos to determine if both types of glia were present in the appropriate numbers and locations within the CNS. In fru^{w12}/fru^{sat15} embryos, the distribution and average number of REPO-positive glial cells (44 ± 4 glia/neuromere, n=4) were not different from that in wildtype embryos (45 ± 4 glia/neuromere, n=4; $P > 0.05$, two sample *t*-test). To label midline glial cells, the AA142 enhancer trap P-element was recombined onto the fru^{sat15} and fru^{AJ96u3} chromosomes (Scholz et al., 1997). *fru* mutant embryos had a wild-type pattern of three to four midline glial cells expressing β–galactosidase (n=5 embryos fru^{w12}/ AA142, fru^{AJ96u3}; n=6 AA142, fru^{sat15}/fru^{AJ96u3} embryos). Since wildtype numbers of glia cells are present in *fru* mutants, it does not appear that *fru* is required for the survival of glial cells. Thus the defects in the FasII and BP102 axonal tracts are not due to a loss of midline or lateral glia.

The expression of specific *UAS-fru* transgenes rescues mutant defects in the CNS of *fru* mutant embryos.

Phenotypic analysis of *fru* mutant embryos along with *fru's* temporal and spatial expression pattern indicates that *fru* functions during neuronal differentiation. To positively demonstrate *fru's* role in the CNS, we rescued *fru* mutant defects by the expression of *UAS-fru* transgenes controlled by a pan-neuronal driver, *scabrous* (*sca*)-*GAL4* (Brand and Perrimon, 1993). The expression pattern of the *sca-GAL4* driver line used was confirmed with a *UAS-lacZ* reporter transgene; uniform β-galactosidase expression was found first in the neuroectoderm, followed by

expression in neuroblasts, GMCs and neurons through stage 16 (Klaes et al., 1994; data not shown). This pattern mirrors FRU's expression pattern in the CNS (see above). We generated three different *fru* constructs that all encode the same BTB and Common sequences but differ in which of the three 3' ZnF domain sequences were included and are designated as *UAS-fruA, UAS-fruB*, and *UAS-fruC* (see Materials and Methods).

The expression of *UAS-fruA* and *fruC* transgenes under the control of a *sca-GAL4* driver was sufficient to rescue the defects in axonal projections in *fru* mutant embryos revealed by anti-FasII and BP102 staining (Fig. 2.6A, 6C, 6D, 6F; Table 2.4A). Embryos from two lines with independent *UAS-fruA* and *UAS-fruC* insertions in the fru^{w12}/fru^{sat15} mutant background were examined and found to have wildtype appearing FasII fascicles (Fig. 2.6A, 6C; Table 2.4A). In both lines of *sca-GAL4/UAS-fruA*; fru^{w12}/fru^{sat15} embryos, the wild-type BP102 pattern was also restored (Fig. 2.6D; Table 2.4A). However, the BP102 pattern was rescued in *UAS-fruC*; *sca-GAL4*; fru^{w12}/fru^{sat15} embryos from only one of the *UAS-fruC* transgenic lines (Fig. 2.6F). In embryos having *UAS-fruC* at a different chromosomal insertion site, 33% of the *fru* mutant embryos had an abnormal pattern of BP102 axonal projections (Table 2.4A).

In contrast to the results with the *UAS-fruA* and *-fruC* transgenes, *sca-GAL4 /UAS-fruB*; fru^{w12}/fru^{sat15} embryos had FasII labeling that was diffuse, indicative of highly defasciculated axonal projections (Fig. 2.6B, Table 2.4A). In regions where FasII axons were bundled, some axons were observed to cross from one fascicle to another in the same hemisegment and to cross over the midline to the opposite side of the segment (Fig. 2.6B). In addition, a higher percentage of *sca-GAL4/UAS-fruB*; fru^{w12}/fru^{sat15} embryos had defects in their FasII fascicles; in those embryos the frequency and severity of defects per hemisegments were more than in fru^{w12}/fru^{sat15} embryos (Table 2.3C, 2.4A). We also noticed that the *sca-GAL4/UAS-fruB*; fru^{-}/fru^{+} sibling embryos (n=20, data not shown) had defective FasII axonal processes. Not surprisingly, the BP102 pattern in both lines of *sca-GAL4/UAS-fruB*; fru^{w12}/fru^{sat15}embryos was severely disrupted (Fig. 2.6E, Table 2.4A). These BP102 pattern defects were also present in the fru^{+} sibling embryos

Figure 2.6. The expression of specific *fru* transgenes rescues the axonal defects in FasII- and BP102-positive axons in *fru* mutants but the expression of male-specific *fru* transgenes disrupts axonal tracts in the CNS.

Filleted CNS of fru^{w12}/fru^{sat15} embryos (st 16) expressing a *UAS-fru* or UAS-*fruM* transgene under the control of the *sca-GAL4* driver and labeled for anti-FasII (A-C and G-I) or BP102 (D-F) followed by HRP cytochemistry.
A) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruA* transgene (*w;sca-GAL4/+;* fru^{w12}/fru^{sat15}, *UAS-fruA*). All segments of A have a normal pattern of FasII tracts.
B) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruB* transgene (*UAS-fruB; sca-GAL4/+;* fru^{w12}/fru^{sat15}). All segments have a more abnormal pattern of FasII tracts (bracket) than does the fru^{w12}/fru^{sat15} mutant embryo alone (see Fig 5B). In addition, many axons project across the midline (arrows).
C) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruC* transgene (*w;sca-GAL4/UAS-fruC;* fru^{w12}/fru^{sat15}). All segments have a normal pattern of FasII tracts.
D) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruA* transgene. All segments have a normal pattern of BP102-positive longitudinal connectives and commissures.
E) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruB* transgene. Many segments have a slightly more abnormal pattern of BP102-positive longitudinal connectives and commissures than wildtype.
F) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruC* transgene. All segments have a normal pattern of BP102-positive longitudinal connectives and commissures.
G) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruMA*. All segments have defasciculated (brackets) and midline crossing (arrows) FasII-positive axons.
H) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruMB*. All segments have defasciculated (brackets) and midline crossing (arrows) FasII-positive axons.
I) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruMC*. All segments have defasciculated (brackets) and midline crossing (arrows) FasII-positive axons.
Anterior is up. Magnification bar 20 μm.

Figure 2.6.

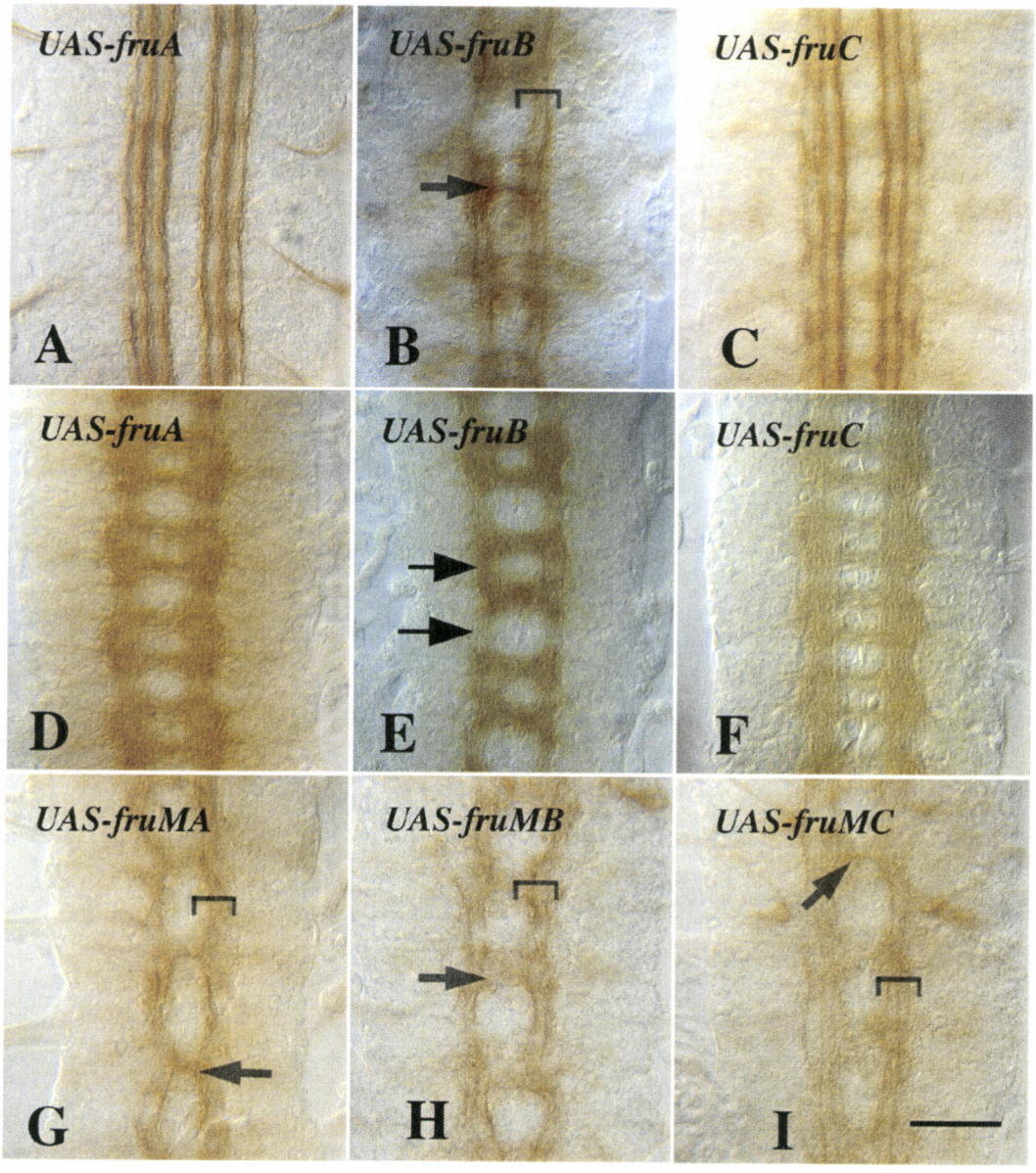

UAS-fruA
A
UAS-fruB
B
UAS-fruC
C
UAS-fruA
D
UAS-fruB
E
UAS-fruC
F
UAS-fruMA
G
UAS-fruMB
H
UAS-fruMC
I

Table 2.4. Pattern of FasII and BP102 axonal tracts in *fru* mutants expressing *UAS-fru* transgenes in the CNS.

A. The percentage of stage 15/16 *w; fru*^{w12}/*fru*^{sat15}; *sca-GAL4; UAS-fru* transgenic embryos with defective FasII or BP102 pattern was determined. This percentage is an average of two independent experiments in which whole mount embryos were evaluated for their mutant phenotype. For most comparisons, data from only one line (except for the second *UAS-fruC* line*, see below) was included in the statistical analysis; the data from the second line (♣) is included.

A one-way ANOVA, which included data from Table 2.3, showed significant differences among genotypes in the number of embryos with abnormal Fas II [$F_{17, 45291} = 281$, $P < 0.0001$] and BP102 tracts [$F_{14, 45539} = 441$, $P < 0.0001$], respectively. Subsequent comparisons (Tukey's HSD) were made between *fru*^{w12}/*fru*^{sat15} mutants, wildtype and *fru* mutants expressing *UAS-fru* transgenes. The FasII patterns of *UAS-fruA/sca-GAL4; fru*^{w12}/*fru*^{sat15} and *UAS-fruC/sca-GAL4; fru*^{w12}/*fru*^{sat15} embryos were not different from wildtype embryos. However, the frequency of the FasII defects in *fru* mutant embryos expressing the transgenes *UAS-fruA* and *UAS-fruC* were significantly different from the frequency of defects found in *fru*^{w12}/*fru*^{sat15} embryos (indicated by # in Table 4). The number of *UAS-fruB/sca-GAL4; fru*^{w12}/*fru*^{sat15} embryos with an abnormal FasII pattern was significantly different from both the number of wildtype and *fru*^{w12}/*fru*^{sat1} embryos.

The percentage of embryos in *UAS-fruA/sca-GAL4; fru*^{w12}/*fru*^{sat15} and *UAS-fruC/sca-GAL4;fru*^{w12}/*fru*^{sat15} with a defective BP102 pattern were not significantly different from that found in wildtype embryos. However, the number of *UAS-fruB/sca-GAL4;fru*^{w12}/*fru*^{sat15} embryos with an abnormal BP102 pattern was significantly different from that found in both wildtype and *fru*^{w12}/*fru*^{sat1} embryos.

In one case, *w; fru*^{w12}/*fru*^{sat15}; *sca-GAL4; UAS-fruC,* the percentage of affected embryos labeled for BP102 were discordant between the two transformant lines. A separate one-way ANOVA was calculated for the analysis of the BP102 staining in this line and showed that there were significant differences among genotypes in the number of embryos with abnormal BP102 tracts [$F_{14, 45292} = 435$, $P < 0.0001$]. Subsequent comparisons (Tukey's HSD) showed that this line was different both from wildtype and from *fru* mutant alone ($p<0.05$)

The frequency of defects in both FasII and BP102 tracts in mutant embryos expressing any of the *UAS-fru* male transgenes were significantly different from the frequency of defects in both wildtype and *fru* mutant embryos.

B. The percentage of stage 16 *elav-GAL4;UAS-fru; fru*^{w12}/*fru*^{sat15} transgenic embryos with defective FasII was determined. None of the *fru* transgene expressed only in postmitotic neurons rescued FasII phenotypes, suggesting that neuronal expression only of transgenes is not sufficient to restore mutant phenotypes to normal.

Table 2.4. Pattern of FasII and BP102 axonal tracts in *fru* mutants expressing *UAS-fru* transgenes in the CNS.

A. The *fru* transgene expression by *scabrous-GAL4*

Phenotypes examined	*UAS-fru* constructs driven by *sca-Gal4*		
	fruA	*fruB*	*fruC*
Percentage of embryos with an abnormal Fas II pattern	7.1 (n=42)[#]	92.6 (n=94)	7.6 (n=105)[#]
	5.9 (n=51)[♣]	96 (n=90)[♣]	7.6 (n=105)[♣]
Percentage of embryos with an abnormal BP102 pattern	4.5 (n=44)[#]	99 (n=116)	2.5 (n=118)[#]
	4.4 (n=45)[♣]	100 (n=34)[♣]	33.3 (n=84)*[♣]
	UAS-fruM constructs driven by *sca-Gal4*		
	fruMA	*fruMB*	*fruMC*
Percentage of embryos with an abnormal Fas II pattern	100 (n=113)	100 (n=123)	93.9 (n=115)
	99 (n=63)[♣]	94 (n=50)[♣]	98.1 (n=53)[♣]
Percentage of embryos with an abnormal BP102 staining	100 (n=115)	100 (n=125)	96.8 (n=156)
	100 (n=36)[♣]	94 (n=47)[♣]	93 (n=43)[♣]

B. The *fru* transgene expression by *elav-GAL4*

Phenotypes examined	*UAS-fru* constructs driven by *elav-Gal4*		
	fruA	*fruB*	*fruC*
Percentage of embryos with an abnormal Fas II pattern	70.6 (n=51)	93.2 (n=59)	67.8 (n=56)

(95% embryos, n=20, figure not shown). Taken together, these results indicate that expression of *fruB* has a dominant negative effect on these axonal phenotypes.

Not all *fru* mutant phenotypes were rescued by the expression of the *UAS-fru* transgenes driven by *sca-GAL4*. The normal pattern of mab22C10 labeling in the cell body of dMP2 and vMP2 pioneer neurons was not rescued by the expression of any of three *UAS-fru* transgenes in mutant embryos (n = 50 hemisegments of *fru* mutant embryo expressing each construct, data not shown).

We also tried to rescue FasII tract mutant phenotypes by using an *elav-GAL4* driver that is exclusively expressed in postmitotic neurons (Lin and Goodman, 1994). None of the *fru* transgenes under control of the *elav-GAL4* driver exhibited normal FasII tracts; rather expression of the transgenes worsened the Fas II tracts phenotypes in that defasciculated FasII tracts extends over entire segments (Table 2.4B; 70.6 % in the neuronal expression of *fruA*, 93.2 % in that of *fruB* and 67.8 % in that of *fruC*), suggesting that *fru* transgene expression only in neurons is not sufficient enough to restore normal axonal phenotype in *fru* mutant embryos. The failure to rescue the mutant phenotype might be due to detrimental effect in *elav* driven neuronal expression of *fru* transgenes which was not found in *sca* driven neuronal expression of *fru* transgenes, due to failure of expression of *fru* transgenes in other *fru* expressing other cell type such as glia or in developmental *fru* expressing neuronal precursors such as NBs and GMCs.

The male-specific transcripts of *fru* derived from the P1 promoter encode an additional N-terminal extension to the common coding sequences of most *fru* transcripts (Ryner et al., 1996; Ito, et al. 1996; Usui-Aoki et al., 2000). To ask if these *fru* transcripts also rescue the defective axonal projections in *fru* mutants, we used three *fru* constructs derived from male-specific *fru* cDNAs (see Materials and Methods). These constructs, designated *fruMA*, *fruMB* and *fruMC*, were made from the sequences encoding the 101 male-specific amino terminus, the BTB and Common coding sequences and one of the three different 3' ends. The FasII- and BP102-positive axonal tracts were defective in virtually all *UAS-fruMA/sca-GAL4;* fru^{w12}/fru^{sat15}, *UAS-fruMB/sca-GAL4;* fru^{w12}/fru^{sat15} and *UAS-fruMC /sca-GAL4;*fru^{w12}/fru^{sat15} embryos (Fig. 2.6G, 6H, 6I, Table 2.4A). In the majority of

embryos, FasII axons did not form fascicles, and in many segments FasII-positive fibers crossed the midline (96 % of *UAS-fruMA*, 100% of *UAS-fruMB* and 78% of *UAS-fruMC* embryos; Table 4A). The male-specific *UAS-fruM* transgenes were, thus, unable to rescue the axonal defects in *fru* mutant embryos and instead interfered globally with axonal patterning in virtually every embryo. These male-specific transgenes differ from the rescuing transgenes by the presence of the male-specific amino terminal domain, suggesting that these proteins are unable to function in the same fashion in the embryo as the *fru* transcripts from the other promoters.

Discussion

fru transcripts and proteins are expressed during embryogenesis

This is the first report showing that *fru* transcripts and proteins are present during embryogenesis (but see Zollman et al., 1994; Lee et al., 2000). The majority of *fru* expression occurs during and after gastrulation and is maintained throughout neurogenesis. FRU proteins are present in neuronal precursors, NBs and GMCs, and in their progeny, neurons and glia. Furthermore, using promoter-specific riboprobes for *in situ* hybridization, we have shown that the *fru* transcripts in the embryo are generated from the P3 and P4 promoters but not from the P1 or P2 promoters. The presence of *fru* transcripts at very early embryonic stages suggests that at least some *fru* transcripts are likely to be produced maternally and then sequestered in the oocyte (M. Foss and B. J. Taylor, personal communication).

The presence of multiple isoforms and tissue-specific patterns of expression are common characteristics of the BTB/POZ ZnF transcription factor family. For example, the *tramtrack* (*ttk*) gene encodes two isoforms, Ttkp69 and Ttkp88, that are expressed in the CNS and PNS. The Ttkp69 protein is expressed in CNS glial cells and Ttkp88 is expressed in the peripheral nervous system (Giesen et al., 1997); Ttkp69 has been implicated in the formation of wildtype axonal tracts in the CNS (Giesen et al., 1997). Another gene with a similar structure to *fru* is the *Broad-Complex* (*BR-C*) in which a family of BTB/POZ ZnF transcription factors is encoded by a single primary transcript that is spliced into four transcripts sharing a common 5' end spliced alternatively to 3' sequences encoding one of four pairs of C_2H_2 zinc-finger domains (Z1, Z2, Z3, and Z4; Bayer et al., 1996). Phenotypic analysis of *BR-C* mutants, in which the expression of individual isoforms is disrupted, has led to the proposal that certain isoforms have specific functions. For example, the Z1 isoform mediates the *reduced bristle on palpus* wildtype function and the Z2 isoform mediates the *broad* wildtype function (DiBello, 1991; Sandstrom et al. 1997). Although all tissues during metamorphosis appear to contain all *BR-C* isoforms, the relative abundance of the difference isoforms is tissue-specific and is thought to contribute to tissue specificity in the response to

ecdysone hormone (Restifo and Merrill, 1994; Bayer et al., 1997; Restifo and Hauglam, 1998).

Unlike these BTB/POZ ZnF genes, *fru* has multiple promoters as well as alternative 3' end splicing. The greater degree of *fru*'s transcript complexity means that tissue- and stage-specific gene expression is regulated at the level of promoter choice as well as alternative splicing. For example, P1 *fru* transcripts are expressed in the CNS from late larva through adult stage and show sex-specific splicing at the 5' end of the primary transcript and sex-nonspecific alternative splicing of the 3' ends (Goodwin et al., 2000; Usui-Aoki et al., 2000; L.C. Ryner, personal communication). In addition, there appears to be regulation at the translational level of the mRNAs from the P1 promoter (Lee et al., 2000; Usui-Aoki et al., 2000).

For the other *fru* transcripts little is known of their regulation. Our results suggest that most or all neurons and glia express transcripts derived from both P3 and P4 promoters. By *in situ* hybridization, *fru* transcripts containing each of the three different 3' ZnF domains are also widely expressed suggesting that neurons and glia probably co-express FRU isoforms with different ZnF domains. If both P3 and P4 transcripts are spliced to use the full range of alternative 3' ends, there may be as many as six different *fru* transcripts in embryos. The primary differences in the FRU proteins encoded by these P3 and P4 transcripts reside in their alternative 3' ends, since these ends encode peptides that differ in size as well as the position and sequence of the ZnF domain (Goodwin et al., 2000; Usui-Aoki et al., 2000; L. C. Ryner, personal communication). Thus, most or all CNS neurons and glia have multiple FRU isoforms, but it is not known whether these proteins will have different functions. Only in a few cell types, such as skeletal muscle, peripheral neurons and glia, was there evidence for the differential presence of A and C ZnF-containing FRU proteins.

Because there was a good correspondence between the cell types that were β-galactosidase positive in the fru^3 and fru^4 P-element lines and the pattern of FRU antibody labeling, the expression of these P-element lines may reveal the location of important enhancers for the control of *fru*'s embryonic expression. The similarity in the pattern of label in the fru^3 and fru^4 lines even though the P-element

insertions are about 40 kb apart indicates that the enhancers driving reporter gene expression are likely to be distributed in this region between the P2 and P3 promoters (Goodwin et al., 2000). By comparison, the number and pattern of labeled cells in the *fru*^{sat} and *fru*^{4} lines are not identical, even though their P-elements are inserted in essentially the same genomic location, suggesting that either intrinsic features of these P-elements or their orientation affect reporter gene expression (Goodwin et al., 2000).

The formation of longitudinal and commissural tracts in the embryonic CNS depends on *fru* function.

We present the first evidence showing that *fru* has sex-nonspecific functions in the development of the embryonic CNS. *fru* mutants that lack most or all *fru* transcript classes formed longitudinal and commissural axonal tracts in which axons either did not coalesce into fascicles, fasciculated with inappropriate partners, or were unable to maintain proper fasciculation. Consistent with the *in situ* hybridization experiments in which P1 and P2 transcripts were not expressed, analyses of *fru* mutants that disrupt P1 or P1 and P2 transcripts but leave P3 and P4 transcripts intact showed that the formation of FasII or BP102 tracts were wildtype in these mutants. By contrast, in *fru* mutants in which P1, P2 and P3 transcripts were disrupted but P4 transcripts were present, the defects in FasII and BP102 axonal tracts were as severe as that in embryos completely lacking *fru* function. Consideration of the axonal phenotypes in these different *fru* mutant genotypes suggests that P3 *fru* transcripts are necessary for the formation of wildtype FasII and BP102 tracts. Even though this explanation is the simplest that accounts for our data, we are unable to assess independently the effects of loss of P2, P3 or P4 transcripts and so are unable to rule out the possibility that elimination of other *fru* transcripts or combinations of transcripts might also result in defective axonal pathfinding.

Even in *fru* nulls, a relatively small fraction of embryos showed defects in their axonal tracts and these defects only involved a few hemisegments. The relative mildness of the mutant phenotype suggests that the activity of other genes

may be able to compensate for the loss of *fru* function. Mutants in other genes such, as *fasII*, *Dlar* and three *receptor protein tyrosine phosphatase* that encode fasciculation and guidance molecules also exhibit mild phenotypes in single mutants but show much stronger phenotypes in double mutants or when heterozygous with mutants that reduce the function of genes that operate in the same developmental pathway (Grenningloh et al., 1991; Seeger et al., 1993; Krueger et al., 1996; Sun et al., 2000; Chapter 4).

fru expression in neurons rescues axonal pathfinding in the embryonic CNS

Our data clearly show that FRU proteins are expressed in neurons as well as lateral and midline glia. All of these cell types have been shown to function during axonogenesis to create the wild-type axonal scaffold in wildtype embryos (Giniger et al., 1994; Hidalgo and Booth, 2000; Klämbt, 1993). The widespread expression of *fru* suggests that its function may be required in each of these three cell types and influence a variety of cellular processes necessary for the formation of a wildtype axonal scaffold. We were able to test whether *fru* expression in neurons would rescue the defects in commissural and connective axonal tracts in *fru* mutants. The expression of specific *fru* transgenes under the transcriptional control of a pan-neuronal driver *sca-GAL4* that is also expressed in the neuronal progenitors led to the formation of wildtype FasII and BP102 tracts. In contrast, expression of *fru* transgenes in post-mitotic neurons by the *elav-GAL4* driver was unable to rescue these phenotypes, suggesting that *fru*'s expression is needed in the progenitors to these neurons or just after the terminal division in order for the axons to make the right axonal pathfinding decisions. The discrepancy in degree of rescue between *sca-* and *elav*-driven *fru* transgene expression leads to the postulation that *fru* expression only in neurons may cause a dominant negative effects on the formation of axonal tracts. In spite of such discrepancy, the ability for *fru* transgenes to rescue *fru* mutant phenotypes strongly support the conclusion that the axonal phenotypes in *fru* mutants are due to the lack of this gene's function in neurons and not the loss of other flanking genes. Furthermore, *fru*'s function in neurons to make proper formation of axonal tracts is supported by our preliminary

observation (Chapter 3); the genetic interaction of *fru* in neurons with genes involved in repulsive signaling mechanism caused defects in FasII axonal tracts, suggesting that along with those genes, *fru* functions for axonal outgrowth in neurons.

The *UAS-fruA* and *UAS -fruC* transgenes provided the most effective rescue of the mutant phenotypes revealed by anti-FasII and BP102 staining, suggesting that these two isoforms of Fru proteins may have very similar functions. By contrast, the *UAS-fruB* transgene did not rescue when expressed with the same pan-neuronal *sca-GAL4* driver but instead led to an increase in the severity and frequency of abnormal FasII and BP102 tracts. The simplest explanation for such failure to rescue along with the appearance of more severe defects may be that *fruB*, unlike *fruA* and *fruC*, is unable to replace other *fru* isoforms and become a dominant negative factor, as result causing severer axonal phenotypes. It may also be due to the mis-expression of *fruB* isoforms at wrong cells and/or in developmental stage, relative levels of *fruB* proteins produced, or other causes. Similar possibilities exist for the interpretation of the expression of the *fru* male-form transgenes.

The initial axonal outgrowth from specific neurons pioneers the path for embryonic longitudinal and commissural tracts (e.g., Hidalgo and Brand, 1997; Hidalgo and Booth, 2000). To address the possibility that *fru* expression in pioneer neurons might be important for the development of the normal axonal tracts, we examined the development of dMP2 and vMP2 neurons in *fru* mutants. We found that most dMP2 and vMP2 neurons in *fru* mutant embryos that lacked all or most *fru* transcripts were not well labeled with the mab22C10 antibody. To rule out the possibility that these neurons were absent, we showed that all dMP2 neurons were present in *fru* mutants by anti-Oddskipped expression. These results suggest that the loss of *fru* function affects the expression of a differentiated neuronal phenotype, not the generation or survival of these neurons. Thus, we infer that it is the expression of the *futsch* gene, that encodes the 22C10 antigen, MAP1B, which is downstream of *fru* gene function. Weak 22C10 labeling has also been described in embryos mutant for the *argos*, *pointed* and *prospero* genes; these genes are

known to be important for establishing neuronal identity and in some cases required for the formation of FasII axonal tracts (Spana and Perrimon, personal communication; Freeman et al., 1992; Klämbt, 1993; Spana and Doe, 1995). It is however not clear whether the loss of mab22C10 expression in dMP2/vMP2 neurons in *fru* mutants affects the outgrowth of their axons or the formation of the FasII fascicles. The *UAS-fruA* and *UAS -fruC* transgenes were able to rescue the FasII axonal pattern without rescuing the mab22C10 expression in dMP2/vMP2 neurons.

Glial cells have been implicated as primary determinants of axonal pathfinding. Glial cells in the CNS can be grouped into two major categories, the midline glia and the lateral glia, according to their position and gene expression profiles in wildtype embryos. Four segmental midline glial cells, closely associated with the developing commissures, are characterized by the expression of the EGF-receptor, *argos* and *pointedP2* (Freeman et al., 1992; Giesen et al., 1997; Klämbt, 1993; review for Jacobs, 2000). The lateral glial cells consist of several subgroup cell types, determined by *pointedP1*, *repo*, and *glial cell missing* (Campbell et al., 1994; Halter et al., 1995; Hosoya et al., 1995; Jones et al., 1995; Klämbt, 1993; Xiong et al., 1994). In our experiments, FRU proteins are detected in lateral glial cells by co-labeling with the REPO antibody. Cell counts in *fru* mutant embryos revealed that there was no change in the number or distribution of REPO-immunoreactive glial cells in these embryos compared with wildtype. Likewise, we could find no defects in the number of midline glial cells in *fru* mutant embryos. Therefore, FRU is not responsible for the survival of glial cells and may only function in the differentiation of lateral and/or midline glial cells.

Glial cells of both subtypes are required for the formation of the axonal scaffold of the ventral nerve cord (Goodman and Doe, 1993; Giesen et al., 1997; Scholz et al., 1997; Hummel et al., 1999). The loss of lateral glial cells has been implicated in defasciculation phenotypes of *tramtrack* and *glial cells missing* mutant embryos (Giesen et al., 1997; Jones et al., 1995). Defasciculation of Fas II axons has also been found in *repo* mutants in which lateral glial cells are largely present, but are in some way unable to support axonal fasciculation (Halter et al.,

1995; Hidalgo and Booth, 2000). Other studies have identified additional mutations in genes involved in midline or glial development that cause defects in FasII and BP102 tracts in the embryonic CNS similar to the phenotypes of *fru* mutants (Freeman et al., 1992; Klämbt, 1993; Giniger et al., 1994; Spana and Doe, 1995; Giesen et al., 1997; Scholz et al., 1997; Thomas, 1998; Hummel et al., 1999). The phenotypic similarity between these mutants and *fru* raises the possibility that *fru* acts somewhere in the same pathway as do these other genes in glial cells. Because the appropriate GAL4 lines are not available, it is not possible to test whether *fru* expression in glial cells alone is sufficient to rescue the FasII and BP102 axonal defects in *fru* mutants.

On the possible function of sex-nonspecific *fru* transcripts in the adult CNS

The adult-specific function of the male-specific FRU proteins in control of male-specific behaviors has been established (Goodwin et al., 2000; Lee et al., 2000; Usui-Aoki et al., 2000; Lee and Hall, 2001; Lee et al., 2001). *fru* transcripts from the P2, P3 and P4 promoters are also widely expressed in the CNS and other tissues in the developing adult nervous system of males and females, as determined by *in situ* hybridization with anti-BTB and anti-Com riboprobes (Ryner et al.,1996; M Foss and B J Taylor, personal communication). It seems likely that these *fru* transcripts might also have the same function in the developing adult CNS as in the embryonic CNS. During metamorphosis, axons of larval neurons being respecified and post-embryonically generated adult-specific neurons form new axonal projections to create new neuronal circuits. By analogy with the embryonic phase of neuronal differentiation, we anticipate that FRU proteins generated from P2, P3 and/or P4 promoters may be required for the formation of wildtype axonal tracts in the adult CNS.

CHAPTER 3

THE *fruitless* GENE IS REQUIRED FOR REPULSIVE SIGNALING IN AXONAL PATHFINDING AND DEVELOPMENT OF GLIA IN THE EMBRYONIC CNS OF *DROSOPHILA*.

Ho-Juhn Song and Barbara J. Taylor

Abstract

One member of the BTB/POZ family of transcription factors, the *fruitless* (*fru*) gene, is required for the proper formation of axonal tracts in the embryonic CNS and is expressed in neurons and glia in the embryonic CNS. To better understand *fru*'s role in axonal guidance and pathfinding in the CNS, double heterozygotes were created between *fru* and other genes known to be involved in axonal guidance. In embryos in which the levels of *fru* and *longitudinal lacking*, *roundabout* or *commissureless* are simultaneously reduced, FasII axons do not form wild-type fascicles and often inappropriately project across the midline. From these experiments, *fru* has been shown to be part of the pathway by which commissural and longitudinal axons respond to repulsive signals secreted from the midline. In a second set of double heterozygotes made between *fru* and genes expressed in lateral glia, *tramtrack* and *glial cells missing*, FasII-positive axons fail to form wild-type fascicles. Thus, *fru* in also involved in the pathway by which lateral glial cells are known to differentiate and acts, perhaps indirectly, on the ability of FasII-positive axons to fasciculate. Finally, *fru* also functions, along with the *single-minded* gene, in the pathway that regulates the development of midline glia. The results of these experiments demonstrate that *fru* is involved in axonal pathfinding through multiple interactions and in more than one cell type.

Introduction

In the developing *Drosophila* ventral nerve cord (VNC), neurons find their correct synaptic targets through the elongation of their axons and their leading edges, the neuronal growth cones. At a series of choice points along its pathway, the growth cone has the opportunity to make decisions as to the direction in which it will grow (for reviews, Goodman, 1996; Tessier-Lavigne and Goodman, 1996). These decisions are made via the binding of attractive or repulsive guidance cues that are present either on the surfaces of surrounding cells or secreted into the extracellular matrix to membrane-bound receptors on the growth cone. The response of the growth cone to these cues in its environment results in activation of signal transduction cascades downstream of the membrane-bound receptor. When these signaling events lead to a change in the direction of the growth cone, the morphology of the growth cone's cytoskeleton is altered so that only a portion of the growth cone continues to extend, resulting in a change in the pathway taken by the axon. It is by a series of directed changes in growth cone morphology that axons chart their course through the central nervous system (CNS).

In general, there are two types of axonal pathways in the CNS. Some neurons, such as embryonic interneurons, have axons that grow ipsilaterally and remain on the same side of the CNS as their cell body. For these axons to continue to grow longitudinally they must either respond to secreted midline repellent factors, such as Slit, or fail to be sensitive to midline attractive factors, such as *Drosophila* Netrin (D-Netrin). Axons from other neurons, such as some motoneurons, project across the midline to form the commissures. Commissural axons are attracted to the midline and cross it. When commissural axons reach the contralateral side of the CNS they must be prevented from re-crossing the midline in order for them to change direction and project longitudinally within the CNS or to exit to innervate targets in the periphery (Tessier-Lavigne and Goodman, 1996). Several molecular and genetic screens have identified many genes and their proteins that are involved in the decision making process during axonal elongation and pathfinding (reviewed in Tear, 1999, Mueller, 1999, Stoker, 2001, and Rusch

and Van Vactor, 2000; Bashaw et al., 2000; Sun et al., 2000; Fritz and vanBerkum, 2000; Hummel et al., 1999).

The cells that form the midline of the CNS play a very prominent role in axonal guidance. Glial cells along the midline secrete factors and provide substrates that serve as intermediate axon guidance targets. These midline glial cells secrete attractive and repulsive chemotactic factors, including Slit, Commissureless (Comm) and D-Netrin (Harris et al., 1996; Mitchell et al., 1996; Tear et al., 1996; Kidd et al., 1999). Another key player in this process is Roundabout (Robo), which is a receptor protein expressed in neurons. One of the ways that axons projecting along longitudinal tracts in the CNS are prevented from crossing the midline is by their response to repulsive cues sent from the midline. Slit proteins are secreted ligands that bind to Robo receptors; the activation of Robo receptors on neuronal axons generally leads to changes in growth cone morphology such that they are directed away from the midline (Brose et al., 1999; Kidd et al., 1999). In *slit* mutants, all growth cones enter the midline but never leave it, resulting in a phenotype in which all axonal tracts have collapsed into the midline (Rothberg et al., 1990; Battye et al., 1999; Kidd et al., 1999). In contrast, in *robo* mutants, FasII-positive axons cross and re-cross the midline (Kidd et al., 1999). Recently two other *robo* genes, *robo2* and *robo3*, have been discovered along with a network of genes that interact with *robo* (reviewed in Rusch and Van Vactor, 2000; Rajagopalan et al., 2000a, 2000b; Simpson et al., 2000a, 2000b).

For axons to cross the midline, a complicated and balanced interaction of both attractive and repulsive cues is essential. Comm proteins mediate the response to repulsive cues. In *comm* mutant embryos, axons that form the commissures do not grow across the midline. Instead, the axons of commissural neurons that normally cross the midline make only ipsilateral projections; these axonal projections appear relatively normal, except for their failure to cross the midline (Seeger, 1993). In axons that are crossing the midline, the activity of the Robo receptor on the growth cone must be reduced, thereby making the decussating axons insensitive to the presence of Slit and enabling them to be attracted to and cross the midline (Tear et al., 1996; Kidd et al., 1998b). It appears that Comm

protein activates removal of Robo from the surface of the growth cone plasma membrane (Tear et al., 1996; Kidd et al., 1998b). Support for this model of Robo and Comm interaction is provided by the phenotype of the *robo*; *comm* double mutant, which is indistinguishable from *robo* mutants. The double mutant phenotype suggests that the ability of axons to cross the midline is the default state, since it occurs in the absence of both proteins and that molecules, such as *robo*, are required for axons to respond differently to the midline (Kidd, 1998b).

Lateral glial cells are another important element in the development of axonal pathways and other aspects of neurogenesis in the embryonic CNS. Proper differentiation of lateral glia is important for the regulation of neuronal apoptosis (for review, Shepherd, 2000 and Granderath and Klämbt, 1999) and for axonal pathfinding (Hidalgo et al., 1995; Hidalgo and Booth, 200; Booth et al., 2000; Kinrade et al., 2001). These glia cells provide substrates on which growth cones can pioneer their pathway. Lateral glial cell development requires the master gene *glial cells missing* (*gcm,* also known as *glide*) that encodes a transcription factor (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). *gcm* acts on a set of downstream target genes to specify glial development and suppress neuronal development. Two target genes, *reverse-polarity* (*repo*) and *pointed* (*pnt*), promote glial differentiation, while two other target genes, *locomotion defects* (*loco*) and *tramtrack* (*ttk*), repress neuronal differentiation (Akiyama et al., 1996; Granderath et al., 2000; Giesen et al., 1997).

The *fru* gene has been shown to affect the differentiation of embryonic neurons. *fru* is expressed in both neuronal and glial cells, and its function is required for the proper formation of axonal tracts in the embryonic CNS and for maintaining neuronal identity (Chapters 2 and 4). The *fru* gene produces a set of sex-specific and sex-nonspecific transcripts generated by differential promoter usage and alternative splicing at the 5' and 3' ends (Ryner et al, 1996; Ito et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000). *fru* transcripts are generated from at least four different promoters, P1, P2, P3 and P4 (Ryner et al, 1996; Ito et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000; Anand et al., 2001). These transcripts encode closely related BTB/POZ (*Bric-a-brac, tramtrack, Broad-*

complex/ Poxvirus and Zinc finger)-Zn finger proteins (ZnF), which may function as transcription factors (Li et al., 1997). *fru*'s sex-specific functions appear to depend on P1 transcripts, which are produced only in the developing adult and adult CNS (Ryner et al, 1996; Ito et al., 1996; Goodwin et al., 2000; Usui-Aoki et al., 2000; Anand et al., 2001). The sex-nonspecific functions of *fru* rely on the products from the other three promoters P2, P3 and P4 in both embryos and adults (Chapter 2; Anand et al., 2001). In *fru* mutants in which transcripts from the P3 and P4 promoters are not present, the differentiation of FasII- and BP102- positive tracts is abnormal. In these *fru* mutants, the axons in these tracts typically fail to maintain the wild-type degree of axonal fasciculation, so that axons take abnormal pathways in the CNS, including longitudinal FasII axons aberrantly projecting across the midline.

These findings inspired me to determine how *fru* might function in the proper formation of axonal tracts during the embryonic CNS development. To begin to define the molecular and genetic mechanisms by which *fru* might function within the genetic cascade needed to establish proper axonal tracts, I searched for candidate genes whose expression pattern and mutant phenotype were similar to those of the sex-nonspecific *fru* transcripts. The complex *fru* expression pattern in neurons as well as lateral and midline glia lead to difficulties in interpreting the cellular basis of *fru*'s function in axonal pathfinding. As a strategy to evaluate both the genetic and potential cellular basis of *fru*'s function, we used embryos doubly heterozygous for *fru* and each redundant candidate gene to look for combinations in which axonal pathfinding was abnormal. These genetic interactions involved producing animals with one wild-type copy of each gene. Even though these embryos have one wildtype copy of each gene, the simultaneous reduction in the level of gene products from two genes in the same pathway has been shown to sensitize developmental systems so that abnormalities occur during development (Kidd et al., 1998b).

Of these candidate genes, I further selected genes that also belonged to *fru*'s family of transcription factors, because the putative role of BTB/POZ motif appears to mediate homo- or hetero-dimerization (Ahmad et al., 1998; Bardwell and

Treisman, 1994; David et al., 1998; Li et al., 1997b, 1999). The candidate genes that best fit these criteria were *longitudinal lacking* (*lola*) and *tramtrack* (*ttk*). Both of these genes belong to the BTB/POZ transcription factor family and are important for the establishment of proper axonal tracts. A family of Lola proteins, derived by alternative splicing, is expressed in all postmitotic neurons and midline glial cells in the embryonic CNS along with other non-neuronal cells (Giniger et al., 1994). By mutant analysis, it has been suggested that *lola* has the ability to modulate axon guidance cues at the transcriptional level (Giniger et al., 1994; Madden et al., 1999). Axonal aberrations caused by the loss of *lola* function are similar to those caused by mutations of *robo* in that FasII axons cross and re-cross the midline (Giniger et al., 1994). The other BTB/POZ domain candidate gene tested for its interaction with *fru* is the *ttk* gene, which is expressed in lateral and midline glial cells. Two different protein isoforms of Ttk proteins are generated by alternative splicing (Giesen et al., 1997). One of the two Ttk protein isoforms, Ttkp69 is directly downstream of *gcm* and acts to repress neuronal differentiation in lateral glia (Giesen et al., 1997).

Once a candidate gene was identified as genetically interacting with *fru*, I also carried out further genetic interaction tests with other genes known to be in the candidate gene's pathway to gain a better understanding of *fru*'s influence in proper formation of axonal tracts in the embryonic CNS. Thus, *fru*'s function in axonal differentiation can be ascribed to its role in two genetic cascades that are important for the formation of longitudinal and commissural axonal tracts.

These studies are the first to show that *fru* genetically interacts with *lola*, *robo* and *comm* and so belongs to the pathway by which the cells in the midline regulate the crossing of commissural axons and prevents longitudinal axons from crossing the midline. In addition, *fru* also interacts with *ttk* and *gcm* suggesting that *fru* function is needed in lateral glial cells for the maintenance of axonal fasciculation in FasII longitudinal tracts. Finally, in midline glia, *fru* interacts with the *single-minded* gene (*sim*) for specification or control of differentiation of these cells (Crews et al., 1988; Nambu et al., 1991).

Materials and Methods

Fly stocks and fly maintenance

Canton-S was used as the wild-type genotype. The following mutant alleles were used: *lola*^{C46} and *lola*^{ORE76} (Giniger et al., 1994), *robo*^{Z14} (Kidd et al, 1998a; provided by E. Giniger), *slit*^{2} (Kidd et al., 1999; Bloomington Stock Center), *comm*^{1} (Seeger et al., 1993; provided by E. Giniger), *pnt*$^{\Delta 88}$ (Scholz et al., 1997; Bloomington Stock Center), *ttk*$^{D2\text{-}50}$ and *ttk*^{02667} (Giesen et al., 1997, provided by C. Klämbt, Bloomington Stock Center), *gcm*$^{N7\text{-}4}$ (Jones et al., 1995; Bloomington Stock Center), *In(3R)fru*^{w12} (*fru*^{w12}, Anand et al., 2001) and *Df(3R)AJ96U3* (*fru*^{AJ96u3}, Chapter 2) for *fru*. For crosses used to generate heterozygous embryos, all mutant alleles were maintained over the Blue Balancer chromosome *TM3, Sb, P[ftz-lacZ]* or *CyO P[ftz-lacZ]*.

Fly stocks were maintained at room temperature on an agar, sucrose, cornmeal and yeast medium with 0.1% nipagin (p-hydroxybenzoic acid methyl ester, Sigma).

Embryonic immunohistochemistry

Timed collections of embryos were prepared by standard techniques as described in Patel (1994). Embryos were staged by morphological criteria (Campos-Ortega and Hartenstein, 1997). Immunohistological protocols for embryo fixation and subsequent antibody incubations were carried out according to standard protocols (Patel, 1994; see Chapter 2 for more details). To label the CNS, the following primary antibodies were used: anti-Fasciclin II (1D4, 1:5, Grenningloh *et al.*, 1991) and mabBP102 (1:20, Seeger *et al.*, 1993). All embryos were also labeled for anti-β-galactosidase (1:10,000, Cappel, NC) to allow the double heterozygous embryos to be distinguished from singly heterozygous siblings. Labeled whole mount and filleted embryos were viewed and photographed with a Sony DKC-5000 digital camera under DIC optics, using an Olympus Vanox-TX microscope. Composite images were assembled in Adobe Photoshop 5.0.

Results

Experimental design and strategy

In wild-type embryos, viewed dorsally, three longitudinal tracts of FasII-positive axons travel the length of the VNC (medial, intermediate and lateral tract; Fig. 3.1A). In *fru* mutant embryos, which lack all or most *fru* function, the FasII-positive axons no longer fasciculate properly to form these tracts and, instead, individual axons and bundles of axons take alternative, abnormal routes through the CNS. These FasII-positive axons may cross between the different FasII fascicles, appearing to project in-between the normal FasII tracts or cross over the midline to the opposite side of the CNS (Fig. 3.1B; Chapter 2). Furthermore, the axonal defects in *fru* mutant embryos are not confined simply to axons forming the FasII pathways but involve most or all axons forming the longitudinal and commissural tracts as visualized by labeling with BP102 antibodies (Fig. 3.1D, 1E; Chapter 2).

To begin to decipher the pathway by which *fru* functions in the formation of wild-type axonal pathways in the CNS, I generated doubly heterozygous embryos, which have one wild-type copy of the *fru* gene and one wild-type copy of another gene that has been shown to be required in neurons or glia for the proper differentiation of axonal tracts in the CNS. The logic of this experimental design is based on the expectation that a reduction in the dosage of two genes that are part of the same developmental pathway will lead to the expression of a mutant phenotype. Candidate genes to be tested for interaction with *fru* were chosen on the basis of the similarity of their expression pattern in the embryonic CNS to *fru*'s gene expression pattern, the similarity of their mutant phenotypes and their involvement in genetic pathways underlying the development of longitudinal and commissural axons tracts. Because *fru* is expressed in all neurons and glia, candidate genes that were expressed in each of these different cells types were chosen.

Figure 3.1. *fru* genetically interacts with *lola* in axonal pathfinding.

Embryos (very late stage 16) were labeled with mAb anti-Fas II (A,B, E and F) or mAb BP102 (C, D, G and H) and visualized by using HRP immunohistochemistry. Anterior to the top

A) In the wild-type CNS, three FasII-positive longitudinal fascicles (medial, intermediate, lateral) are present on each side.

B) In the fru^{w12}/fru^{sat15} CNS, the FasII axons frequently defasiculate (bracket) and do not stay within the same fascicle. The medial tract in one hemisegment (arrow) has crossed the midline.

C) In the wild-type CNS, BP102-positive axons have a regular ladder with uniform longitudinal connectives and commissures.

D) In the fru^{w12}/fru^{sat15} CNS, BP102-positive axons do not form regular longitudinal tracts, and in some segments there are no commissural axons (arrow). The variation in the size of the tracts suggests that in some tracts more axons than in wildtype or fewer axons than in wildtype are present.

E) In a $lola^{C46}/+; fru^{AJ96u3}/+$ CNS, the pattern of FasII tracts is only slightly abnormal in which axons in the lateral and intermediate fascicle have defasciculated. Some FasII axons have crossed the midline (arrow) and were found to project between these two fascicles (arrowheads)

F) In the $lola^{C46}/+; fru^{AJ96u3}/+$ CNS, the pattern of FasII tracts is severely disrupted and no well-defined fascicle can be seen. Many axons cross the midline (arrow).

G) In a $lola^{C46}/+;fru^{AJ96u3}/+$ CNS, BP102 positive commissural and longitudinal tracts show normal patterns in wild-type embryos but they are relatively thinner.

H) In a $lola^{ORE76}/+;fru^{AJ96u3}/+$ CNS, BP102-positive commissural tracts are aberrant. In some cases, no commissure forms (arrow), an extremely thin commissure (arrowhead) forms, or axons do not form a continuous longitudinal tract (bracket).

Figure 3.1.

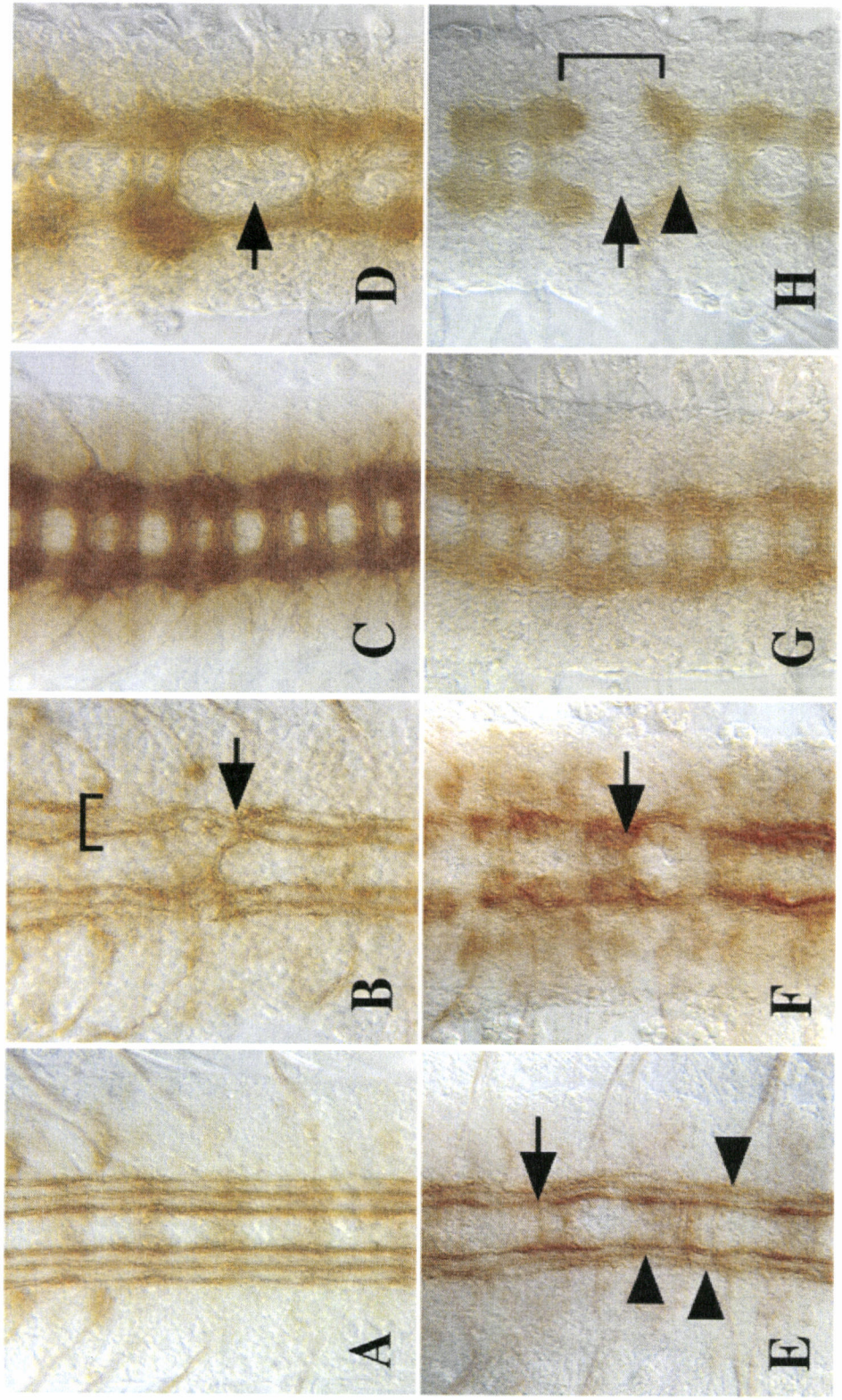


Table 3.1 Phenotypic analysis of FasII- and BP102-positive axonal tracts in *fru*, *lola* and *robo* double heterozygous embryos.

For genetic interaction studies with one of the candidate genes above, standard genetic crosses were made between fru^{AJ96u3}/*Bal* animals and *lola*/*Bal* or *robo*/*Bal* animals to make double heterozygous embryos. The alleles of all three genes were null alleles (See Material and Methods). Embryos were collected, fixed and labeled with anti-FasII or BP102 antibody. The morphology of the FasII-positive fascicles or BP102 tracts was scored in late stage 16 embryos of double heterozygotes and sibling embryos. The number of embryos examined is given in parentheses. The appropriate Balancer chromosome is denoted by *Bal* (see Materials and Methods) and + refers to a wild-type chromosome.

A. The first column gives the percentage of embryos with defasciculated FasII axons. The second column reports the percentage of embryos with both defasciculated and midline crossing FasII-positive axons; these embryos were a subset of the embryos that had defasciculated FasII axons and reported in the first column. All *robo*/+; *fru*/+ embryos that showed defasciculation also had midline crossing axons. The third column gives the percentage of embryos with wildtype FasII axonal tracts.

B. The first column gives the percentage of embryos with any defect in the BP102-positive longitudinal or commissural tracts. In the second column is the percentage of embryos with defects in the BP102-positive longitudinal tracts; these defects include the presence of thinner or thicker longitudinal tracts between or within segments, or tracts that appear discontinuous, as compared to those of wild-type or sibling control embryos. The third column gives the percentage of embryos with aberrant commissural tracts; these defects include commissures that are thinner (denoted by T), or that are thicker tracts with fused anterior and posterior commissures (denoted by U), as compared to that of wildtype or sibling control embryos. In the last column is the percentage of embryos with wildtype BP102 tracts.

Table 3.1. Phenotypic analysis of FasII- and BP102-positive axonal tracts in double heterozygous embryos with *fru* and genes expressed in neurons, *lola* or *robo*.

A. Percentage of embryos with defective FasII-positive fascicles

Genotype	Percentage of embryos with FasII axonal defasciculation	Percentage of embryos with axonal defasciculation and midline crossing axons	Percentage of embryos with wildtype FasII axonal tracts
$lola^{C46}$/+; fru^{AJ96u3}/+	30.5 (n=48)	49.2	69.5
$lola^{ORE76}$/+; fru^{AJ96u3}/+	40.3 (n=48)	7	59.7
$lola^{C46}$/+; +/*Bal* or +/*Bal*; fru^{AJ96u3}/*Bal*	5.3 (n=38)	0	94.7
$robo^{Z14}$/+; fru^{AJ96u3}/+	30.3 (n=33)	100	69.7
$robo^{Z14}$/+;+ /*Bal* or +/*Bal*; fru^{AJ96u3}/*Bal*	4.8 (n=42)	0	95.2

B. Percentage of embryos with defective BP102-positive tracts

Genotype	Percentage of embryos with defects in BP102 tracts	Percentage of embryos with aberrant longitudinal tracts	Percentage of embryos with aberrant commissural tracts	Percentage of embryos with wildtype BP102 tracts
$lola^{C46}$/+; fru^{AJ96u3}/+	21.2 (n=33)	21.2	0	78.8
$lola^{ORE76}$/+; fru^{AJ96u3}/+	30.7 (n=39)	25.6	5.1 [T]	69.3
$lola^{C46}$/+; +/*Bal* or +/*Bal*; fru^{AJ96u3}/*Bal*	0 (n=29)	0	0	100
$robo^{Z14}$/+; fru^{AJ96u3}/+	12.8 (n=47)	0	12.8 [U]	87.2

fru genetically interacts with *lola*, a transcription factor expressed in neurons and midline glia.

One candidate gene that fits all of the criteria for testing with the *fru* locus is the *longitudinal lacking* (*lola*) gene. The *lola* gene is similar to the *fru* gene in that it encodes a family of BTB/POZ ZnF transcription factors. *lola* is expressed during embryonic neurogenesis beginning in the neuroectoderm and then restricted to all postmitotic neurons and midline glial cells during late embryonic development (Giniger et al., 1994). This pattern of expression is very similar to that of *fru*'s P3 and P4 transcripts and FRU proteins (Chapter 2). In *lola* mutant embryos, FasII-positive axons, which normally project longitudinally, cross the midline, suggesting that one of *lola*'s wild-type functions is to suppress midline crossing in FasII neurons even though its mechanism of action is still unknown (Giniger et al., 1994).

Doubly heterozygous $lola^{-}/+;fru^{-}/+$ embryos were examined for the formation of FasII-positive axonal tracts. In this experiment, *fru* and *lola* null alleles were used, and the double heterozygotes were expected to have around half the wild-type level of each gene product. The fru^{AJ96u3} mutation is a deficiency, which removes all *fru* coding regions and at least one other ORF. The defects in the differentiation of FasII and BP102 axons have been shown to depend entirely on the loss of *fru* function and not on the loss of the other ORF uncovered by the fru^{AJ96u3} deficiency (Chapter 2). Two different *lola* null alleles, $lola^{C46}$ and $lola^{ORE76}$, were used to eliminate all known *lola* isoforms (Giniger et al., 1994). In 40% of the $lola^{-}/+;fru^{-}/+$ double heterozygous embryos, there were striking defects in the development of the FasII-positive tracts ($lola^{C46}/+;fru^{AJ96u3}/+$, n=48; $lola^{ORE76}/+;fru^{AJ96u3}/+$, n= 49; Table 3.1A; Fig. 3.1E, 1F). The most common defect was the failure of FasII-positive axons to fasciculate into the three tracts found in wild-type embryos (Fig 3.1A). In embryos that showed the most severe phenotype, there was no evidence for any bundling of axons into separate longitudinal fascicles (Fig. 3.1F). In less severely affected embryos, the medial fascicle appeared to have the greatest number of fasciculated axons, since these fascicles were heavily

labeled in these preparations. The lateral and intermediate fascicles were less distinct, indicating that fewer axons were included in the fascicle (Fig. 3.1E).

In some *lola*/+; *fru*/+ embryos, in addition to the generalized defasciculation phenotype, FasII axons in the medial fascicle were found to cross the midline and project to the contralateral side of the CNS ($lola^{C46}$/+; fru^{AJ96u3}/+, 15.3 %; $lola^{ORE76}$/+; fru^{AJ96u3}/+, 2.8 %, Fig. 3.1E, 1F; Table 3.1A). In addition, all three FasII-positive axons were found to project between these fascicles (arrowheads in Fig. 3.1E). The differences in the number of the *lola*/+; *fru*/+ double heterozygotes that had axons crossing the midline may reflect differences in the genetic background in these two crosses. This phenotype, the inappropriate crossing of the midline by axons in the medial fascicle, also occurs in both *fru* and *lola* mutant embryos (Fig. 3.1B; Chapter 2; Giniger et al., 1994; Seeger et al., 1993; Simpson et al., 2000a; Rajagopalan et al., 2000b). However, *fru* and *lola* mutants varied in the number of axons that crossed the midline; in *fru* mutants, the medial FasII axons crossing the midline were highly fasciculated, whereas in *lola* mutants individual or smaller groups of medial axons crossed the midline. In the double heterozygote $lola^{C46}$/+; fru^{AJ96u3}/+ embryos, it appears that nearly all medial axons in the fascicle approach and cross the midline, a phenotype similar to that in *fru* mutants.

To see whether these axonal phenotypes were common to most axons in the embryo, I labeled $lola^{-}$/+; fru^{-}/+ embryos with the BP102 antibody to examine the differentiation of the commissural and longitudinal tracts (Fig. 3.1G, 1H). The longitudinal and commissural tracts were present, but in many embryos (21 % of $lola^{C46}$/+; *fruAJ96u3*/+, n= 33, or 25.6 % of $lola^{ORE76}$/+; fru^{AJ96u3} embryos, n=39; Table 3.1B), the tracts appeared smaller than the ones found in wild-type or in sibling embryos labeled in the same experiment (Fig. 3.1C, 1G, 1H). In a small number of $lola^{ORE76}$/+; fru^{AJ96u3} embryos (5.1%; Table 3.1B) the commissural tracts were missing in some segments (Fig. 3.1H).

These results suggest that the simultaneous reduction in the level of FRU and Lola proteins results in at least some FasII-positive axons being unable to bundle together to create a fascicle or, if able to initiate the formation of a fascicle,

being unable to maintain fasciculation throughout the full length of the longitudinal tract. In addition, the presence of midline crossing axons suggests that many of the FasII-positive axons in these *lola*$^-$/+; *fru*$^-$/+ embryos either responded inappropriately to attractive midline cues or did not respond to the repulsive cues at the midline and so grew toward and subsequently crossed the midline. Because FRU and Lola are both BTB/POZ ZnF proteins that are thought to be transcription factors, it is possible that these proteins may be involved in neuronal differentiation by acting together within neuronal precursors, neurons and/or midline glial cells.

fru interacts with *robo*, which encodes a receptor involved in the repulsion of axons from the midline.

Because of common phenotypes between *fru* mutants and *lola*$^-$/+;*fru*$^-$/+ double heterozygotes where FasII axons cross the midline inappropriately and BP102-positive axons have an abnormal distribution of axons into longitudinal and commissural tracts, it seemed likely that *fru* might function in the response of axons to signals generated at the midline. Robo is a membrane bound receptor on axons that responds to repulsive signals diffusing from the midline, which acts to prevent those axons from responding to attractive midline signals and, thus, is largely responsible for keeping axons in longitudinal tracts in the CNS (Kidd et al., 1999). To determine whether *fru* might be part of the same developmental pathway as the *robo* gene, I generated *robo*^{Z14}/+; *fru*^{AJ96u3}/+ embryos to test this possibility. *robo*^{Z14} is an amorphic allele, so the double heterozygotes would be expected to have half the wild-type levels of both Robo and FRU products.

As was the case for *lola*$^-$/+; *fru*$^-$/+ heterozygotes, in 30% of the *robo*^{Z14}/+; *fru*^{AJ96u3}/+ embryos (n=33), the FasII-positive axons had clearly defasciculated (Figs 2A, 2B; Table 1A). FasII-positive axons in the intermediate fascicle were the most likely to be defasciculated. By contrast, the medial and lateral fascicles were usually fairly distinct. In these embryos, FasII axons in the lateral fascicle were also observed to project towards the lateral margin of the CNS almost 90° opposite to their normal direction (arrowheads in Fig. 3.2A, 2B). In all of the embryos in which axons were defasciculated, axons in the medial FasII fascicles also crossed the

midline. The degree of midline crossing of FasII-positive axons was not as extreme as in *robo* mutant embryos, but the pattern of axons in the medial fascicles crossing the midline was similar to *robo* and *fru* mutant embryos (Kidd et al., 1998b; Fig. 3.1B).

In order to determine whether axons of most neurons were disrupted in these embryos, I next examined commissural and longitudinal tracts in the *robo*$^-$/+;*fru*$^-$/+ embryos using the BP102 antibody. In general, the intensity of BP102 label in commissural and longitudinal tracts was normal in doubly heterozygous embryos, but the degree of separation of anterior and posterior commissures was reduced, likely implying more axons cross the midline, consequently leading to thicker anterior and posterior commissures (Fig. 3.2C, 13 %, n=47; Table 3.1B). Taken together, the phenotype of many *robo*$^-$/+; *fru*$^-$/+ embryos was similar to that of *fru* and *robo* mutants suggesting that the reduction in the levels of FRU and Robo proteins leads to FasII axons crossing the midline and failing to properly fasciculate into wild-type longitudinal tracts. Because Robo is predominantly expressed in post-mitotic neurons, which also express FRU proteins, it seems likely that *fru* and *robo* may be part of a pathway operating within neurons to keep axons that form longitudinal fascicles projecting away from the midline through the response of these neurons to repulsive midline signals.

Figure 3.2. *fru* genetically interacts with *robo* and *comm* in axonal pathfinding.

Embryos (very late stage 16) were labeled with mAb anti-Fas II (A, B, D and E) and mAb BP102 (C, F) visualized for HRP histochemistry.
A) In a $robo^{Z14}$/+; fru^{AJ96u3}/+ CNS, FasII-positive axons in the lateral and intermediate tract (bracket) have defasciculated and axons in the medial tract cross the midline (arrow). A few axons from the lateral fascicle project to the lateral margins of the CNS. Arrowhead (A and B) indicates axons in the lateral fascicle projecting lateral into an inappropriate region of the CNS.
B) In a $robo^{Z14}$/+; fru^{AJ96u3}/+ CNS, FasII-positive axons have severe defects with no apparent intermediate fascicles. Medial axons cross over the midline (arrow).
C) In a $robo^{Z14}$/+; fru^{AJ96u3}/+ CNS, BP102 positive axons in the anterior and posterior commissures are not properly separated (bracket) but the longitudinal tracts have normal size.
D In a $slit^{2}$/+;fru^{AJ96u3}/+ CNS, the FasII-positive fascicles are wildtype (compare to Fig 1A).
E) In a $comm^{1}$+/ + fru^{AJ96u3} CNS, axons in all three FasII fascicles are defasciculated.
F) In a $comm^{1}$+/ + fru^{AJ96u3} CNS, the BP102-positive tracts have a normal pattern but the tracts are thinner than wildtype tracts. The BP102 labeling in these double heterozygotes had lower intensity than in other heterozygotes (Figs 2A, 2D) or wildtype (Fig. 1C).
G) In the wildtype CNS of stage 14, BP102-positive axons have a regular ladder with uniform longitudinal connectives and commissures.
H) In a $comm^{1}$+/ + fru^{AJ96u3} CNS of earlier stage than stage 14 (G), BP102 positive longitudinal tracts are aberrant and commissural axons are thinner (arrowhead) or do not form (arrow).

Figure 3.2.

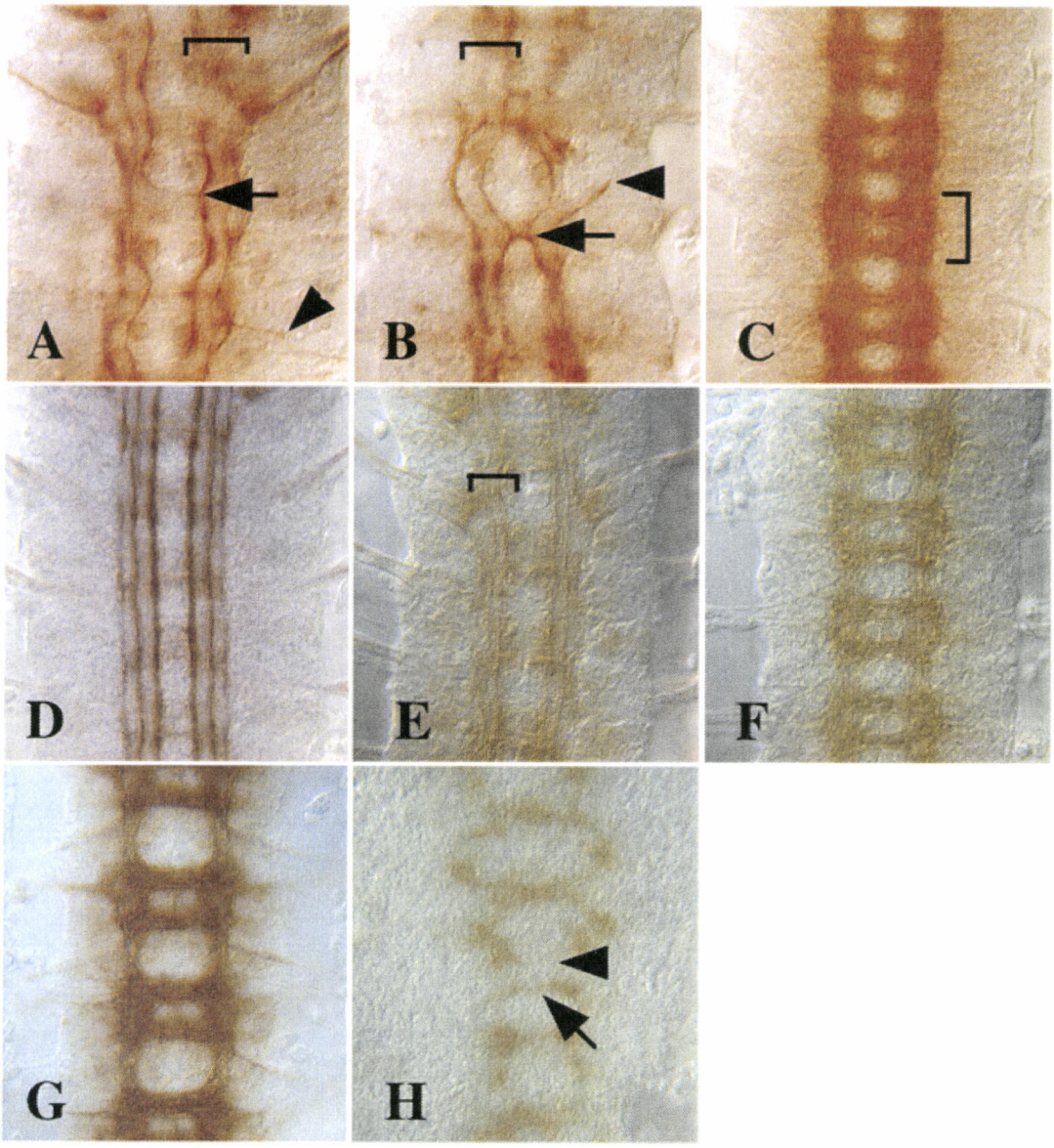


Table 3.2. Phenotype analysis of FasII and BP102 positive axonal tracts in double heterozygous embryos with *fru* and genes expressed in neurons, *comm,* and midline glia *sim* and *slit*.

A. Percentage of embryos with defective FasII-positive fascicles

Genotype	Percentage of embryos with FasII axonal defasciculation	Percentage of embryos with axonal defasciculation and midline crossing axons	Percentage of embryos with wildtype FasII axonal tracts
sim^2+/ + fru^{AJ96u3}	12 (n=20)	20	88
$slit^2$/+; fru^{AJ96u3}/+	1.7 (n=58)	0	98.3
$slit^2$/+ ; +/*Bal* or +/*Bal*; fru^{AJ96u3}/*Bal*	5.4 (n=39)	0	94.6
$comm^1$+/ + fru^{AJ96u3}	57.1 (n=70)	0	42.9
$comm^1$+/ *Bal* or + fru^{AJ96u3}/ *Bal*	0 (n=30)	0	100

B. Percentage of embryos with defective BP102-positive tracts

Genotype	Percentage of embryos with defects in BP102 tracts	Percentage of embryos with aberrant longitudinal tracts	Percentage of embryos with aberrant commissural tracts	Percentage of embryos with wildtype BP102 tracts
sim^2+/ + fru^{AJ96u3}	32.1 (n=28)	10.7	10.7 ^{S}/10.7 ^{U}	67.9
$slit^2$/+; fru^{AJ96u3}/+	0 (n=37)	0	0	100
$slit^2$/+ ; +/*Bal* or +/*Bal*; fru^{AJ96u3}/*Bal*	0 (n=26)	0	0	100
$comm^1$+/ + fru^{AJ96u3}	31.6 (n=57)	31.6	0	68.4
	18.8 (n=18)*	0	18.8 ^{T}	81.2

Standard genetic crosses were made between fru^{AJ96u3}/*Bal* animals and *sim*/*Bal*, *slit*/*Bal* or *comm*/*Bal* animals to make double heterozygous embryos. The analysis and notation are the same as in Table 3.1. In sim^2+/ + fru^{AJ96u3} embryos, two different phenotypes were found: some embryos had a *sim*-like phenotype (S) in which all axonal tracts collapsed into midline. This phenotype was not found in any other genotype. The $comm^1$+/ + fru^{AJ96u3} embryos were scored at both late stage 16 (upper row) and in stage 13 (bottom row *).

fru does not interact with the *slit* gene, the repulsive signal secreted by midline glia.

Midline glial cells are central to the formation of the longitudinal and commissural tracts through the secretion of both repulsive and attractive signals. The repulsive signals are used to keep axons projecting along longitudinal tracts from crossing the midline, while the attractive signals are used to direct the growth cones of commissural fibers to grow toward and then cross the midline. My previous results showed that FRU proteins were expressed in midline glial cells and, further, that the wildtype complement of midline glial cells was present in *fru* mutants (Chapter 2). The expression of *fru* in midline glia suggested that one possible role for *fru* might be to regulate the production of the repulsive ligand Slit.

To test this hypothesis, I generated *fru* and *slit* doubly heterozygous embryos, using the amorphic $slit^2$ allele. Surprisingly, $slit^2/+; fru^{AJ96u3}/+$ embryos showed wildtype development of the FasII-positive longitudinal fascicles (n=57, Fig. 3.2D; Table 3.2A) and BP102-positive longitudinal and commissural tracts (n=37; Table 3.2B). These findings suggest that *fru* is unlikely to be involved in establishing the proper gradient of secreted Slit ligands. This result was somewhat unexpected because 30-40% of $slit^2+/+$ *robo* embryos had FasII-positive axons that inappropriately crossed the midline (Kidd et al., 1999).

fru interacts with the *comm* gene to generate a surprising axonal phenotype.

Another possible mechanism that would account for the midline crossing by FasII axons and the BP102 commissural phenotypes in *fru* mutants involves the production or function of the *comm* gene. In wildtype embryos, Comm protein is a membrane bound receptor, expressed in neurons and midline glial cells (Tear et al., 1996; M. Seeger, unpublished data). Both *robo* and *comm* are dosage sensitive and the loss of *fru* function might affect the relative balance of the expression of these two genes leading to defects in axonal pathways in the CNS (Kidd, et al., 1998).

To test this hypothesis, I used a strong hypomorphic allele of *comm* to create $comm^1+/+fru^{AJ96u3}$ embryos. These embryos yielded surprising results. First, the most striking phenotype in the heterozygous embryos was that FasII-positive axons had defasciculated. The axons in the lateral and intermediate FasII-

positive fascicles were the most affected whereas the medial fascicles had relatively normal morphology (57%, n=68; Fig. 3.2E; Table 3.2A). These results suggest that the reduced dosage of FRU and Comm proteins in these heterozygous embryos leads to a reduced ability of FasII-positive axons to become or remain fasciculated.

Since Comm appears to reduce the number of Robo receptors and thus blunt the axonal response to the Slit ligand, fewer axons would be expected to cross the midline resulting in thinner commissures. BP102-positive axons did form commissures in *comm*$^{-}$+/ + *fru*$^{-}$; however, they were somewhat less intensely labeled at the midline than in wild type as though not all axons growing toward the midline actually crossed. The primary effect was changing the thickness of the BP102 longitudinal tracts (31%, n=57; Fig. 3.2F; Table 3.2B) in stage late 16; but the difference was not very great, compared to control sibling embryos. On the other hand, nearly 17% *comm*^{1}+/ + *fru*^{AJ96u3} of stage 13 embryos, the stage when commissures are first forming, did show aberrant commissural tracts (n=18, stage 13 embryos; Fig. 3.2H), compared to that of wildtype (Fig. 3.2G). This suggests that the axons pioneering the commissures may not pioneer those pathways in the same way as do wild type axons but that later follower's axons growing across the midline obscure this defect. Taken together, these results indicate that the *fru* interaction with *comm* is more pronounced in the FasII-positive longitudinal tracts than in the commissural tracts.

fru interacts with *tramtrack* and *glial cells missing* in lateral glial cells.

The proper differentiation of lateral glia cells is also important for proper fasciculation and axonal projection of longitudinal axonal tracts (Hidalgo et al., 1995; Halter et al., 1995; Kinrade et al., 2001). *fru* is expressed in lateral glial cells but is not necessary for the survival of these lateral glia (Chapter 2). The *ttk* gene was chosen as a candidate gene to test for an interaction with *fru* because it encodes a pair of BTB/POZ proteins and is expressed in lateral and midline glia in a similar pattern to *fru* gene expression (Giesen et al., 1997). The Ttk69P protein is expressed in all non-neuronal cells in the CNS and its primary role is to repress the expression of neuronal differentiation genes in lateral glial cells (Giesen et al.,

Figure 3.3. *fru* genetically interacts with *ttk*, *gcm* and *sim*.

Embryos (very late stage 16 A-E or stage 14 F) were labeled with mAb anti-Fas II (A, C and D) and mAb BP102 (B, E and F) visualized for HRP histochemistry.
A) In a ttk^{02667}+/ + fru^{AJ96u3} CNS, FasII axonal tracts are defasciculated, especially intermediate and lateral (bracket). Some axons appear to join adjacent tracts (arrow).
B) In a ttk^{02667}+/ + fru^{AJ96u3} CNS, BP102-positive axons have wildtype appearing anterior and posterior commissures but longitudinal tracts show an uneven distribution with fewer axons as thinner regions (arrow) and more axons as thicker regions (arrowhead) connectives.
C) In a gcm^{N7-4}/+;fru^{AJ96u3}/+ CNS, axons in all three FasII fascicles have defasciculated (bracket).
D) In a sim^{2}+/ + fru^{AJ96u3} CNS, the embryos double transheterozygous for *sim* and *fru* show defasciculated all three fascicles (bracket) along with midline crossing axons (arrows).
E) In a sim^{2}+/ + fru^{AJ96u3}CNS, BP102-positive axons have formed nearly fused anterior and posterior commissures (bracket).
F) In a different sim^{2}+/ + fru^{AJ96u3} CNS, all axons have collapsed into the midline. This more severe phenotype is similar to that found in *sim* mutant embryos.

Figure 3.3

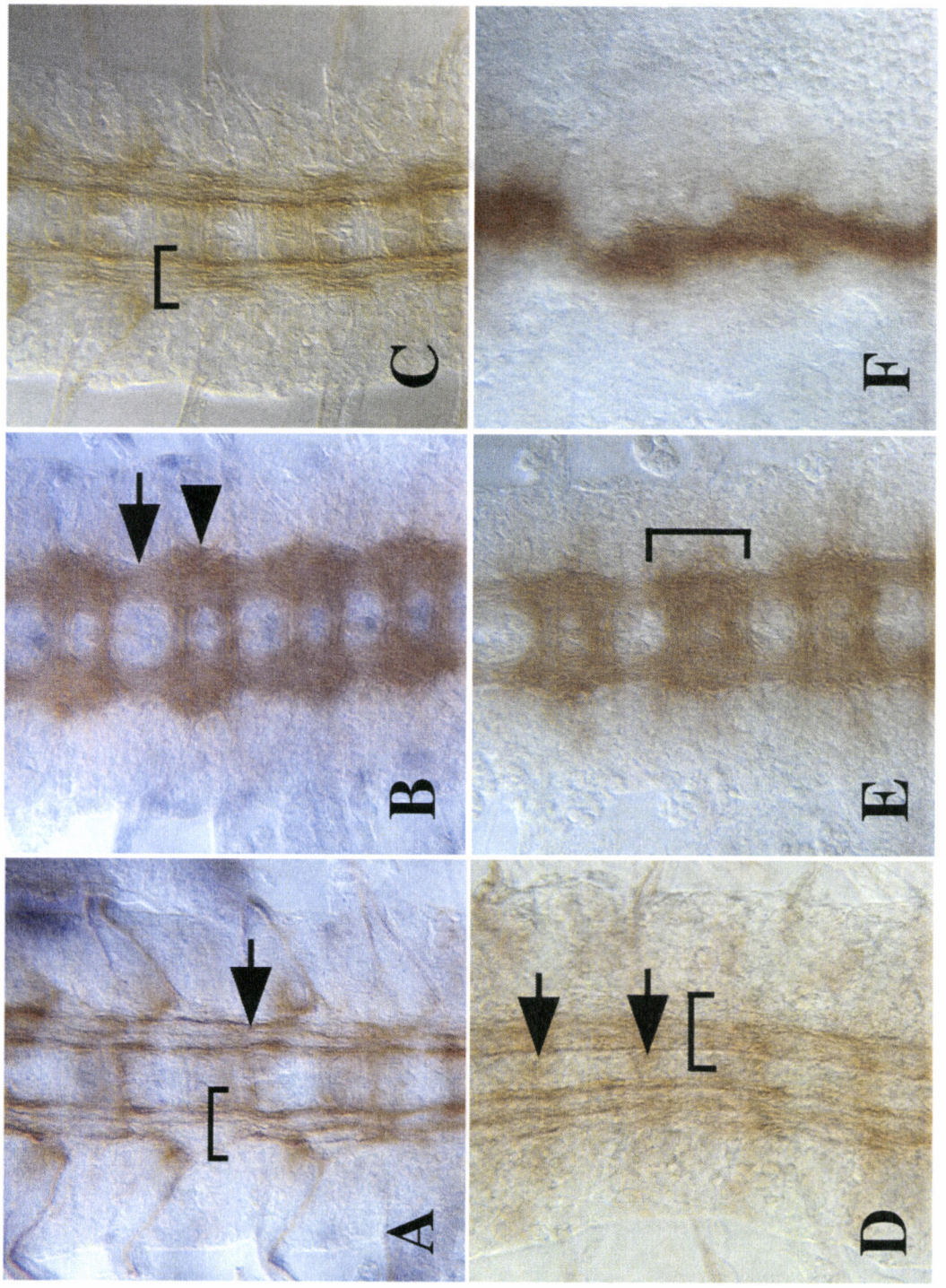


Table 3.3. Phenotypic analysis of FasII and BP102 positive axonal tracts in double heterozygous embryos with *fru* and genes expressed in lateral glia, *ttk*, *gcm* or *pnt*.

A. Percentage of embryos with defective FasII-positive fascicles

Genotype	Percentage of embryos with FasII axonal defasciculation	Percentage of embryos with axonal defasciculation and midline crossing axons	Percentage of embryos with wildtype FasII axonal tracts
ttk^{02667}+/ + fru^{AJ96u3}	33.3 (n=24)	0	66.7
$ttk^{D2\text{-}50}$+/ + fru^{AJ96u3}	20.7 (n=29)	0	79.3
ttk^{02667}+/*Bal* or + fru^{AJ96u3}/ *Bal*	4 (n=54)	0	96
$gcm^{N7\text{-}4}$/ +; fru^{AJ96u3}/+	19.6 (n=55)	0	80.4
$pnt^{\Delta 88}$+ / + fru^{AJ96u3}	2.9 (n=35)	0	97.1
$pnt^{\Delta 88}$+ /*Bal* or + fru^{AJ96u3}/*Bal*	4.9 (n=41)	0	95.1

B. Percentage of embryos with defective BP102-positive tracts

Genotype	Percentage of embryos with defects in BP102 tracts	Percentage of embryos with aberrant longitudinal tracts	Percentage of embryos with aberrant commissural tracts	Percentage of embryos with wildtype BP102 tracts
ttk^{02667}+/ + fru^{AJ96u3}	26.7 (n=30)	26.7	0	73.3
$ttk^{D2\text{-}50}$+/ + fru^{AJ96u3}	93.6 (n=31)	93.6	0	4.4
ttk^{02667}+/*Bal* or + fru^{AJ96u3}/*Bal*	2.6 (n=31)	2.6	0	97.4
$ttk^{D2\text{-}50}$+/*Bal* or + fru^{AJ96u3}/*Bal*	9 (n=33)	9	0	91

Standard genetic crosses were made between fru^{AJ96u3}/*Bal* animals and *ttk*/*Bal*, *gcm*/*Bal* or *pnt*/*Bal* animals to make double heterozygous embryos. The analysis and notation are the same as in Table 3.1. Note that nearly all $ttk^{D2\text{-}50}$+/ + fru^{AJ96u3} embryos had abnormal longitudinal connectives and thus highly penetrant for this phenotype.

1997). For the genetic interaction test, I used both a hypomorphic and an amorphic allele, ttk^{02667} and ttk^{D2-50} (Table 3.3).

Between 20-30% of the ttk^{02667}+ / + fru^{AJ96u3} embryos had defasciculated FasII-positive longitudinal fascicles (33% ttk^{02667}+ / + fru^{AJ96u3}, n=24; 20.7% ttk^{D2-50} + / + fru^{AJ96u3}; n= 9; Fig. 3.3A; Table 3.3A). The tracts most affected were the lateral and intermediate tracts, since axons were found to project between these two fascicles (arrow in Fig. 3.3A). The medial tracts were more wild-type but were not uniform in size. No FasII axons or fascicles were found to cross the midline. The BP102-positive longitudinal connectives and commissural tracts were also not uniform in size with the connectives being thinner between segments and thicker next to the commissures (26.7% ttk^{02667}+ / + fru^{AJ96u3}, n=30; 93.6% ttk^{D2-50} + / + fru^{AJ96u3}; n= 31, Fig. 3.3B; Table 3.3B). These findings show that reduced dosage of Ttk and FRU proteins lead to the defasciculation of FasII-positive axons. This defasciculation may be due to the inappropriate differentiation of lateral or midline glia cells or a weakening of the proper glia-neuronal interaction that occurs during axonal pathfinding. Since the *ttk* gene functions in the repression of neuronal gene expression in lateral glia cells, we infer that *fru* might be involved in the same genetic cascade.

To further define *fru*'s role in lateral glia, another gene in *ttk's* genetic pathway was tested for its interaction with *fru*. *ttk* is known to be under the direct control of master regulator of glial cell fate, *glial cells missing* (*gcm*), which is expressed in the progenitors of lateral glial cells such as neuroglioblasts within neuroectoderm (Giesen et al., 1997). About 20% of the gcm^{N5-7}+ / + fru^{AJ96u3} embryos had defasciculated Fas II-positive tracts (n=55, Fig. 3.3C, Table 3.3B). The degree and morphology of the FasII-positive fascicles in these double heterozygotes was very similar to that of the ttk^{-}+ / + fru^{-} heterozygotes described above. The medial fascicles were thinner but the lateral and intermediate fascicles were completely defasciculated without any noticeable axons that cross the midline (Fig. 3.3C). This result indicates that *fru* functions somewhere in the genetic pathway involving *gcm* and *ttk*.

In the development of lateral glia, *gcm* has the dual role of suppressing neuronal gene expression through a *ttk* branch and enhancing the expression of glial genes through the activity of a different transcription factor, *pointed* (*pnt*; Kleas et al., 1994). *pnt* encodes two ETS isoforms: *pointP1* is only expressed in lateral glia and acts as a constitutively active transcription factor, and *pointP2* is found only in midline glia and requires phosphorylation by MAPkinase to become a potent transcriptional activator (Klämbt, 1993; Brunner et al., 1994; O'Neill et al., 1994). Since *pnt* is downstream of *gcm* but in a genetic pathway independent of *ttk*, I expected that the embryos doubly heterozygous for *pnt* and *fru* would have wild-type FasII-positive fascicles (Giesen et al., 1997). The double heterozygotes were made using an amorphic *pnt* allele, $pnt^{\Delta 88}$. As expected, the doubly heterozygous *pnt* and *fru* embryos ($pnt^{\Delta 88}$+ / + fru^{AJ96u3}, n=35; Table 3.3A) showed normal differentiation of Fas II positive tracts (Figure not shown). This finding suggests that *fru* does not appear to interact with *pnt* in either lateral or midline glia. Thus, *fru* is likely in the *gcm* pathway but may only be involved in the *ttk* branch to suppress neuronal differentiation and not in the *pnt* branch to initiate glial differentiation.

fru may be required for the specification of midline glia.

Since FRU proteins are expressed in mesectodermal cells and later midline glia and appeared to be involved in the gcm pathway, I wanted to determine whether fru also might have functions at earlier stages of midline glial cell determination (Chapter 2). The *single-minded* (*sim*) gene is an important regulator of midline glial cell fate. *sim* is expressed in the mesectoderm but becomes restricted only to midline glia. Based on mutant analysis, the *sim* gene has been considered a master regulatory gene of mesectodermal lineage (Crews et al., 1988; Nambu et al., 1991).

To examine *fru*'s function in midline glia, transheterozygotes were made with *fru* and the *sim* gene. Since embryos mutant for genes, such as *pnt* and *karussell*, involved in the development of midline glia showed *robo*-like mutant phenotypes in which FasII-positive axons crossed the midline, I examined FasII-

positive fascicles in the doubly heterozygous $sim^{-}+/+fru^{-}$ embryos (Hummel et al., 1999). In 12 % of the doubly heterozygous embryos ($sim^{2}+/+fru^{AJ96u3}$, n=20; Fig. 3.3D; Table 3.2A) showed defasciculated FasII fascicles in which there were no longer three obvious fascicles. In these heterozygotes, there were midline crossing FasII projections, which means that there was robo-like phenotype. Therefore, these finding is consistent with the hypothesis that proper formation of midline glia is important to proper axonal projections (Hummel et al., 1999).

To further analyze *fru*'s role, I found that 39 % of $sim^{2}+/+fru^{AJ96u3}$ embryos had defective longitudinal and commissural axonal pathways as observed by BP102 staining (n=28; Table 3.2B). Several different defects were found in these embryos. In some embryos, all of the axons were confined to a single band along the midline (11%, Fig. 3.3F; Table 3.2B, S notation); these were the only embryos to have this phenotype. The inability of axons to escape from the midline is a common phenotype of *sim* loss-of-function mutants and has been found in a small fraction of *fru* mutant embryos (Seeger et al., 1993). Another common phenotype in $sim^{-}+/+fru^{-}$ embryos was that the anterior and posterior commissures appeared to fuse (21%, Fig. 3.3E; Table 3.2B). The presence of fused commissures has also been found in mutants in which the migration of midline glia cells between the two commissures is abnormal (Hummel et al., 1999). The similarity of the phenotypes in these heterozygous embryos and *fru* and *sim* mutants suggests that *fru* likely functions in the same genetic pathway with *sim* to specify the cell fate of midline glia or the differentiation of developing midline glia, but apparently not via production or secretion of the Slit gradient.

Figure 3.4. A model of *fru*'s role and genetic hierarchy in guiding contralateral axonal projections with genes expressed in neurons.

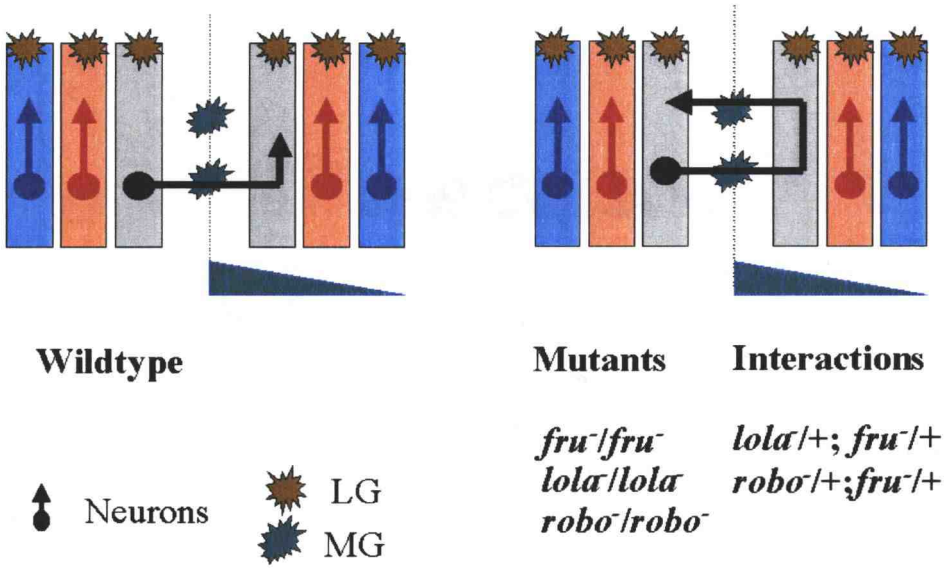
A. In wildtype (left panel), the axonal projection of a contralateral neuron (in black) crosses the midline and extends its process on the other side of the CNS due to the activity of Robo receptors responding to Slit repulsive signal (Reverse triangle in green). In *fru*, *lola* or *robo* mutants and in embryos doubly heterozygous for *lola* or *robo* and *fru*, the processes of contralateral neurons re-cross the midline likely due to reduced activity of the Robo receptor and consequently a reduction in the response to the Slit repulsive signal (right top). Note that both *fru* mutants and doubly heterozygous embryos have a normal Slit gradient. Ipsilateral neurons (colored circles with arrow) form three longitudinal fascicles; the lateral fascicle is sky blue, the intermediate fascicle is pink and the medial fascicle is gray. LG, lateral glia in brown. MG, midline glia in green. Dotted line, midline. Symbols and abbreviation are the same until specified.

B. Genetic hierarchy in neurons

Three genetic models would account for the data. In all cases, Robo, a receptor protein, is considered to be a downstream target gene In Case I, *fru* is downstream of *lola* but upstream of *robo*. In Case II, *fru* is upstream of *lola*. In Case III, *fru* and *lola* work synergistically to control the activity of *robo*. A question mark is used to indicate which interactions are proposed.

Figure 3.4.

A. Model of *fru*'s role in guiding contralateral axonal projections with genes expressed in neurons



B. Genetic hierarchy in guiding contralateral axonal projections with genes expressed in neurons

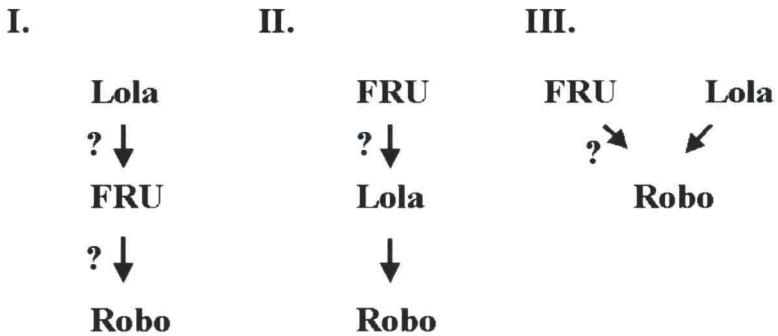


Figure 3.5. A model of *fru*'s role and genetic hierarchy in guiding longitudinal axonal projections with genes expressed in neurons and midline glia.

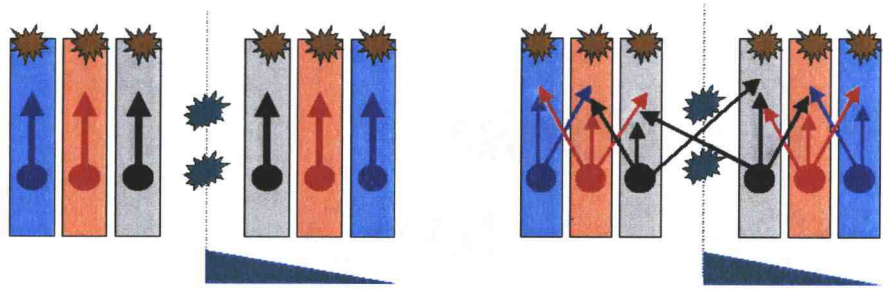
A. In the wildtype embryonic CNS (left panel), three longitudinal FasII fascicles are formed by the appropriate axonal projections of ipsilateral neurons (arrows). In *fru*, *lola* or *robo* mutants and in the embryos doubly heterozygous for *fru* and *lola*, *comm* or *robo*, axons in each fascicle take an inappropriate pathway between fascicles or between hemisegments. The subsequent result is defasciculation. Note that in the embryos doubly heterozygous for *fru* and *comm*, midline crossing axons were not found and defasciculation in longitudinal tracts has not been reported in *comm* mutant embryos.

B. Genetic hierarchy in neurons

In a similar fashion to that proposed for pathfinding by contralateral neurons, the reduced activity of Robo in ipsilateral neurons is suggested to cause their growth cones to take inappropriate pathways likely due to their insensitivity to long-range repulsive signals and consequently interfere with the formation of FasII fascicles. Since Comm and Robo form a complex that leads to the clearance of Robo from the cell surface of axons (M. Seeger, unpublished data), Comm protein activity is in the same level as Robo. Thus similar models of genetic hierarchy in neurons may be working for defasciculation shown in Fig. 3.4B. T bars between Comm and Robo indicate downregulation of Robo activity through the clearance of Robo from the cell surface.

Figure 3.5.

A. Model of *fru*'s role in guiding longitudinal axonal projections with genes expressed in neurons and midline glia



Wildtype **Mutants** **Interactions**

Neurons LG MG

fru⁻/fru⁻ ***lola⁻/+; fru⁻/+***
lola⁻/lola⁻ ***robo⁻/+;fru⁻/+***
robo⁻/robo⁻ ***comm⁻ +/+ fru⁻***

B. Genetic hierarchy in guiding longitudinal axonal projections with genes expressed in neurons

I.

Lola
?↓
FRU
? ↙ ↘ ?
Comm ⊣ Robo

II.

FRU
?↓
Lola
? ↙ ↘ ?
Comm ⊣ Robo

III.

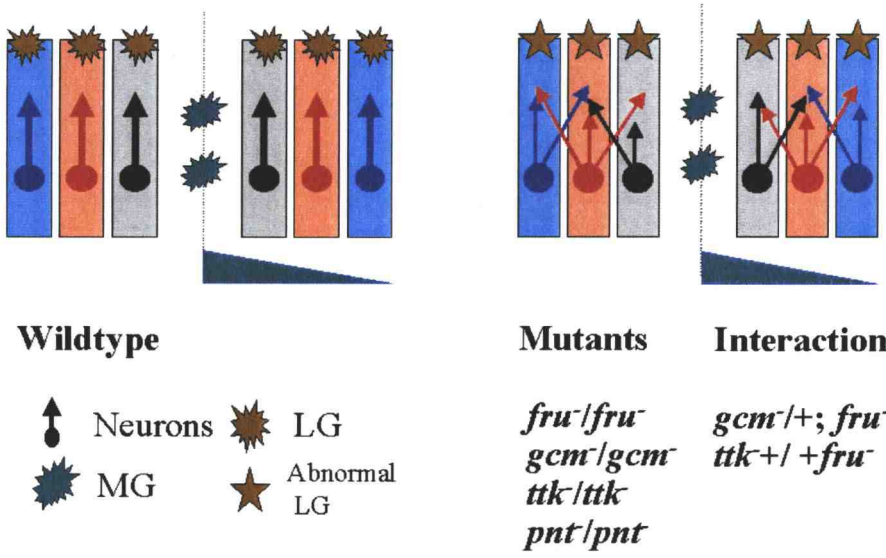
FRU **Lola**
? ↘ ↙ ?
Comm ⊣ Robo

Figure 3.6 A model of *fru*'s role and genetic hierarchy in guiding longitudinal axonal projections with genes expressed in lateral glia.

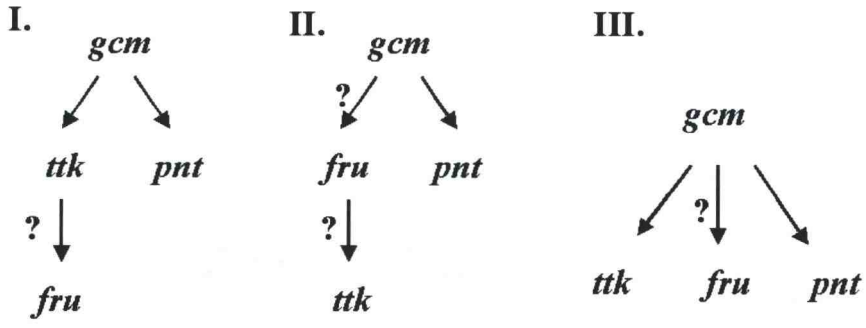
A. In wildtype embryos, lateral glia (brown starburst) enwrap three FasII axonal fascicles. In *fru*, *gcm* or *ttk* mutants and in embryos doubly heterozygous for *fru* and *gcm* or *ttk*, lateral glia may develop abnormally (brown stars) and consequently lose their ability to wrap the axonal fascicles, resulting to axonal defasciculation. Alternatively, abnormal glia may not act as guideposts for growth cone elongation of neurons and as a result axons take inappropriate pathways, consequently leading to FasII axonal defasciculation.
B. Potential genetic hierarchy in lateral glia
Because of the central role of *gcm* in the differentiation of lateral glia and since lateral glia cells are present in *fru* mutants, I postulate that *fru* is downstream of *gcm* and in the pathway with the same pathway as *ttk* but in a different pathway from *pnt*. However, *ttk* may be directly downstream of gcm and upstream of *fru* (case I). Alternatively, *fru* may be the direct target of *gcm* and upstream of *ttk*. (case II). Finally, *fru* may be in a separate pathway from *ttk* and would then work synergistically with *ttk* on downstream targets.

Figure 3.6.

A. Model of *fru*'s role in guiding longitudinal axonal projections with genes expressed in lateral glia



B. Genetic hierarchy in guiding longitudinal axonal projections with genes expressed in lateral glia



Discussion

fru functions in controlling axonal guidance across the midline along with *robo* and *comm.*

By examining doubly heterozygous embryos, these studies show that the simultaneous reduction of the wildtype dose of *fru* and the dose of the genes *robo* and *comm* leads to axonal pathfinding defects. In these embryos, FasII-positive axons, which should travel longitudinally in the CNS, grow across the midline as well as defasciculate. Both Robo and Comm are part of the molecular mechanism by which neurons receive and respond to Slit, a repulsive signal secreted by glia cells along the midline. In the current model, the Robo receptor protein on the surface of growth cones and axons projecting in longitudinal pathways binds the Slit ligand and causes growth cones to avoid turning to cross the midline and so continue along a longitudinal pathway (Kidd et al., 1999; Simpson et al., 2000a, 2000b; Rajagopalan et al., 2000a; 2000b). In addition, commissural axons express the Robo receptor on their growth cones after they have crossed the midline, which acts to prevent the axons from re-crossing the midline (Kidd et al., 1998b). Based on this model, the expectation is that when levels of Robo protein on axons is reduced or fewer Robo receptors are activated, those axons will more likely cross the midline or re-cross the midline. As these phenotypes are observed in the *robo*$^-$/+; *fru*$^-$/+ embryos, it is suggested that there has been a reduction in Robo activity in these embryos. A growth cone with reduced Robo activity is, thus, expected to be rendered less sensitive to the Slit ligand, so that the axons cross or re-cross the midline (Fig. 3.4A, 4B).

There are several possible explanations for how the reduction in FRU might lead to the reduction in Robo activity. The first possibility is that because Robo and FRU are both expressed in neurons, the reduction in the amount of FRU protein leads to a reduction in the levels of Robo protein in neurons below the threshold required to prevent axons from inappropriately crossing the midline. Since FRU likely functions as a transcription factor and Robo is a membrane bound receptor, FRU may regulate Robo activity by directly or indirectly controlling the

transcription of the *robo* gene (Fig. 3.4B; Kidd et al., 1998a). However, it is unlikely that by itself FRU functions as a major controller of Robo transcription or activity, since even *fru* null mutants do not have as severe a midline crossing phenotype as that found in *robo* mutants. It is striking that a higher percentage of double heterozygous embryos of both genotypes, *robo*$^-$/+; *fru*$^-$/+ and *comm*$^-$+/ + *fru*$^-$, had defects, and more segments in these embryos were affected than found for *fru* null mutants. These results suggest that FRU is a partner that helps to fine-tune the expression of genes in axonal patterning, such as *robo* and *comm*. From the data presented here, other molecular mechanisms by which FRU might control the level of Robo protein or its activity within the same cell type cannot be ruled out.

The second possibility for why there are midline crossing FasII-positive axons is that FRU is involved in the production or secretion of the Slit ligand or has some role in the differentiation of midline glial cells. The expectation is that a reduction in the amount of Slit secreted would lead to more axons crossing the midline. However, in *slit*$^-$/+; *fru*$^-$/+ heterozygotes, the FasII- and BP102-positive axonal tracts were wildtype indicating that *fru* is unlikely to be involved in the production or release of the Slit ligand. FRU proteins do, however, appear to have a role in the specification of the MECs, since *sim*$^-$+/ + *fru*$^-$ embryos have defects, thus *fru* may affect the development of the midline glia but does not seem to affect the ability of these cells to generate a Slit repulsive signal.

The third possibility is a more complicated one in which FRU might regulate the level of Comm protein. Comm protein is expressed in neurons and midline glia and along with the secreted attractive signals, such as the D-Netrin ligand, is involved in allowing commissural axons to cross the midline (Kidd et al., 1996; Kidd et al., 1999; Harris et al., 1996; Mitchell et al., 1996; M. Seeger, unpublished data). It is thought that Comm and Robo form a complex that leads to the clearance of Robo from the cell surface of axons (M. Seeger, unpublished data). For the observed phenotypes in the *robo*$^-$/+; *fru*$^-$/+ heterozygotes, a reduction in FRU would need to result in an increase in the level or activity of the Comm protein. In this way, the combination of a reduction in Robo due to the presence of a single *robo*$^+$ gene in the *robo*$^-$/+; *fru*$^-$/+ heterozygote coupled with the increase in

Comm would result in more axons crossing the midline. The failure to find any FasII axons that crossed the midline in the *comm*$^-$+/ + *fru*$^-$ does not support the hypothesis that there was an increase in Comm protein in these heterozygotes. Instead, these *comm*$^-$+/ + *fru*$^-$ heterozygotes had an extreme defasciculation phenotype showing that there was an effect of reducing Comm function in these doubly heterozygote embryos (see below). Since Robo activity on cell surface of axons depends on Comm protein, the reduced dose in Comm protein then leads to less clearance of the Robo receptor and subsequently makes the growth cone much sensitive to the Slit repulsive signal. As a result, an defasculated phenotype was found in the *comm*$^-$+/ + *fru*$^-$ heterozygotes. Therefore, it is suggested that like that of Robo protein, the expression of Comm is likely dependent on *fru* expression (Fig. 3.5B). However, the mutation used to reduce Comm function is a strong hypomorph but not an amorphic allele and it remains possible that the level of Comm protein was not reduced sufficiently so that unlike the absence of commissural tracts in comm mutant embryos commissural axons was found in the *comm*$^-$+/ + *fru*$^-$ heterozygotes embryos as observed by BP102 staining (Tear et al., 1996).

The only other genotype of heterozygotes in which FasII axons incorrectly crossed the midline was the *lola*$^-$/+; *fru*$^-$/+. These heterozygotes had defects in FasII and BP102 axonal tracts that were similar to the phenotypes in both *lola* and *fru* mutant embryos (Chapter 2; Giniger et al., 1994). *lola* and *fru* are both potential transcriptions factors that are also both expressed in neurons and midline glia (Chapter 2; Giniger et al., 1994). There are two types of mechanism by which Lola and FRU proteins might interact to control transcription of downstream genes involved in axonal pathfinding. In one, FRU proteins might directly regulate the transcription of the *lola* gene or Lola proteins might regulate transcription from P3 and/or P4 *fru* promoters (Fig. 3.4B-I). Alternatively, FRU and Lola proteins might form hetero-dimers through their BTB/POZ domains, and thus might both bind to the promoter of the same downstream target genes (Fig. 3.4B-III). From the *robo*$^-$ /+; *fru*$^-$/+ results, one target gene is *robo*. This hypothesis is supported by the evidence that the double heterozygous *robo* and *lola* embryos show *robo* mutant-

like phenotype and also that *lola* mutant embryos have an approx. 40-50% reduction of the expression of the Robo protein (E. Giniger, personal communication). Another interesting finding is that a small percentage of $lola^{ORE76}/+; fru^{AJ96u3}$ embryos had segments in which no commissures were formed. Considering the midline crossing phenotypes of *robo*, *comm*, *lola* and *fru* heterozygotes, it appears that FRU is involved in regulating Robo function and that this regulation may be in conjunction with the *lola* gene.

fru functions in controlling axonal fasciculation of FasII axons along with genes expressed in neurons and lateral glia.

The other phenotype found in all but two of the heterozygote genotypes was defasciculation of the FasII axons. Defasciculated FasII axons were found in the same $robo^-/+; fru^-/+$, $comm^-+/+fru^-$ and $lola^-/+; fru^-/+$ embryos in which FasII axons aberrantly crossed the midline. The defasciculation phenotype in $lola^-/+; fru^-/+$ embryos was expected since *fru* and *lola* mutant embryos have defasciculated FasII axons (Fig. 3.5A, 5B; Chapter 2, Giniger et al., 1994; Madden et al., 1999). Since FasII axons travel in the lateral parts of the neuropil, lateral glia and neurons are likely to be largely responsible for the cellular interactions that facilitate the adhesion of longitudinal axons as they elongate their axonal processes. If *lola* and *fru* are functioning in the same cell, then it is likely that they will act as transcription factors in neurons regulating the ability of FasII axons to recognize other FasII axons or for FasII axons to recognize glia and promote fasciculation.

The presence of defasciculated FasII axons in $robo^-/+; fru^-/+$ and $comm^-+/+fru^-$ embryos was entirely unexpected, since *robo* and *comm* functions have only been established for how axons respond to midline signals (Tear et al., 1996; Kidd et al., 1998a, 1998b). A review of the published data does show defasciculation in $robo^1$, $robo^2$ and $robo^3$ mutant embryos (refer to Fig. 3 in Rajagopalan et al., 2000a and 2000b). The proper formation of wildtype longitudinal fascicles can be controlled by the coordinated expression of Robo1, Robo2 and Robo3 proteins in neurons (Rajagopalan et al., 2000a and 2000b; Simpson et al., 2000a and 2000b); as like axonal behavior of contralateral neurons mediated by Robo responsive to

Slit repulsive protein, that of ipsilateral neurons that form longitudinal fascicles are Robo-mediated. Thus, it is possible that the reduced level of Robo in ipsilateral neurons of embryos doubly heterozygous for *fru* and *robo* leads to their axons aberrantly projecting to inappropriate fascicle pathway due to their insensitivity to long-range repulsive signals and consequently interfere with the formation of FasII fascicles (Fig. 3.5A, 5B). Since *robo* is transiently expressed in lateral glia in stage 13 (Kinrade et al., 2001), it may be that the simultaneous reduction in Robo and FRU proteins in lateral glia may affect the ability of FasII axons to fasciculate. Alternatively, reduction in the level of Robo in lateral glia and FRU in neurons may lead to a defasciculation phenotype or midline-crossing axon phenotype.

A similar situation might happen to explain the phenotype of *comm*$^-$+/ + *fru* embryos. Since Comm is required for the removal of Robo proteins on cell surface and its activity is associated with that of Robo proteins (M. Seeger, unpublished data), the reduced Comm proteins in *comm*$^-$+/ + *fru* embryos leads to axons with more Robo proteins that are much sensitive to Slit signals and then less commissural axons cross the midline and consequently these commissural axons interfere with longitudinal projection of Fas II fascicle forming ipsilateral neurons. Alternatively, Comm may be transiently expressed in other cell types during neurogenesis (Tear et al., 1996). It is not known whether *comm* mutants have defasciculated FasII axons. It is interesting that the primary defect in *robo*, *comm*, *lola* and *fru* heterozygotes is the defasciculation of FasII axons. Further, a higher percentage of heterozygotes have defasciculated FasII axonal tracts than do *fru* mutants.

fru's function in the development of lateral glia.

A surprising finding in this study is that when the dose of *fru* was reduced along with the dose of the genes, *gcm* and *ttk*, which are expressed in lateral glia, FasII-axons were unable to maintain wild-type fasciculation (Fig. 3.6A, 6B). Since FRU proteins are expressed in lateral glial cells, it is possible that all three genes are part of a regulatory hierarchy controlling the glial cell development or aspects of glial differentiation that affects the way glial cells mediate axonal properties.

gcm is a master regulator of lateral glial cell fate specification (Hosoya et al., 1995; Jones et al., 1995) and *ttk* is a downstream target gene of *gcm* and required for the survival of lateral glia and to repress neuronal fates in cells destined to become glia (Giesen et al., 1997). In contrast with *fru*'s interaction with *ttk*, *pnt* + / + *fru* heterozygous embryos had wildtype FasII and BP102 tracts. Since pnt is also known to be a downstream target gene of *gcm* and required for normal differentiation of lateral glia by activating glia specific function, the finding that *fru* does not genetically interact with *pnt* suggests that *fru* and *pnt* is not in the same genetic pathway (Fig. 3.6B).

In parallel with its role in the expression of the *ttk* gene, *gcm* might be a transcriptional regulator of *fru* gene transcription from the P3 and P4 promoters, which generate the relevant *fru* transcripts. If that were the case, then FRU proteins might act as partners with Ttk proteins through their BTB/POZ domains to regulate the transcription of downstream genes in glial cells that affect the ability of FasII-positive axons to fasciculate or the development of lateral glia (Fig. 3.6B). Alternatively, FRU proteins might directly or indirectly regulate *ttk* gene expression or TTK proteins might regulate *fru* transcription (Fig. 3.6B). Another possible mechanism to explain the interaction is that FRU function might be required in neurons and Gcm and Ttk functions are required in glial cells and that the mutant phenotypes arise because of the reduction in these genes in the two different cell types affect their cell-cell communication. One possible mechanism is that when *ttk* function is reduced in glial cells, these cells may express neuronal proteins on their membranes that provide an adhesive surface for FasII axons to grow along and thus compete with the normal fasciculation partners of FasII axons. In *fru* mutants, *even-skipped* (*eve*) expression was occasionally not maintained, which may affect the ability of these cells to maintain their neuronal identity (Chapter 4). Thus, in *fru* mutants and in these double heterozygotes between *fru* and *gcm* or *ttk*, it is likely that lateral glia cells fail to effectively repress the expression of neuronal signals or adopt an appropriate glial fate leading to the inability of FasII axons to properly fasciculate.

fru functions in the development of midline glia along with the *sim* gene.

Another interesting finding is that *fru* was involved in the regulation of the development of midline cells. *sim* plays a crucial role as a master regulator of mesectodermal lineages including midline glia specification (Crews et al., 1989; Nambu et al., 1991; for review, Jacobs, 2000). The genetic interaction tests between *fru* and *sim* revealed a *sim*-like phenotype and other defects in commissural tracts. While the loss of *sim* function leads to loss of midline glia, interestingly the loss of *fru* function is not required for the survival of those cells (Chapter 2). Since both *fru* and *sim* are expressed in mesectodermal cell and later midline glial cells, these findings do not indicate at which stages of midline glia development *fru* and *sim* functions are required. If *fru* functions early during the period when the mesectodermal cells are specified, *fru* may then be part of either the *snail* and *twist* pathway or the Notch pathway that initiates the transcription of the *sim* gene (Kosman et al., 1991; Morel and Schweisguth, 2000). If *fru* functions later then it may partner with the *Tango* protein, which is known to dimerize with Sim to activate downstream gene expression in midline glia (Sonnenfeld et al., 1997). It should be noted that *fru* is not involved in the differentiation of midline glia through the activity of Pointed transcription factor or in the production of the Slit signal. In doubly heterozygous *pnt* + / + *fru* or *slit*/+; *fru*/+ embryos, the commissures were wildtype. On the other hand, *lola*, which also interacts with *fru*, genetically interacts with *slit* (E. Giniger, personal communication), suggesting that *lola* may have a different role from *fru* in the midline glial differentiation. Thus, *fru* has a role in the development of midline glia in a pathway that does not include *pnt* and *slit*.

fru's function in axonal pathfinding

In this study, I have demonstrated that *fru* is involved with genes that are known to function in neurons and glia and to control axonal pathfinding and fasciculation in the embryo. Many questions still remain. First, although Fru itself is a putative transcription factor, I as yet have no evidence as to whether Fru primarily act as a transcription factor along with others, such as Lola, Ttk, and Sim,

for controlling downstream target gene expression in neurons, lateral and midline glia. Moreover, I have only examined the phenotypes in several double heterozygotes and not tested whether or not the expression of a interacting gene is affected in *fru* mutant background or vice versa. To distinguish function of *fru* isoforms derived from either P3 or P4 promoter, I need to use other *fru* alleles that take out the transcripts from P3 promoter and/or other techniques such as RNA interference to remove specific *fru* isoforms. In addition, I need use different alleles of genetic interactors to ascertain whether there is allelic difference. It remains possible that *fru* affects the expression of other guidance proteins and other transcription factors participating in the differentiation of neurons, lateral glia and midline glia. In this study, FRU reveals that it is involved with multiple guidance factors and acts in more than one cell type to orchestrate the delicate balance of particular guidance decisions.

CHAPTER 4

THE *fruitless* GENE IS REQUIRED FOR MAINTAINING NEURONAL IDENTITY IN *evenskipped*-EXPRESSING NEURONS IN THE CNS DURING DROSOPHILA EMBRYOGENESIS.

Ho-Juhn Song and Barbara J. Taylor.

Abstract

We have previously shown that the *fruitless* (*fru*) gene functions in the formation of wildtype FasII and BP102 axonal tracts during embryonic CNS development in *Drosophila*. To address whether *fru* may also have another role in the embryonic CNS, I examined the development of Eve-expressing ganglion mother cells (GMCs) and neurons in *fru* mutant embryos. In *fru* mutant embryos the positioning of Eve-positive GMCs was abnormal with respect to their normal anterior-posterior position in a segment. These Eve-positive GMCs produce Eve-positive neurons, but many of the these neurons were unable to maintain Eve expression. In addition, other neurons that normally do not express Eve became Eve-positive in *fru* mutants. These defects were rescued in *fru* mutant embryos that expressed *fru* transgenes under the control of a pan-neuronal *sca*GAL4 driver. From these phenotypic analyses and rescue experiments, I conclude that *fru* gene function is required to maintain neuronal identity rather than establishing neuronal fate.

Introduction

Development of the embryonic central nervous system (CNS) in *Drosophila* is characterized by the sequential production of precursors called neuroblasts (NBs), which arise from the neuroectoderm (reviewed in Goodman and Doe, 1993; Bhat, 1999; Skeath, 1999). At the first step in this process of neurogenesis, neuroblasts delaminate from the neuroectoderm, chosen through an active process that mediates the balance of proneuronal and neurogenic gene activity within the presumptive NB and its neighbors (for review, Skeath, 1999; Bhat, 1999; Campos-Ortega, 1995). Each NB emerges from among a small cohort of equivalent neuroectodermal cells. The expression of the proneuronal genes, such as those in the *achaete-scute* complex, in a cluster of about six neuroectodermal cells, thus defines the equivalence group, whereas, the neurogenic genes, such as Delta and Notch, antagonize proneuronal gene function and lead to NB formation. Competition between neuroectodermal cells for the ligand, Delta, which binds to the Notch cell surface receptor, results in the selection of one cell becoming the NB from each cluster (for review, Skeath, 1999; Bhat, 1999; Skeath and Carroll, 1992; Martin-Bermudo et al., 1995).

Once delaminated, each NB produces a series of intermediate precursors, called ganglion mother cells (GMCs), from repeated asymmetric division. During each division, specific proteins and mRNAs, such as Numb, Miranda, Staufen and Prospero, are differentially segregated into the GMC (Spana et al., 1995; Shen et al., 1997; Li et al., 1997; Broadus et al., 1998; Doe et al., 1998). It is likely that the action of intrinsic determinants distributed at this division contributes to the specification of each GMC's fate. The last step of neurogenesis is the division of each GMC to produce a pair of postmitotic neurons or glia. In most cases, two sibling neurons differ in their gene expression and/or axonal projections and are thus identifiable (Spana and Doe, 1996). The mechanisms by which GMCs divide to produce sibling neurons with different identities are not well understood, but the distribution of cellular determinants, such as that occurring in GMCs, is likely to be involved (Buescher et al., 1998; Skeath and Doe, 1998).

Once generated, neurons differentiate by process outgrowth to form both dendrites and axons (Schmid et al., 1999; Spana and Doe, 1996). Depending on the neuron, its axons may grow out along a tract ipsilateral to its cell body or cross the midline to innervate targets located on the side contralateral to its cell body. Thus growing axons must recognize, grow toward and then choose between crossing or not crossing the embryonic midline (Tear, 1999). These choices for process outgrowth are made with by coordinated interactions between the axonal growth cone and lateral and midline glia or other neuronal processes (Booth et al., 2000; Hidalgo and Booth, 2000). The end result is the formation of a neuronal typical morphology. The organization of these axonal tracts and the resulting axonal scaffold within the *Drosophila* CNS can be visualized by several different neuronal markers (Goodman and Doe, 1993).

There are many genes involved in making or keeping the proper organization of the axonal scaffold in the embryonic CNS. Among the most important genes are those expressed in neurons and associated with axonal pathfinding (for review, Rusch and Van Vactor, 2000 and Tear, 1999), genes expressed in lateral glia and involved in maintaining the scaffold structure (Giesen et al., 1997; for reviews, Granderath and Klämbt, 1999; Lemke, 2001), and genes expressed in midline glia encoding chemo-attractive or -repulsive factor that guide axons toward or away from the midline (for review, Jacobs, 2000; Harris et al., 1996; Mitchell et al., 1996; Hiramoto et al., 2000; Brose and Tessier-Lavigne, 2000).

One of the genes that plays a role in axonal patterning in the embryonic CNS is the *fruitless* (*fru*) gene (Chapter 2 and 3). The *fru* gene produces a complex set of sex-specific and sex-nonspecific transcripts generated by differential promoter usage and alternative splicing at the 5' and 3' ends (Ryner et al, 1996; Ito et al., 1996; Goodwin et al., 2000; Anand et al., 2001; Usui-Aoki et al., 2000; Ryner et al., in preparation). *fru* transcripts are generated from four promoters, P1, P2, P3 and P4, and encode a related set of BTB/POZ ZnF (Broad complex, Tramtrack, and Bric-a-brac /Poxvirus and Zinc finger) proteins (Ryner et al, 1996; Ito et al., 1996; Goodwin et al., 2000; Anand et al., 2001; Usui-Aoki et al., 2000;

Ryner et al., in preparation). Transcripts from the P1 promoter are produced in CNS neurons starting in the late third instar larval stage and continue through the adult (Ryner et al, 1996; Ito et al., 1996; Lee et al., 2000; Usui-Aoki et al., 2000). These P1 transcripts are translated only in male animals and control male-specific functions (Ryner et al, 1996; Ito et al., 1996; Lee et al., 2000; Usui-Aoki et al., 2000). Transcripts from the other promoters control adult viability and morphology and are expressed throughout development (Chapter 2; Lee et al., 2000; Usui-Aoki et al., 2000; Foss et al., in preparation). From mutant analysis and molecular studies, *fru* transcripts, derived from mainly the P3 and/or P4 promoters, are translated in both sexes and expressed in a variety of cell types including many neurons, glia and imaginal discs, from early embryogenesis to pharate adults (Chapter 2; Lee et al., 2000; Usui-Aoki et al., 2000; Foss et al., in preparation). The temporal and spatial expression of FRU proteins originated from the P3 and/or P4 promoter throughout fly development and the range of mutant phenotypes, which results from the disruption of these transcripts, indicate that they are multifunctional (Anand et al., 2001; Chapter 2).

During embryogenesis, FRU proteins are expressed during all stages of neurogenesis throughout the CNS (Chapter 2). Immunohistochemical analysis of axonal pathways in the CNS of *fru* mutants showed that neurons were unable to maintain their normal pattern but defasciculate and grow along different pathways (Chapter 2). Further, the axonal defects in *fru* mutant embryos were rescued by the expression of *fru* transgenes in neuronal cells or their precursors (Chapter 2). These data suggested that there might be defects in the differentiation of neurons responsible for their inability to form normal appearing axonal pathways.

In order to assess the development of neurons in *fru* mutants, I chose to look at the development of a well-characterized set of neurons, the Even-skipped (Eve)-positive neurons in the ventral CNS. A regular pattern of Eve-positive neurons is found in each abdominal hemisegment in stage 13-17 embryos as follows: the aCC, pCC and RP2 neurons are located adjacent to the dorsal midline; three ventro-medial U neurons and two or three lateral U neurons are arrayed from the ventral midline to more lateral positions; a cluster of 8-10 Eve lateral (EL) neurons are

found dorso-laterally (Broadus et al., 1995; Spana et al., 1995; Skeath and Doe, 1998). The neuroblast and ganglion mother cell precursors for all of these Eve-positive neurons are also well known (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999). Neuroblast 1-1 and its first GMC generates aCC and pCC, neuroblast 4-2 produces the RP2 neuron, neuroblast 3-3 produces the ELs and neuroblast 7-1 produces the U neurons. Since FRU proteins are also detected in all neuroblasts, GMCs and neurons it is likely that the absence of *fru* function may affect in some way the specification of neuronal cell fate, which may result in the abnormal neuronal differentiation.

In this study we report that in specific *fru* mutants Eve-positive neurons fail to maintain Eve expression and that other neurons ectopically express Eve. These phenotypes are unlikely to stem from defects in initial cell fate determination in NBs and GMCs because other markers were expressed in these cells albeit with a developmental delay. Using a *scaGAL4* driver to express *fru* transgenes panneuronally in *fru* mutant embryos, I found that the wildtype Eve-expression pattern was completely restored. Thus, *fru* likely plays a crucial role in maintaining neuronal identity and properties rather than in establishing neuronal fate.

Materials and Methods

Fly stocks and fly maintenance

Canton-S was used as the wildtype genotype. The following *fru* mutations were used: *In(3R)fru*^{1} (*fru*^{1}); *Df(3R)Cha*^{M5}(*Cha*^{M5}); *Df(3R)fru*^{440} (*fru*^{440}): *In(3R)fru*^{w12} (*fru*^{w12}); *Df(3R)AJ96*^{U3} (*fru*^{AJ96u3}); *Df(3R)fru*^{w24}(*fru*^{w24}); *Df(3R)fru*^{sat15} (*fru*^{sat15}); *Df(3R)P14* (*P14*) (Ryner et al., 1996; Ito et al., 1996; Anand et al., 2001; Chapter 2). All *fru* mutant alleles were maintained over the 'Blue Balancer' chromosome *TM3, Sb, P[ftz-lacZ]* which permitted *fru* mutant embryos to be distinguished from control siblings. A deficiency (*w; Df(3L)XDI98* [65A02-65E1]) was used to analyze the other inversion breakpoint of the *fru*^{w12} allele (Chapter 2).

For *fru* expression studies, we used lines in which one of six different *UAS-fru* transgenes (*UAS-fruA, UAS-fruB, UAS-fruC, UAS-fruMA, UAS-fruMB* and *UAS-fruMC*) had been crossed into either the *fru*^{w12} or *fru*^{sat15} mutant background (Chapter 2). In order to express *fru* in the CNS, we used *scabrous-GAL4* (*sca-GAL4*; Klaes et al., 1994). Standard crosses were used to introduce the chromosomes with the *GAL4* driver constructs into a *fru*^{w12} or *fru*^{sat15} mutant background.

Fly stocks were maintained at room temperature on an agar, sucrose, cornmeal and yeast medium with 0.1% nipagin (p-hydroxybenzoic acid methyl ester, Sigma).

Embryonic immunohistochemistry

Timed collections of embryos were prepared by standard techniques as described in Patel (1994). Embryos were staged by morphological criteria (Campos-Ortega and Hartenstein, 1997) or at early stages by the distinct Engrailed (En)-positive cell pattern in the embryonic head during neuronal specification (Schmidt-Ott and Technau, 1992). Immunohistological protocols for embryo fixation and subsequent antibody incubations were carried out according to standard protocols (Patel, 1994). To label neurons in the CNS, the following primary antibodies (except where not noted these antibodies were a gift from N.

Patel) were used: anti-*Even-skipped* (2B8, 1:30, Patel et al., 1994), anti-*Engrailed/Invected* (4D9, 1:10, Patel et al., 1989), anti-*Runt* (1:20, Dormand and Brand, 1998, from M. Bate), anti-*Fasciclin II* (1D4, 1:5, Grenningloh *et al.*, 1991), mab22C10, (1:200, Fujita *et al.*, 1982), mabBP102 (1:20, Seeger *et al.*, 1993) and anti-*Hunchback* (1G10, 1:5, Kambadur et al., 1998). All embryos were also labeled with the anti-β-galactosidase antibody (1:10,000, Cappel, NC) to identify *fru* mutant embryos (unlabeled) from control siblings (labeled).

Whole-mount and filleted embryos were viewed and photographed with a Sony DKC-5000 digital camera under DIC optics, using an Olympus Vanox-TX microscope. Composite images were assembled in Adobe Photoshop 5.0.

Statistics

The frequency of defective embryos in various *fru* mutant genotypes and wildtype embryos were analyzed statistically by one-way ANOVA using the SAS program (version 6.12; SAS Institute) and post-hoc analysis by Tukey HSD comparison.

Results

In *fru* mutants, NB and GMCs generating Eve-positive neurons are aberrant.

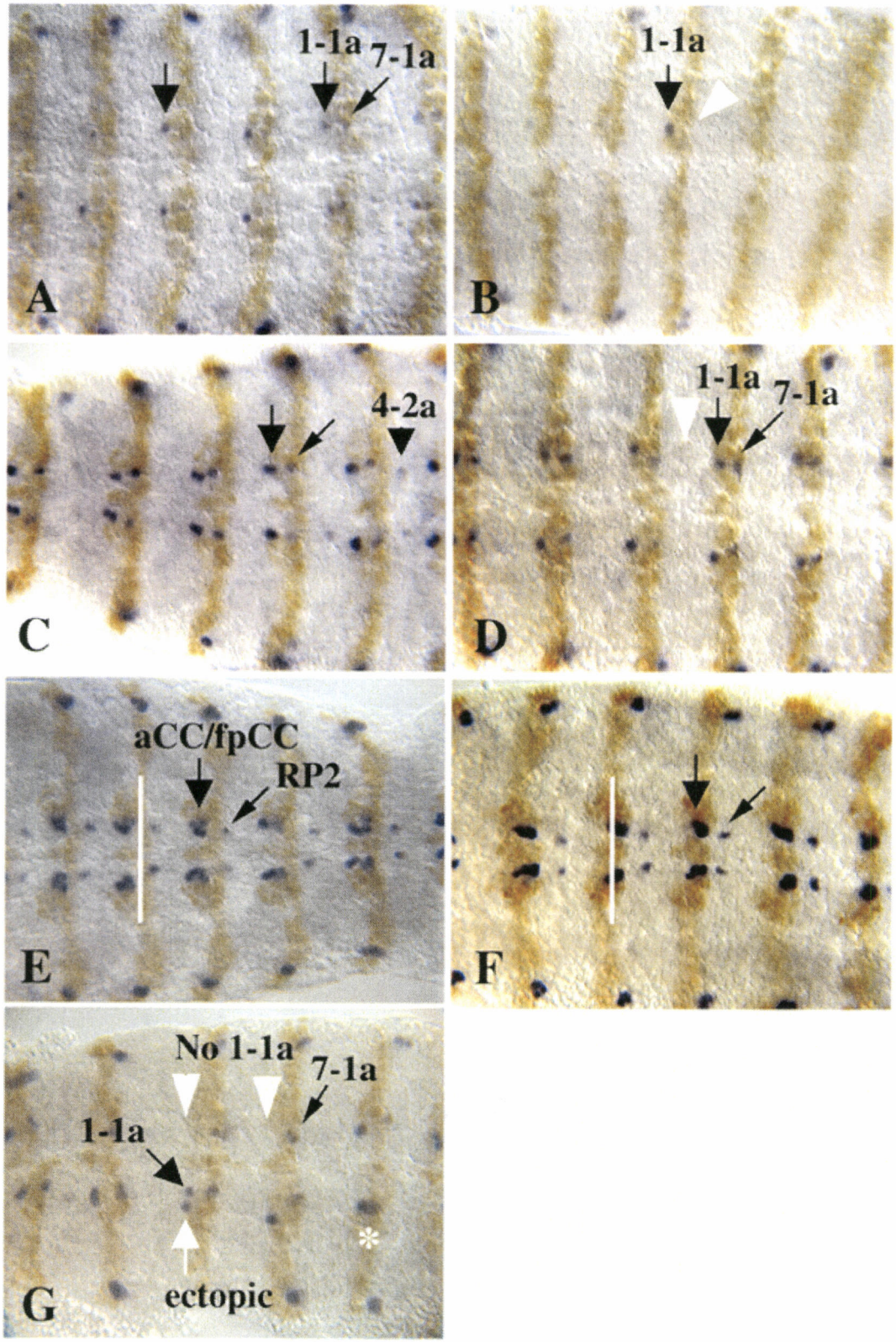
Having found that *fru* transcripts and proteins were widely expressed in the developing CNS, we used the well-established pattern and development of the *eve*-positive neurons in *fru* mutant embryos to show that *fru* functions in both early and later differentiation events in these neurons (see also Chapter 2). To follow the first stage of neurogenesis, I used an anti-Hunchback (Hb) antibody to label delaminating neuroblasts in *fru* mutant embryos (Kambadur et al., 1998). At stage early 9 when NBs start to express Hb proteins in wildtype, few NBs were labeled in *fru* mutant embryos of genotype fru^{w12}/fru^{sat15} (n=10 wildtype and n=10 *fru* mutant). At slightly later stage 9 NBs are normally expressing Hb in *fru* mutant embryos (Figure not shown).

To determine whether the delay in expression of hunchback protein in NBs in *fru* mutants might lead to subsequent changes in their progeny, I used anti-Eve antibody to label specific GMCs in stage 10-11 *fru* mutant embryos. Three GMCs are Eve-positive: GMC1-1a divides to produce the Eve-positive sibling neurons, aCC and pCC; GMC4-2a divides to create the Eve-positive RP2 and Eve-negative RP2 sibling and GMC7-1a divides to produce two Eve-positive medial U neurons. In wildtype embryos, Eve- expression is first detected in GMC1-1a and GMC7-1a at stage 10 and in GMC4-2a at stage 11 (Fig. 4.1A, 1C; Broadus et al., 1995). In fru^{w12}/fru^{sat15}mutant embryos, very few GMCs were Eve-positive at stage 10 as only a few GMC1-1a and no GMC7-1a were labeled (Fig. 4.1B). However, a little later in development, early stage 11, both GMC1-1a and GMC7-1a were Eve-positive in every hemisegment (Fig. 4.1D). GMC4-2a also has delayed Eve-expression because none are detected at stage 11 but were present by stage 12 (Fig. 4.1D). The delay in the onset of Eve-expression indicates that either the divisions to generate these GMCs from their NB is delayed or that the interval between GMC birth and Eve expression is longer in *fru* mutant embryos compared to that in wildtype embryos.

Figure 4.1. Eve expression pattern in GMCs and neurons in wildtype and *fru* mutant embryos.

Wildtype (A, C and E) and fru^{w12}/fru^{sat15} (B, D, F and G) embryos were double labeled with anti-Eve (purple, AP histochemistry) and anti-En (brown, HRP histochemistry). Anterior is to the right.
A) Stage 10 wildtype embryo, every GMC1-1a (large arrow) but only some GMC7-1a (small arrow) are Eve-positive.
B) Stage 10 *fru* mutant embryo with only few GMC1-1a (large arrow) and no GMC7-1a that are Eve-positive.
C) Early stage 11wildtype embryo, GMC1-1a (large arrow), GMC7-1a (small arrow) and most GMC4-2a (arrowhead) are Eve-positive.
D) Early stage 11*fru* mutant embryo, every GMC1-1a (large arrow) and GMC 7-1a (small arrow) is Eve-positive but no GMC4-2a expresses Eve. White arrowhead indicates the approximate location within the segment for GMC4-2a. GMC1-1a is located within the En-positive region that marks the posterior part of the hemisegment.
E) Midstage 12 wildtype embryo, GMC1-1a has generated the Eve-positive neurons, aCC and fpCC (large arrow) and GMC4-2a has produced RP2 (small arrow). Another Eve-positive neuron, friend of pCC (fpCC), is also found in some segments (Jacobs and Goodman, 1989; Goodman and Doe, 1993). The white bar indicates the middle of the En-positive stripe of cells.
F) Midstage 12 *fru* mutant embryo, the wildtype complement of Eve-positive neurons, aCC, pCC, fpCC neurons (large arrow) and RP2 (small arrow), are present. Both aCC and pCC neurons are located more posteriorly within the En-positive stripe than in the wildtype embryo. With respect to the white bar drawn through the middle of the En-positive stripe, aCC and pCC are posterior in *fru* mutant embryos and anterior in wildtype embryos.
G) Early stage 11 *fru* mutant embryo in which an additional Eve-positive GMC is present (large white arrow). Other defects are visible in this CNS including the absence of labeled GMC1-1a (white arrowheads) and GMC7-1a (small arrow) in some segments and abnormally positioned GMC1-1a (asterisk).

Figure 4.1.


1-1a
7-1a
A
1-1a
B
4-2a
C
1-1a
7-1a
D
aCC/fpCC
RP2
E
F
No 1-1a
7-1a
1-1a
ectopic
G

The Eve-positive GMCs have a characteristic anterior-posterior location within the hemisegment. Cells in the posterior compartment of each segment are En-expressing. This expression pattern provides a marker for assessing the relative positions of Eve-expressing GMCs. In each hemisegment, GMC1-1a is located just anterior to the En-positive cells of the posterior segment compartment and GMC7-1a lies within the posterior compartment (Fig. 4.1A; Broadus et al., 1995). However, in fru^{w12}/fru^{sat15} mutant embryos, the GMC1-1a was found within the posterior compartment (Fig. 4.1B, 1D). In contrast, in *fru* mutant embryos, GMC7-1a remained En-positive and located within the posterior compartment, which is similar to its location in wildtype embryos (Fig. 4.1C, 4.1D). In about 5% of the neuromeres (5/90 hemisegments; Fig 4.1G), additional Eve-positive GMCs were found in an ectopic location. The fate of these ectopic GMCs is not known.

In *fru* mutants, Eve-expression is delayed in newly born neurons.

Consistent with the delay in Eve-expression in the GMCs, there was a delay in the appearance of Eve-positive neurons in *fru* mutants. By stage 12/3 (mid-stage 12), the neuronal progeny of the Eve-positive GMCs1-1a and GMCs4-2a, (aCC, pCC and RP2) were present in wildtype embryos (n=100 hemisegments, n=10 embryos; Broadus et al., 1995). Eve-positive neurons were present in fru^{w12}/fru^{sat15} mutant embryos at late stage 12 (12/1, later than stage 12/3) (Fig. 4.1F; n=90 hemisegments). Except for the delay in the onset of expression, the wildtype complement of Eve-expressing neurons was present in *fru* mutant in stage 12-13 fru^{w12}/fru^{sat15} and fru^{AJ96u3}/fru^{sat15}. All RP2, aCC/pCC and EL neurons are normally expressing Eve in stage 13 (n = 70 hemisegments of filleted CNS of fru^{w12}/fru^{sat15} and fru^{AJ96u3}/fru^{sat15}). In addition, no ectopic Eve-positive cells were detected. The failure to find ectopic Eve-positive neurons in stage 12-13 embryos suggest that the ectopic Eve-positive GMCs do not produce Eve-positive neurons. There are ectopic Eve-positive neurons in older embryos (see below).

Table 4.1. Genetic analysis of Eve-expressing cells with the CNS

The presence and location of Eve-positive neurons (+) were scored in wildtype and *fru* mutant embryos (st 15-16), labeled in two independent experiments. For each phenotype, a one-way ANOVA was calculated with subsequent comparisons using Tukey's HSD for contrast. For the data presentation, I organized the *fru* mutants into groups. In the B group, the *fru* mutants were viable and these mutations eliminated or affected the production of P1 and P2 transcripts. In the C group, the *fru* mutants die as late pupae and only truncated transcripts are generated from the P1, P2 and P3 promoters but full length P4 transcripts are made (Anand, et al., 2001). In the D group, the mutants die early in the pupal stage and these deficiencies remove *fru* coding sequences. ND, not determined.

[a] The same number of whole mount embryos were examined for the organization of dorsal Eve-positive neurons and the presence of Eve-positive EL clusters.

[b] To determine whether there were any additional defects in the Eve pattern, embryos that had obvious defect in their *eve* neuronal pattern were filleted and examined for the changes in dorsal Eve-positive neurons. Abdominal hemisegments A2-A7 were counted. The same number of hemisegments (N) were examined for the presence of Eve-positive RP2, aCC/pCC neurons and additional Eve-positive cells.

[c] Comparisons of embryos examined for the presence of abnormally positioned Eve-positive neurons revealed significant differences among genotypes [$F_{11, 1408} = 58.45$, $P < 0.0001$]. All embryos in C and D groups (with the exception of fru^{AJ96v3}/fru^{w24}) were significantly different (all Ps < 0.05) from wildtype or viable *fru* (group B) mutants.

[d] Comparisons among embryos examined for the absence of EL Eve-positive neurons revealed significant differences among genotypes [$F_{11, 478} = 45.84$, $P < 0.0002$] with groups C and D significantly different (all Ps < 0.05) from wildtype or viable *fru* (group B) mutants

[e] Comparisons among embryos examined for the absence of Eve-positive staining in RP2 neurons found genotype differences [$F_{8, 205} = 13.1$, $P < 0.005$]. Subsequent comparisons between revealed that group C and D genotypes were different (all Ps < 0.05) from wildtype or fru viable genotypes.

[f] Comparisons among embryos examined for the absence of Eve-positive staining in aCC and/ or pCC showed significant genotype differences [$F_{8, 132} = 10.91$, $P < 0.007$].

[g] Comparisons among embryos examined for the presence of additional Eve-positive neurons found significant differences among genotypes [$F_{8, 388} = 15.84$, $P < 0.0001$]. Subsequent comparisons revealed that C and D group *fru* mutants (all Ps < 0.05) had significantly more ectopic Eve-positive neurons compared to wildtype or viable *fru* (group B) mutants.

Table 4.1. Genetic analysis of Eve-expressing cells with the CNS

Phenotypes / Genotypes	Percentage of embryos with abnormally positioned dorsal Eve (+) neurons [a]	Percentage of embryos lacking Eve (+) EL clusters [a]	Percentage of hemisegments lacking Eve (+) RP2 neurons[b]	Percentage of hemisegments lacking Eve (+) aCC/pCC[b]	Percentage of hemisegments with additional Eve(+) cells[b]
A. Wildtype	0.4 (n=251)	0.8	1.9 (n=152)	0	0.6
B. *fru* genotypes with reduced P1or lacking P1 and P2 transcripts but producing P3 and P4 transcripts.					
fru^{1}/fru^{w24}	0.9 (n=107)	0.9	3.3 (n=150)	0	1.3
fru^{440}/fru^{sat15}	2.3 (n=129)	0	0.8 (n=130)	0	2
$fru^{440}/P14$	1.2 (n=162)	1.2	0.9 (n=120)	0	2.6
C. *fru* genotypes lacking P1, P2 and P3 transcripts.					
fru^{w12}/fru^{AJ96u3}	14 (n=135) [c]	9.6 [d]	13 (n=102)[e]	12 [f]	13 [g]
fru^{w12}/fru^{sat15}	15 (n=130) [c]	10.7 [d]	9 (n=101) [e]	6 [f]	11 [g]
fru^{w12}/fru^{w24}	32 (n=146) [c]	12.3 [d]	15 (n=109)[e]	12.7 [f]	8 [g]
$fru^{w12}/P14$	14 (n=139) [c]	7.9 [d]	ND	ND	ND

Table 4.1. Continued

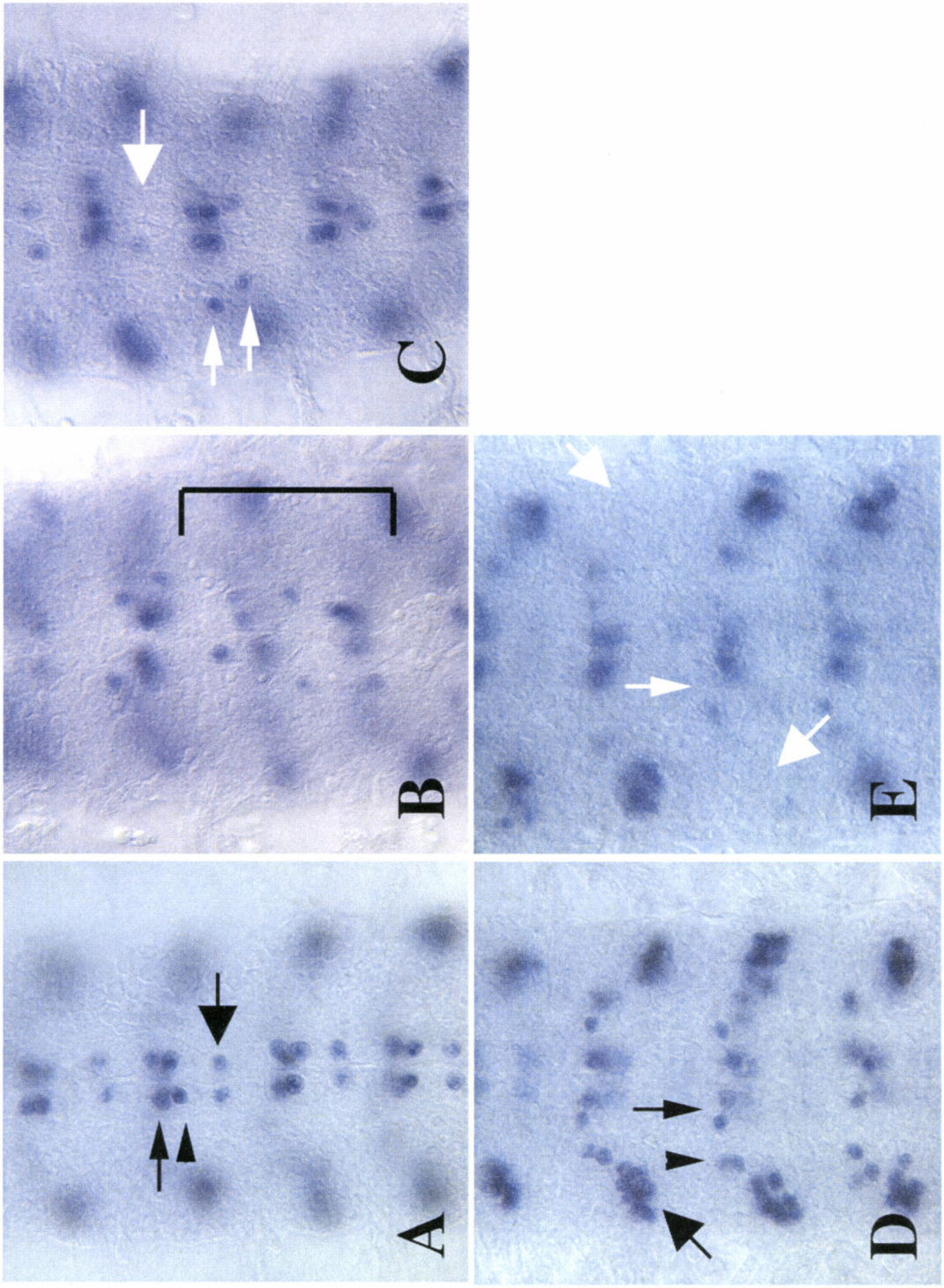
D. *fru* genotypes producing no *fru* transcripts.

fru^{AJ96u3}/fru^{sat15}	13 (n=112) [c]	8.9 [d]	8 (n=100) [e]	4 [f]	9 [g]
fru^{sat15}/fru^{w24}	10 (n=128) [c]	9.4 [d]	6 (n=100) [e]	5 [f]	5 [g]
fru^{AJ96u3}/fru^{w24}	17 (n=106) [c]	1.9	ND	ND	ND
$fru^{AJ96u3}/P14$	10 (n=89) [c]	7.9 [d]	ND	ND	ND

Figure 4.2. Eve-positive neurons in wildtype and *fru* mutant stage 16 embryos.

Wildtype (A, D) and fru^{w12}/fru^{sat15} (B, C, E) embryos were labeled with anti-Eve antibodies, processed for alkaline phosphatase immunohistochemistry and filleted to expose the CNS. Anterior is to the top.
A. Wildtype CNS with the typical pattern of dorsal Eve-positive RP2 (large arrow) and aCC (small arrow) and pCC (arrowhead) neurons.
B) fru^{w12}/fru^{sat15} mutant CNS in which dorsal Eve-positive neurons are found in abnormal locations. In the bracketed area, there are six neurons but they are no longer in their wildtype positions making it difficult to distinguish RP2 from aCC or pCC.
C) fru^{w12}/fru^{sat15} mutant CNS with additional dorsal Eve-positive neurons present more laterally (small white arrows) and in which no Eve-positive neuron is labeled in the position expected for an RP2 neuron (large white arrow).
D) Wildtype CNS with the typical pattern of ventro-lateral Eve-positive medial U (arrowhead), lateral U neurons (small arrow) and cluster of EL (large arrow) neurons.
E) fru^{w12}/fru^{sat15} mutant CNS in which there are no Eve-positive EL (large white arrows) in two hemisegments or U neurons (small white arrow).

Figure 4.2.



In *fru* mutants, Eve-positive neurons are located in aberrant positions in the CNS.

To further analyze the development of Eve-positive neurons, I examined stage 15-16 embryos in *fru* null mutants. The distribution and pattern of Eve-positive neurons showed three types of defects (Table 4.1D). The most obvious defect in fru^{w12}/fru^{sat15} mutant embryos was a change in the location of the dorsal Eve-positive neurons, aCC, pCC and RP2. These neurons are juxtaposed to the midline in wildtype CNS (Fig. 4.2A), but in *fru* mutants they are not located along the midline but shifted more laterally or in different anterior/posterior and dorsal/ventral positions. In 15 % of the fru^{w12}/fru^{sat15}mutant embryos (n=130), the abnormally positioned dorsal Eve-positive neurons were found (Fig 4.2B). In wildtype embryos, the progeny of GMC1-1a, the aCC and pCC neurons, were located posterior to En stripes and are not on En-positive (vertical line Fig. 4.1C in 1E). However, in fru^{w12}/fru^{sat15} mutant embryos, aCC and pCC neurons were located within the En-positive stripe (vertical line in Fig. 4.1F). The relative position of the RP2 neuron is likewise shifted with respect to the En-positive stripe compared to its location in wildtype embryos (vertical line in Fig. 4.1E, 1F). These changes in neuronal position may reflect in part earlier shifts in the position of their GMCs, GMC1-1a and GMC4-2a, in germband extended embryos, and the subsequent abnormal location of aCC and pCC within the posterior region of the segment in germband retracted embryos. The finding that neurons from two different GMCs and their progeny neurons are shifted with respect to their normal positions suggests that there may also be abnormality in the organization of the CNS during germ band retraction, probably accompanying the dislocated dorsal Eve-positive neurons found in later stage during embryogenesis (Fig. 4.2B).

The second defect involves the loss of Eve-expression in neurons that were Eve-positive at late stages of neurogenesis (Fig. 4.2B, 2C, 2E compare with Fig. 4.2A, 2D). Many embryos had at least one hemisegment in which there were no labeled cells in the position of the dorsal Eve-positive neurons, aCC, pCC or RP2, or the EL neurons with the anti-Eve antibody. In those hemisegments in which the EL neurons were unlabeled, the entire group of 10 EL neurons often failed to label (Fig. 4.2E; Table 1). In hemisegments in which the EL neurons were not labeled,

Eve-positive dorsal neurons as well as ventral U neurons were usually present (Fig. 4.2E). Since different GMCs generate various Eve-positive neurons, these findings suggest that the loss of Eve-expression might be occurring at the GMCs or neuron level but does not represent a change in the property of the entire hemisegment.

An alternative explanation for the failure to find Eve-expressing neurons is that the particular neuron was not produced and the loss of an Eve-expressing cell would mean that the cell was not produced. To address this possibility, I labeled embryos with additional markers for RP2, pCC and the EL neurons. To identify RP2 and pCC neurons, I labeled stage 16 fru^{w12}/fru^{sat15} mutant embryos with mab22C10 to detect the Futsch protein (Fujita *et al.*, 1982; Hummel et al., 2000). All possible combinations of antibody staining patterns were found in mutant embryos colabeled with anti-Eve and 22C10. In about 5% of the hemisegments in fru^{w12}/fru^{sat15} mutant embryos, neurons in the position in the CNS where an RP2 neuron would be expected were not labeled by either anti-Eve or mab22C10 antibodies (white arrow in Fig. 4.3C, n=150 hemisegments). In about 7.7% of these hemisegments, the RP2 neurons were mab22C10-positive but Eve-negative (arrows in Fig. 4.3C) suggesting that these RP2 neurons were present and differentiating. Approximately 12.7 % of these hemisegments were found to have a single Eve-positive neuron in dorsal-anterior area where aCC and pCC are normally located (n= 19/150 hemisegments). Since these Eve-positive cells were also mab22C10-positive, these neurons are identified as pCC neurons. These data suggest that the daughters of the GMC1-1a, aCC rather than the pCC neurons are more likely to be Eve-negative. The ectopic Eve-positive cells in dorsal locations (5.3%, n=150 hemisegments) were not labeled by the mab22C10 antibody (figure not shown). In order to determine whether EL neurons were present even though no Eve-positive neurons were present laterally, fru^{w12}/fru^{sat15} mutant embryos were labeled for the transcription factor Runt, which labels the neurons in the EL cluster (Dormand and Brand, 1998). In all hemisegments, the appropriate number of lateral neurons were Runt-positive suggesting that EL neurons are present but are not always Eve-positive (n=36 hemisegments).

Figure 4.3. Wildtype and *fru* mutant embryos doubly labeled for axonal tracts and Eve.

Embryos were labeled with anti-Eve antibodies, processed for alkaline phosphatase (purple) and then labeled either with anti-Fas II, anti-BP102 or mab22C10 and processed for HRP (brown).

A) fru^{w12}/fru^{sat15} stage 16 CNS double labeled for Eve and FasII. In one hemisegment (brackets), the three FasII tracts are disrupted and the dorsal Eve-positive neurons (slightly out of the plane of focus) are not juxtaposed to the midline. Axons from the medial tract have defasciculated and crossed the midline (arrow) to join the opposite medial tract.

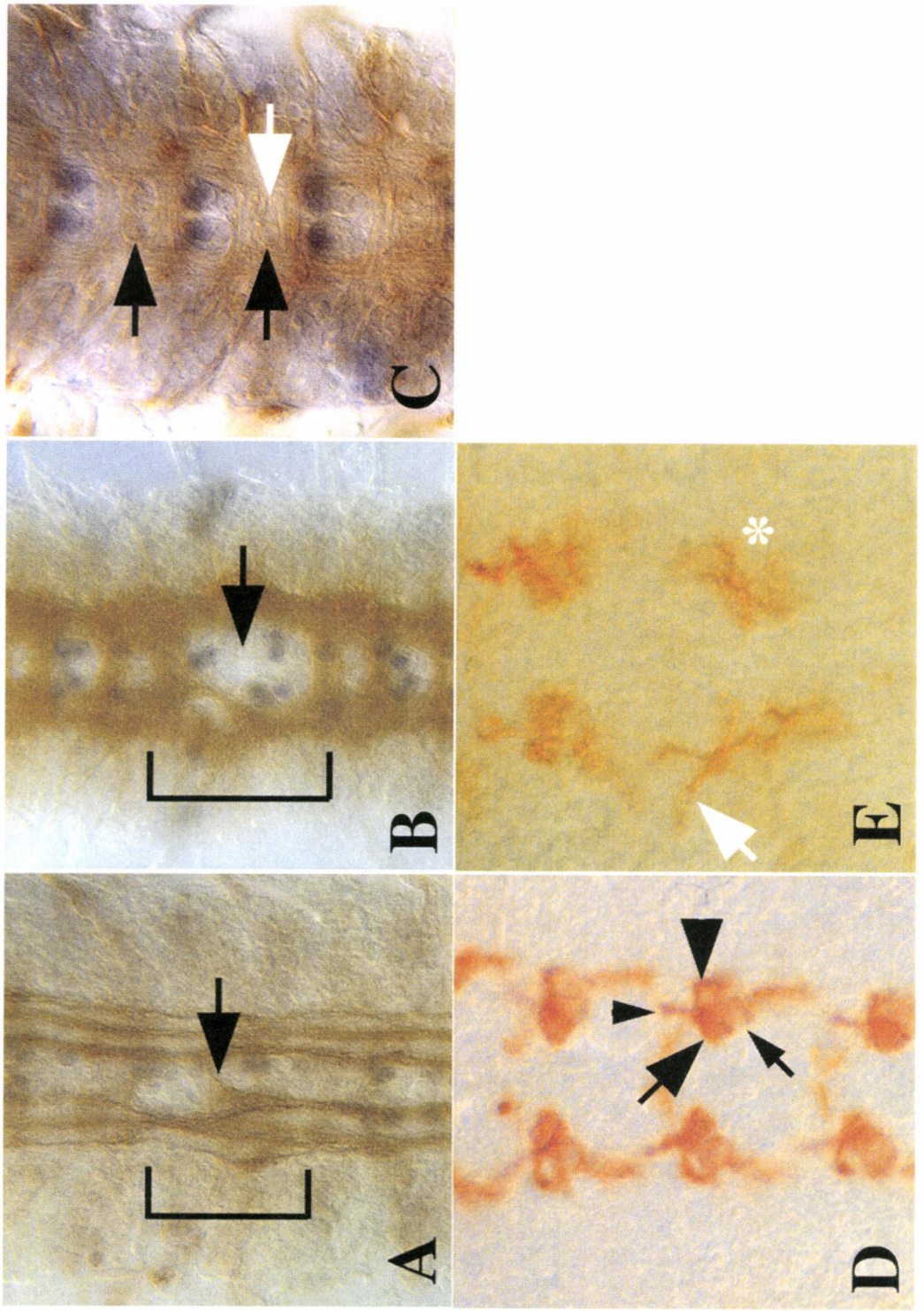
B) fru^{w12}/fru^{sat15} stage 16 CNS double labeled for Eve and BP102. In one segment (brackets), the anterior and posterior commissures have not formed and the longitudinal connectives are more separated than in adjacent segments. The dorsal Eve-positive neurons (arrow) in this segment are also out of alignment.

C) fru^{w12}/fru^{sat15} stage 16 CNS double labeled for Eve and mAb 22C10. All six RP2 neurons are Eve-negative but have 22C10-positive (arrows) or 22C10-negative processes (white arrow).

D) Wildtype stage 12 embryo labeled for FasII. Both aCC (large arrow) and pCC (small arrow) neurons strongly express Fas II. aCC extends its axonal growth cone dorsolateral (large arrowhead) and pCC extends its growth cone anteriorly (small arrowhead).

E) fru^{w12}/fru^{sat15} 12 embryo labeled for FasII. The aCC and pCC neurons (asterisk) and their growth cones are weakly FasII-positive (white arrow) making it difficult to distinguish the pattern of growth cone outgrowth. The growth cones do not fasciculate as closely as in the wildtype (Fig 4D). The separation of the pair of aCC/pCC neurons is greater across the midline than in wildtype embryos (see 4B and 4D).

Figure 4.3.



Surprisingly, I also found that in *fru* mutant embryos, additional Eve-positive cells were found in ectopic locations (Fig. 4.2C). In hemisegments with the normal complement of Eve-positive neurons, these additional Eve-positive cells must represent cells that normally do not express *eve* and not displaced Eve-positive cells. While I also found ectopic Eve-positive GMCs, it is not clear whether the ectopic Eve-positive neurons are derived from these GMCs because no ectopic Eve-positive cells were found at earlier stages (st 12-13) of neurogenesis (see above). Taken together, these results suggest that the disruption of *fru* function leads to the loss of Eve staining rather than loss of Eve-expressing neurons. In summary, *fru* functions are necessary for maintaining Eve expression in Eve-positive neurons and probably for inhibiting Eve expression in neurons that do not normally express Eve. Therefore, *fru* plays a role in regulating Eve-expression and likely has some role in sustaining some aspects of neuronal identity.

By analysis of Eve-positive neurons in other *fru* genotypes, I confirmed that these defects in Eve-expressing neurons resulted from the loss of *fru* function. The fru^{w12} is transheterozygotes in an inversion that separates the P1, P2 and P3 promoters from the *fru* coding regions, but allow full length P4 transcripts to be made (Anand et al., 2001). The same frequency of defects were found in fru^{w12}/fru^{-} embryos as in the *fru* null embryos showing that it is the loss of *fru* function that is responsible for the defects in eve neuronal expression. Since fru^{w12} is an inversion with two chromosomal breaks, one in the *fru* locus and the other mapped cytologically to 65C-D, we examined *eve* neuronal phenotypes in these embryos. The full complement of Eve-positive neurons was present and no abnormal phenotypes were found (n=10 embryos) indicating that the relevant chromosomal break is in the *fru* locus. Additional mutant analysis of embryos in which P1 and P2 *fru* transcripts are reduced or eliminated but P3 and P4 transcripts are still made (e.g. fru^{440}/fru^{sat15}) the pattern and expression of Eve-positive neurons shown in wildtype (Table 4.1). In summary, these findings suggest that P3 but not P1, P2 or P4 transcripts are involved in controlling the expression of *eve* in neurons or in their distribution in the CNS.

Coordinate defects in Eve-expression and axonal tracts in many segments in *fru* mutant embryos.

I next examined whether or not dislocations of dorsal Eve-positive neurons observed in *fru* mutant embryos occurred in the same segments as did other neuronal defects, such as the formation of FasII and BP102 axonal tracts (Chapter 2). fru^{w12}/fru^{sat15} embryos were double labeled with anti-Eve and anti-FasII or BP102 (Fig 4.3A, 3B). In more than half of the hemisegments in which FasII axons had defective fascicles, the dorsal Eve-positive neurons were also shifted in the position (Fig. 4.4A, n=13/20). In a similar fraction of segments in which BP102-positive fibers did not form normal longitudinal connectives and commissures, the dorsal Eve-positive neurons were dislocated (Fig. 4B, n=6/12). In 12 % of the fru^{w12}/fru^{sat15} mutants labeled for both anti-Eve and anti-FasII antibodies, aCC and pCC neurons produced abnormal axonal projections (n=100 hemisegments; Fig. 4.3D, 4.3E). The fact that both Eve-expression and FasII and BP102 tracts are affected in about half of segments suggests that many aspects of neuronal differentiation rely on *fru* function.

The expression of *fru* transgenes restore normal Eve staining patterns in *fru* mutant embryo.

To determine whether these neuronal defects found in *fru* mutants are due to the loss of *fru* function, *fru* transgenes were expressed in fru^{w12}/fru^{sat15} mutant embryos under the control of the *scabrous-GAL4* driver. This *GAL4* driver is expressed in the similar pattern as the *fru* gene in embryos (Chapter 2). In stage 15-16 embryos, the expression of either *UAS-fruA* or *UAS-fruC* rescued the full pattern and distribution of dorsal and ventral Eve-neurons (Fig. 4.4A, 4.4B; Table 4.2). In fru^{w12}/fru^{sat15} embryos expressing the *UAS-fruB* transgene, the percentage of embryos with abnormally positioned dorsal Eve-positive was significantly higher than that found in wildtype embryos, but was also significantly different from that in the *fru* mutant embryo

Figure 4.4. The expression of specific *fru* transgenes rescues the Eve phenotype in *fru* mutant embryos.

Stage 16 fru^{w12}/fru^{sat15} embryos either expressing the *UAS-fruC* transgene (A-C;*UAS-fruC; sca-GAL4/+;* fru^{w12}/fru^{sat15}) or *UAS-fruM* transgene driven by *sca-GAL4* were stained with anti-Eve (A-B, D-E) followed by AP cytochemistry or anti-Fas II antibody (C, F) followed by HRP cytochemistry.
A) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruC* transgene. All segments have a normal pattern of dorsal Eve-positive neurons.
B) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruC* transgene (*UAS-fruC; sca-GAL4/+;* fru^{w12}/fru^{sat15}). All segments have a normal pattern of ventral Eve-positive neurons.
C) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruC* transgene. All segments have a normal pattern of FasII tracts.
D) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruMC* transgene (dorsal view). All segments have an abnormal pattern of Eve-positive neurons (dorsal view). Most dorsal Eve-positive neurons (bracket) are not aligned along the midline. In a region in which an RP2 neuron would be expected no Eve-positive neuron is present (white arrow). The disruptions are greater than in the fru^{w12}/fru^{sat15} mutant embryo.
E) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruMC* transgene (ventral view. EL clusters are abnormal with fewer EL neurons (4 Eve-positive neurons, small white arrow), two distinct groups of EL neurons (bracket). In the region where the U neurons are located, no Eve-positive neuron is present (small white arrowhead).
F) fru^{w12}/fru^{sat15} mutant embryo expressing the *UAS-fruMC* transgene. All segments have an abnormal pattern (defasciculation) of FasII tracts (bracket) and many axons project across the midline (arrows).

Figure 4.4.

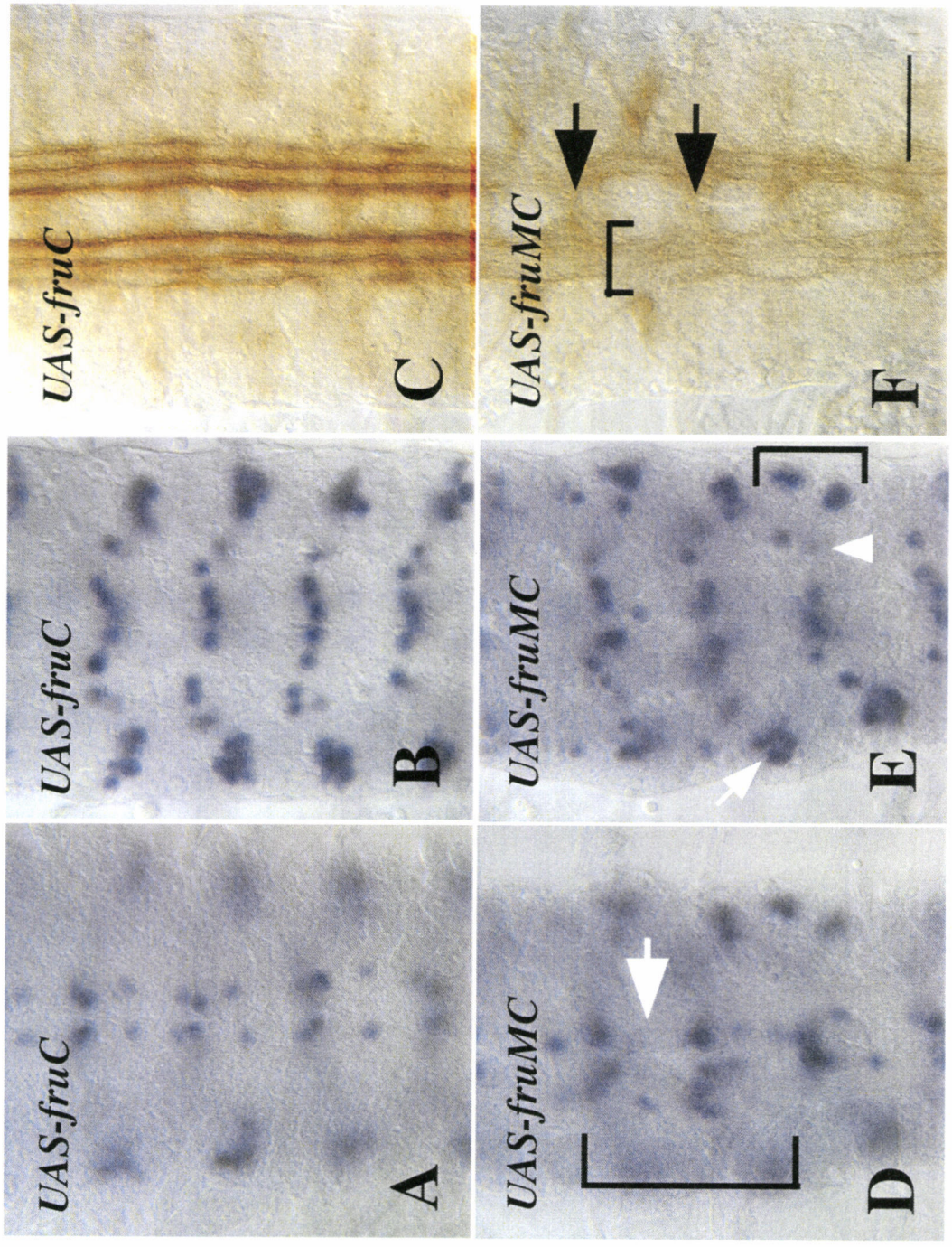

UAS-fruC
A
UAS-fruC
B
UAS-fruC
C
UAS-fruMC
D
UAS-fruMC
E
UAS-fruMC
F

Table 4.2. Genetic analysis of Eve-positive neurons in *fru* mutant embryos expressing *fru* transgenes

The percentage of stage 15/16 *w; fru^{w12}/fru^{sat15}; sca-GAL4; UAS-fru* transgenic embryos (n = number of embryos or hemisegment analyzed) with defects in their Eve-positive neuronal pattern was determined. This percentage is an average of two independent experiments in which whole-mount embryos were evaluated for their mutant phenotype. The analysis in the lack of Eve staining in RP2 and aCC/pCC was performed as described in Table 1. More than 50 abdominal hemisegments were examined for the presence of Eve-positive RP2 and aCC/pCC neurons. In all *fru^{w12}/fru^{sat15}; sca-GAL4; UAS-fru* embryo every RP2 and aCC/pCC neuron was Eve-positive.

A one-way ANOVA, which included data from Table 1, was used to analyze each phenotype subsequent comparisons (Tukey's HSD) were made between *fru^{w12}/fru^{sat15}* mutants, wildtype and *fru^{w12}/fru^{sat15}* mutants expressing *UAS-fru* transgenes. The one-way ANOVA with genotypes as the main effect revealed significant differences among genotypes scored for abnormally positioned dorsal Eve-positive cells [$F_{13,4392} = 74$, $P < 0.0001$] and for the lack of Eve-positive EL neurons [$F_{13,523} = 13.57$, $P < 0.0001$], respectively.

[ac] The number of *UAS-fruA/sca-GAL4;fru^{w12}/fru^{sat15}* and *UAS-fruC/sca-GAL4; fru^{w12}/fru^{sat15}* embryos with abnormally positioned Eve-positive neurons or Eve-positive EL clusters were not different than wildtype embryos but were significantly different from the *fru^{w12}/fru^{sat15}* embryos (P<0.05).

[b] The number of *UAS-fruB/sca-GAL4;fru^{w12}/fru^{sat15}* embryos with abnormally positioned Eve-positive neurons was significantly different from wildtype (P<0.05), but not from *fru^{w12}/fru^{sat15}* embryos. For the comparison of Eve-positive EL clusters, *UAS-fruB/sca-GAL4;fru^{w12}/fru^{sat15}* embryos were not different than wildtype embryos but were significantly different from the *fru^{w12}/fru^{sat15}* embryos (P<0.05).

[de] The number of *UAS-fruMA/sca-GAL4;fru^{w12}/fru^{sat15}* and *UAS-fruMB/sca-GAL4;fru^{w12}/fru^{sat15}* embryos with abnormally positioned Eve-positive neurons was significantly different from the number in wildtype (P<0.05) and *fru^{w12}/fru^{sat15}* embryos(P<0.05).

[f] The number of *UAS-fruMC/sca-GAL4;fru^{w12}/fru^{sat15}* embryos with abnormally positioned Eve-positive neurons was significantly different from the number in wildtype (P<0.05) but not *fru^{w12}/fru^{sat15}* embryos.

[gi] The number of *UAS-fruMA/sca-GAL4;fru^{w12}/fru^{sat15}* and *UAS-fruMC/sca-GAL4;fru^{w12}/fru^{sat15}* embryos lacking Eve-positive EL neurons was significantly different from the number in wildtype (P<0.05) but not *fru^{w12}/fru^{sat15}* embryos.

[h] The number of *UAS-fruMB/sca-GAL4;fru^{w12}/fru^{sat15}* embryos with abnormally positioned Eve-positive neurons was not significantly different from the number in wildtype but was different from *fru^{w12}/fru^{sat15}* embryos(P<0.05).

Table 4.2. Genetic analysis of Eve-positive neurons in *fru* mutant embryos expressing *fru* transgenes

Phenotypes	*UAS-fru* constructs driven by *sca-GAL4*		
	fruA	*fruB*	*fruC*
Percentage of embryos with abnormally positioned dorsal Eve (+) cells	0.9 (n=107)[a]	8.9 (n=96)[b]	2.2 (n=135)[c]
Percentage of embryos lacking Eve (+) EL clusters	0 (n=107)[a]	1 (n=96)[b]	0.8 (n=135)[c]
Percentage of hemisegments lacking Eve (+) RP2 neurons	0 (n=52)	0 (n=64)	0 (n=50)
Percentage of hemisegments lacking Eve (+) aCC/pCC neurons	0 (n= 52)	0 (n= 64)	2 (n= 50)
	UAS-fruM constructs driven by *sca-GAL4*		
	fruMA	*fruMB*	*fruMC*
Percentage of embryos with abnormally positioned dorsal Eve (+) cells	30.2 (n=116)[d]	40.4 (n=109)[e]	20.5 (n=117)[f]
Percentage of embryos lacking Eve (+) EL clusters	6 (n=116)[g]	0 (n=109)[h]	5.1 (n=117)[i]

(Table 4.2). The discrepancy in the ability of the *UAS-fruA*, *UAS-fruB* and *UAS-fruC* transgenes to rescue the dorsal Eve pattern may be due to the nature of each transgene product because two independent insertion lines of each transgene gave similar results (data not shown). In stage 15-16 embryos, the expression of either *UAS-fruA, UAS-fruB* or *UAS-fruC* rescued the full pattern and distribution of EL Eve-positive neurons (Table 4.2). In addition, no ectopic Eve-expressing cells were found (Table 4.2). Taken together, these results strongly indicate that the defective phenotypes in Eve-positive neurons found in *fru* mutants are due to loss of *fru* function and *fru* function is necessary for maintaining proper position of dorsal Eve neurons and Eve-expression.

<u>The misexpression of *fru* male form transgene increase the severity of defective phenotypes in *fru* mutants.</u>

I also examined whether the expression of *fru* male transgenes, *fruMA*, *fruMB* and *fruMC*, would rescue mutant Eve phenotypes in fru^{w12}/fru^{sat15} mutant embryos (Chapter 2). Since the expression of *fru* male transgene increased the severity and frequency of defective axonal phenotype to include virtually all *fru* mutants and sibling fru^{+} embryos (Chapter 2), I expected that there would be a commensurate level of defects in Eve staining patterns. For most Eve mutant phenotypes, the expression of the *UAS-fruM* transgenes either failed to rescue these phenotypes or caused more embryos to show defects in their Eve-positive neurons (Fig. 4.4D, 4E). The percentage of fru^{w12}/fru^{sat15} mutant embryos with a defective dorsal Eve pattern and expressing *UAS-fruMC* (about 20 %) is similar to that in the *fru* mutant embryos, suggesting a failure to rescue. The percentage of embryos with defects in *fru* mutants expressing *UAS-fruMA* (about 40 %) or *UAS-fruMB* (about 31%) is significantly higher than that in *fru* mutants (Fig. 4.4D, 4.4E; Table 4.2). All EL neurons were Eve-positive in fru^{w12}/fru^{sat15} mutant embryos expressing the *UAS-fruMB* transgenes but not rescued in embryos expressing *UAS-fruMA* or *UAS-fruMC* (Fig. 4.4D, Table2). More ectopic Eve-positive cells were found in all *UAS-fruM* transgenes in *fru* mutants (20%, n=50 for each genotype; Fig. 4.4E indicated by small arrow).

Discussion

An early function of *fru* is involved in the positioning of GMCs and their progeny.

Eve-positive GMCs and neurons are a well-described population that have allowed me to analyze *fru*'s role in neuronal identity. The earliest *fru* phenotype I have found is the aberrant localization of Eve-positive GMCs in relation to the En-positive strip of cells that marks the posterior compartment. The GMCs such as GMC 1-1a and 7-1a and their neuronal progeny were also abnormally distributed in the CNS, these differences in cell location may affect the differentiation of the neurons. This phenotype was fully rescued by the expression of two *fru* transgenes, *UAS-fruA* and *UAS-fruC*, driven by *scabrous-GAL4*, suggesting that the expression of *fru* transgenes in cells in the neuroectoderm, neuronal precursors and neurons was sufficient to restore normal phenotype in *fru* mutant embryos. Expression of the *UAS-fruB* transgene did partially rescue mutant phenotypes.

The abnormal position of Eve-expressing GMCs may also indicate that a major structural event occurs during embryogenesis where the CNS elongates in germ band extension and then coalesces during germ band retraction. The abnormal position of dorsal Eve-positive neurons may reflect earlier abnormalities in development. However, abnormalities in germ band retraction have also been found in mutants of other genes that affect neuronal fate determination, such as *prospero* (Doe et al., 1991), in mutants of genes associated with lateral and midline glial cell development (Halter et al., 1995; Giesen et al., 1997) and in those of genes involved in amnioserosa (Lamka and Lipshitz, 1999; Yip et al., 1997).

fru functions to maintain neuronal identity of Eve-expressing neurons and other neurons.

To see whether genes that function to define the identity of neurons, such as Eve, are altered in *fru* mutants, I examined the pattern of Eve-positive neurons. In *fru* mutant embryos that lack all *fru* transcripts, the normal pattern of Eve-expressing cells was not maintained. At early stages (stage 12-13) all Eve-expressing neurons were present, but by stage 15-16, fewer Eve-positive neurons

were present and the RP2, aCC, pCC or EL neurons were not labeled by the anti-Eve antibody in some hemisegments. These data indicate that FRU function is not required for the initial expression of Eve in neurons that are normally Eve-expressing but rather FRU is needed for the maintenance of Eve-expression. By examining Eve-expression in a variety of *fru* mutant embryos, I found that embryos in which P1 and P2 *fru* transcripts are not made but P3 and P4 *fru* transcripts are present had wildtype Eve-expressing neurons. In *fru* mutants in which only P4 transcripts are made but P1, P2 and P3 transcripts are not produced, the Eve neuronal phenotypes are similar to those in *fru* null mutants. These data suggest that *fru* transcripts from the P3 promoter have a major role in the ability of Eve-neurons to retain their Eve-expression. In addition, the expression of all *fru* transgenes by *sca-GAL4* restored normal neuronal identity in Eve-positive and Eve-negative neurons in *fru* mutant embryos.

In order to assess whether or not neurons failing to express Eve were present in mutant embryos, additional markers were used. Both pCC and RP2 express the Futsch protein, which is the antigen for the mab22C10 (Fujita *et al.*, 1982; Hummel et al., 2000). In *fru* mutant embryos, RP2 and pCC neurons co-labeled with Eve and mab22C10 in about 75% of the hemisegments. In about 20% of the hemisegments, neurons were labeled with only one of the markers and in about 5% of the hemisegments, no cell was detected by either antibody. This shows that Futsch expression does not require Eve expression. The additional marker for half of the neurons in an EL cluster was the transcription factor, Runt. In all hemisegments in *fru* mutants, Runt expression was present showing that EL neurons were present but not all EL clusters continued to express Eve. Although Runt has been shown to be necessary for Eve expression (Dormand and Brand, 1998), it is interesting that in *fru* mutants some Runt-positive neurons were unable to maintain Eve-expression.

The loss of *fru* function and the expression of *fru* transgenes did not result in the same phenotypes as found for genes that have been demonstrated to be important in establishing neuronal fate in *eve* expressing neurons. For example, the RP2 neuron is derived from neuroblast 4-2; *prospero* , *pdm-1*, *fushi tarazu* and

huckebein (*hkb*) are all required to activate *eve* expression in GMC 4-2a and its progeny, RP2 and RP2sib (Doe et al., 1991; Yeo et al., 1995; Bhat and Schedl, 1994; Chu-LaGraff et al., 1995). Over-expression of *hkb* for instance gives rise to a cell fate change of NB 4-2 and its progeny, GMC 4-2a, resulting in duplicated RP2 neurons. Expression of pair-rule gene, *runt*, is also necessary for the expression of *eve* in the EL neurons derived from NB 3-3. The loss-of-function of the *runt* gene leads to a complete loss of Eve expression in the entire EL cluster, but over-expression of *runt* generates twice the number of Eve expressing cells in the EL neurons (Dormand and Brand, 1998). Although the loss of *fru* function leads to the absence of staining in RP2 neurons and EL clusters, the expression of *fru* transgenes did not result in duplicated number of RP2 and EL neurons. Therefore, *fru* function is necessary to maintain Eve expression in *eve*-expressing neurons rather than to establish cell fate of *eve*-expressing neurons.

Surprisingly, ectopic Eve-positive neurons were also found in *fru* mutants. The presence of these cells raises the possibility that FRU protein(s) may act like a negative regulator in some cells. Other BTB/POZ transcription factors act as transcriptional repressors. For example, *tramtrack* represses the segmentation genes, *eve*, *hairy*, *odd-skipped*, and *runt* (Brown and Wu, 1993). Taken together, fru^+ appears to function to maintain neuronal differentiated fates in both Eve-positive and some Eve-negative neurons. In this capacity, FRU might act to promote *eve* transcription in Eve-positive neurons but suppress *eve* transcription in Eve-negative neurons.

Comparison of *fru* function for the differentiation of axonal tracts and Eve-expression.

Having analyzed two different types of phenotypes in *fru* mutants, I was interested in whether segments that had defects in one phenotype also had defects in the other phenotype. About half of the segments with defects in FasII or BP102 axonal tracts also had defects in Eve-expression. If the defects in axonal pathfinding and Eve-expression were due to early defects in the development of the nervous system, then it would be expected that both types of phenotypes would

occur in the same segment. However, the level of correspondence found in about 50 % defects in axonal tract vs that in Eve pattern suggests that these phenotypes are more likely independent events.

I have found, however, that the expression of the *UAS-fruA* and *UAS-fruC* transgenes by the *sca-GAL4* driver are sufficient to rescue both axonal tract and Eve-expression phenotypes (Chapter 2). These data may suggest that the primary transcript classes involved in *fru*'s function in the embryonic CNS are the P3 transcripts that are spliced to the A or C 3' end. By contrast, expression of the *UAS-fruB* transgene was partially successful in rescuing Eve expression phenotypes but caused a higher frequency of defective axonal phenotypes, suggesting that *fru* transcripts using the B 3' end are not able to replace *fru* function in *fru* mutant embryos.

The expression of the *fru* male transgenes failed to rescue or caused an increase in the number of segments with defective Eve-phenotypes. As was true for the axonal tract phenotypes (Fig. 4.4C compared to Fig. 4.4F), these *fru* male transgenes are not able to replace the common *fru* transcripts and in fact exacerbate abnormal development of Eve-positive neurons and axonal tracts (Chapter 2). It is worthy noticing the difference in severity between two phenotype analyses, axonal phenotype and Eve phenotype. Even though the phenotypical severity was increased in both cases, the frequency of defective Eve phenotypes (approx. average 30 %) was much less than that of defective axonal tracts (approx. average 98 %) in *fru* mutants and sibling fru^+ embryos with the expression of *fru* male form transgenes by *sca-GAL4* driver. Such difference might be due to the ability of *fru* male form transgenes; *fru* male form transgenes might be preferentially involved in axonal pathfinding rather than in maintaining neuronal identity or property. This hypothesis might be supported by the finding that all tested sibling fru^+ embryos with the expression of *fru* male form transgenes by *sca-GAL4* also showed no longer typical three longitudinal scaffold but somewhat appear in normal Eve pattern. This difference leads to the speculation that *fru* male form works in opposite way to normal fru^+ function to guide axonal projections. Therefore we infer that *fru* male form is able to alter guidance of axonal outgrowth.

A similar situation might happen in the adult CNS. The sex non-specific proteins are assumed to function in a similar way to that found in embryonic CNS to establish normal adult CNS formation. These proteins need to maintain a neuronal identity or property for proper axonal projection during the neuronal differentiation. On the other hand, normal expression of *fru* male form protein (FRU^M) in male CNS might be necessary to alter neuronal property or identity in adult male CNS for proper and specific wiring of neuronal circuits associated with male sexual behavior by my assumption that the *fru* male form may work in opposite way to fru^+ function. Since all FRU^M expressions are argued to restrict to the postmitotic neurons essential for adult sex behavior (Lee et al., 2000), FRU^M proteins likely function in neurons to wire neuronal circuitry for male sexual behaviors. Nevertheless, the questions remain to be determined on whether fru^+ function in the proper formation of embryonic CNS are relevant to adult CNS formation, how the neuronal property for FRU^M positive neurons is determined, and how FRU^M expressing neurons make putative male specific neuronal wiring.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

The overall objective of my research is to understand the developmental function of the *fruitless* (*fru*) gene, especially its sex-nonspecific functions. Beginning with a series of experiments to describe *fru*'s pattern of expression in the embryo, I have shown, by *in situ* hybridization and immunohistochemistry, that FRU proteins are expressed in neurons, glia and their progenitors. In order to determine whether *fru* had a role in the development of the embryonic CNS, I examined the full range of *fru* mutants for defects in the formation of longitudinal and commissural axonal tracts labeled by several different markers. In *fru* null alleles, FasII and BP102-positive axons were not wildtype in that axons did not remain bundled together and did not take their normal routes through the CNS but rather pioneered new pathways. By genetic analysis of a variety of *fru* mutants in which different subsets of *fru* transcripts were eliminated or reduced, I was able to conclude that transcripts from P3, and perhaps the P4, *fru* promoters are required for the proper formation of the FasII and BP102 axonal tracts. In a parallel set of experiments, I examined *fru* mutants for changes in the expression of the *even-skipped* gene, which is involved in determining the identity of a small number of neurons in the embryonic CNS. In the same *fru* mutants that had defects in their axonal pathways, Eve-expression was also abnormal. Again, these data suggest that *fru* transcripts from the P3 and perhaps, the P4 promoters are required for the retention of Eve-expression in neurons. These findings are consistent with the localization data for *fru* RNA and protein expression.

One way to conclusively demonstrate that the axonal phenotypes in *fru* mutants were due to the loss of *fru* function is to restore *fru* expression to *fru* mutants. Given the structural complexity of the *fru* gene, it was necessary to use a battery of transgenes in order to test adequately the role of different *fru* transcripts. By expressing three different *fru* transgenes that had the same 5' end but different 3' ends, it was possible to provide cells with FRU proteins that would be similar to the

full range of FRU proteins encoded by the P2, P3 and P4 transcripts. By using a pan-neuronal driver with a similar pattern of expression to the *fru* gene itself, FRU proteins could be added back to mimic *fru*'s wildtype function. Expression of two of these transgenes rescued the defects in their FasII and BP102 axonal tracts and Eve-expression in *fru* mutant embryos. Further, it was interesting that the expression of *fru* transgenes by a elav-GAL4 driver that is only expressed after neurons have been born is unable to rescue the axonal defects. One explanation for these findings is that *fru* has functions in other cell types, such as glia, as well as neurons in order to regulate axonal guidance or Eve-expression. Alternatively, *fru* may need to be expressed earlier in neurons during differentiation before the *elav* driver is active. Not only did the expression of the *fru* transgenes under the control of the *elav* driver fail to rescue, but there was an increase in the number of embryos with abnormal axonal pathways. This increase in the number of embryos with axonal defects was greater than that found in the *fru* mutant genotype alone, suggesting that temporal *fru* expression in neurons is capable of affecting the ability of axons to find their normal pathways.

Given the findings with Eve-expressing neurons, it is possible that the defects in axonal projection found in late embryogenesis was not due to change in neural fate but rather due to a failure to maintain neuronal identity. Therefore, one explanation for the inappropriate axonal projections in *fru* mutant embryos is that there is a loss of neuronal identity, which leads to inappropriate neuronal differentiation. To take this idea further, then, *fru* functions originating from the P3 or/and P4 promoter would be required to maintain neuronal identify. These findings are supported by the discovery of the genetic interactions between *fru* and other genes involved in axonal pathfinding and genes involved in the development of the glia.

Among the genes that are needed for axonal pathfinding, *fru* was found to interact with *robo*, which encodes a repulsive receptor in neurons, and with *comm*, which encodes a novel transmembrane protein produced by neurons and midline glia. These findings suggest that *fru* function is required for neurons to respond properly to the repulsive signaling mediated by Robo and Comm activity. Since

the defects in *fru* mutants alone are less extreme than in the robo mutants or in the double heterozygotes, it suggests that *fru* is not the main regulator of robo or comm expression but likely works along with other proteins to regulate their function. One mechanism for how FRU proteins might control expression of robo or comm is through dimerization between FRU's BTB/POZ domain and that of other BTB/POZ domain proteins, such as Lola. Indeed, it was found that *fru* interact genetically the *lola* gene, which is required for *robo1* expression. In *lola* mutants, the levels of Robo1 protein is reduced by 40-50 % (E. Giniger, personal communication). Therefore, I infer that the expression of *robo1* and *comm* partially depends on that of the *fru* gene in neurons and midline glia.

fru function is also required for the proper development of both lateral and midline glia. In *fru* mutant embryos, Repo positive lateral glia and AA162 (midline glia specific marker) positive midline glia are both normal with respect to their number and position. However, studies with double heterozygotes revealed that *fru* interacts with genes, *ttk* and *gcm*, that have been shown to be required in glia cells for the repression of neuronal differentiation (Giesen et al., 1997). A similar mechanism to that proposed for FRU and Lola proteins would also account for how FRU and Ttk, a BTB/POZ domain protein, might interact. Thus these genetic interaction studies lead to the hypothesis that *fru* functions as a transcriptional activator or repressor in neurons and glia in contact with other BTB/POZ proteins. Taken together, *fru* functions are required for axonal pathfinding and for the development of lateral and midline glia; all *fru* functions are associated with proper formation of embryonic axonal architecture.

Many questions still remain from these studies. First, though FRU itself may function as a transcription factor, there is as yet no evidence as to the molecular mechanisms by which FRU proteins act. Further, there is no evidence for whether FRU is interacting with Lola, Ttk or Sim directly or indirectly through a longer chain of events leading to the regulation of proteins, such as Robo, that mediate axonal adhesion. Moreover, I have only identified some genetic interactors, and not shown whether the expression pattern or level of these genes is changed in *fru* mutants or whether *fru*'s expression pattern is changed in other

mutant backgrounds. A better way to distinguish the function of *fru* isoforms derived from either P3 or P4 promoter would require the use of other *fru* alleles that only take out individual classes of *fru* transcripts. Other techniques, such as RNA interference, may provide a more precise analysis of the functions of specific *fru* isoforms. In addition, we need use different alleles of genetic interactors to ascertain whether there is allelic difference. It remains possible that *fru* affects the expression of other guidance proteins and other transcription factors participating in differentiation of neurons, lateral glia and midline glia.

Putative Functions of Sex-Specific and Sex-Non Specific *fru* in Adult CNS

From an analysis of vertebrate sexual behavior through the action of steroid hormone (androgens and estrogens), an Organizational vs. Activational hypothesis of sexually dimorphic behaviors was elaborated several decades ago by Young and his colleagues (Pheonix et al., 1959). This hypothesis proposes that during development, the sex steroid hormones organize or establish the components of the nervous system that will be needed for subsequent male- or female-specific behaviors. Thus, the organizational part of the hypothesis defines how the major differences in function and structure between male and female brains becomes established. The activational part of the hypothesis, proposes that in adult animals, these same hormones activate, modulate, or inhibit the function of these existing neuronal circuits. Further, the Organizational Concept emphasized that the male phenotype was hormonally induced organization while the female phenotype was the neutral or default condition (Nelson, 1995).

A similar conceptual hypothesis for sexual behavior by gonadal hormones can be used to describe the action of a single gene or genes that control sex-specific behavior. In the organizational role, a gene might function during development to build into the CNS the potential for a behavior. In this case, a gene's activity would specifically construct the circuitry that subserves a behavior. In the activational role, a gene or genes might control the actual manifestation of a behavior as it occurs. That is, action of genes would be necessary to induce the establishment or organization of the male neuronal phenotype, with the female

neuronal phenotype being the neutral or default condition (Baker et al., 2001). The *fru* expression pattern during metamorphosis, a time of major developmental change, leads us to postulate that the *fru* gene has an organizational role with male-specific functions in adult CNS formation. During metamorphic transition from the vermiform larvae to the winged adult, sexual differentiation in the CNS and other tissues, such as sex-specific internal and external genitalia, occurs through sex-determination hierarchy (Truman et al., 1993; reviews by Yamamoto et al., 1998, Arthur et al., 1998, and Schutt and Nothiger, 2000). During metamorphosis, male specific FRU proteins and sex-non specific FRU proteins are widely expressed in developing CNS and male FRU proteins remain expressed in few cells of adult male CNS (Lee et al., 2000; Usui-Aoki et al., 2000; M. Foss and B.J. Taylor, unpublished data). Therefore, *fru* expression during metamorphosis is likely associated with establishing neuronal circuitry to subserve sexual behaviors rather than with controlling the actual manifestation of the behavior as it occurs.

From our understanding of *fru*'s function during the formation of embryonic CNS, I postulate that these sex-nonspecific *fru* RNAs and proteins that are widely expressed in adult CNS are required for proper axonal projection and CNS formation in both sexes. This would mean that they are expressed in the same cell types in adults as in embryos. These neurons are waiting to be investigated in the adult male and female CNS. Nevertheless, it is to be expected that the genetic mechanisms for axonal pathfinding in the adult CNS might be similar to that found in the embryonic CNS. Thus, the sex-nonspecific FRU proteins might be considered to function to establish certain basic features of adult CNS formation in both sexes during metamorphosis. Even so, the question arises as to how male specific FRU proteins establish male specific neuronal circuitry for behaviors. Indirect evidence was found in the misexpression of a *fru* male form transgene in embryos, which caused more severe defects in axonal projections than that found in *fru* mutant embryos. Even the sibling control embryos were severely affected. These findings suggest that male FRU proteins function in opposite to the sex-nonspecific FRU proteins. Likewise, function of male specific FRU protein may oppose that of the sex-nonspecific *fru* protein if they are present in cells where sex-

nonspecific *fru* proteins are expressed during metamorphosis. Therefore, during metamorphosis, this function of male specific FRU protein might underlay the development of male specific neuronal circuits specific to male behavior. Another possibility is that male forms of FRU protein may control, in opposite way to sex-nonspecific *fru* proteins, genes associated with male adult CNS formation, leading to male specific circuits. In this way, the genetic action mediated by male FRU proteins would induce the establishment or organization of the male CNS phenotype, with the sex-nonspecific CNS phenotype being the neutral or default condition.

BIBLIOGRAPHY

Ahmad, K.F., Engel, C.K. and Prive, G.G. (1998). Crystal structure of the BTB domain from PLZF. Proc Natl Acad Sci USA. 95, 12123-8.

Akiyama, Y., Hosiya, T., Poole, A.M. and Hotta, Y. (1996). The gcm motif: a novel DNA binding motif conserved in *Drosophila* and mammals. Proc Natl Acad Sci USA. 93, 14912-14916.

Akiyama-Oda, Y., Hosoyam, T. and Hotta, Y. (1999). Asymmetric cell division of thoracic neuroblast 6-4 to bifurcate glial and neuronal lineage in *Drosophila*. Development 126, 967-974.

Albagli, O., Dhordain, P., Deweindt, C., Lecocq, G. and Leprince, D. (1995). The BTB/POZ domain: a new protein-protein interaction motif common to DNA- and actin-binding proteins. Cell Growth Differ. 6, 1193-98

Anand, A., Villella, A., Ryner, L.C., Carlo, T., Goodwin, F.G., Song, H.-J., Gailey, D.A., Morales, A., Hall, J.C., Baker, B.S., and Taylor, B.J. (2001). Molecular genetic dissection of the sex-specific and vital functions of the *Drosophila melanogaster* sex determination gene *fruitless*. Genetics 158, 1569-95.

Arthur, B.I. Jr., Jallon, J.M., Caflisch, B., Choffat, Y. and Nothiger, R. (1998). Sexual behavior in *Drosophila* is irreversibly programmed during a critical period. Curr Biol. 8, 1187-1190.

Auld, V. (1999).Glia as mediators of growth cone guidance: studies from insect nervous systems. Cell Mol Life Sci. 55, 1377-85.

Bainbridge, S.P. and Bownes, M. (1981). Staging the metamorphosis of *Drosophila* melanogaster. J. Embryol Exp Morphol. 66, 57-80.

Baker, B.S., Taylor, B.J. and Hall, J.C. (2001). Are complex behaviors specified by dedicated regulatory genes? Reasoning from *Drosophila*. Cell 105, 12-24.

Balakireva, M., Stocker, R.F., Gendre, N. and Ferveur, J.F. (1998). *Voila*, a new *Drosophila* courtship variant that affects the nervous system: behavioral, neural, and genetic characterization. J Neurosci. 18, 4335-43.

Bardwell, V.J. and Treisman, R. (1994). The POZ domain: a conserved protein-protein interaction motif. Genes Dev. 8, 1664-1677.

Bashaw, G.J. and Goodman, C.S. (1999). Chimeric axon guidance receptors: the cytoplasmic domains of slit and netrin receptors specify attraction versus repulsion. Cell 97, 917-26.

Bashaw, G.J., Kidd, T., Murray, D., Pawson, T. and Goodman, C.S. (2000). Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. Cell 101, 703-715.

Bate, C.M. (1976). Pioneer neurons in an insect embryo. Nature 260, 54-55.

Battye, R., Stevens, A. and Jacobs, J.R. (1999). Axon repulsion from the midline of the *Drosophila* CNS requires *slit* function. Development 126, 2475-2481.

Battye, R., Stevens, A., Perry, R.L. and Jacobs, J.R. (2001). Repellent signaling by Slit requires the leucine-rich repeats. J Neurosci. 21, 4290-4298.

Bayer, C.A., von Kalm, L. and Fristrom, J.W. (1997). Relationships between protein isoforms and genetic functions demonstrate functional redundancy at the Broad-Complex during *Drosophila metamorphosis*. Dev Biol. 187, 267-82.

Bernardoni, R., Kammerer, M., Vonesch, J.L. and Giangrande, A. (1999). Gliogenesis depends on glide/gcm through asymmetric division of neuroglioblasts. Dev Biol. 216, 265-75.

Bhat, K.M. (1999). Segment polarity genes in neuroblast formation and identity specification during *Drosophila* neurogenesis. Bioessays 21, 472-85.

Bhat, K.M. and Schedl, P. (1994). The *Drosophila miti-mere* gene, a member of the POU family, is required for the specification of the RP2/sibling lineage during neurogenesis. Development 120, 1483-1501.

Booth, G.E., Kinrade, E.F. and Hidalgo, A. (2000). Glia maintain follower neuron survival during *Drosophila* CNS development. Development 127, 237-244.

Bossing, T. and Technau, G.M. (1994). The fate of the CNS midline progenitors in *Drosophila* as revealed by a new method for single cell labeling. Development 120, 1895-1906.

Bossing, T., Udolph, G., Doe, C.Q. and Technau, G.M. (1996). The embryonic central nervous system lineages of *Drosophila melanogaster*. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. Dev Biol. 179, 41-64.

Bouchard, T.J.Jr. 1994. Genes, environment, and personality. Science 264, 1700-1701.

Brand, A.H. and Perrimon, N. (1993). Targeted gene expression as a mean of altering cell fate and generating dominant phenotypes. Development 118, 401-415.

Breedlove, S.M. (1986). Cellular analyses of hormone influence on motoneuronal development and function. J Neurobiol. 17, 157-76.

Broadus, J. and Doe, C.Q. (1995). Evolution of neuroblast identity: *seven-up* and *prospero* expression reveal homologous and divergent neuroblast fates in *Drosophila* and *Schistocerca*. Development 121, 3989-96.

Broadus, J.B, Skeath, J.B., Spana, E.P., Bossing, T., Technau, G. and Doe. C.Q. (1995). New neuroblast markers and the origin of the aCC/pCC neurons in the *Drosophila* central nervous system. Mech Dev. 53, 393-402.

Broadus, J.B., Fuerstenberg, S. and Doe, C.Q. (1998). Staufen-dependent localization of prospero mRNA contributes to neuroblast daughter cell fate. Nature 391, 792-795.

Brose, K. and Tessier-Lavigne, M. (2000). Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. Curr Opin Neurobiol. 10, 95-102.

Brose, K., Bland, K.S., Wang, K.H., Arnott, D., Henzel, W., Goodman, C.S., Tessier-Lavigne, M. and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell 96, 795-806.

Brown, J.L. and Wu, C. (1993). Repression of *Drosophila* pair-rule segmentation genes by ectopic expression of *tramtrack*. Development 117, 45-58.

Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H. and Klämbt, C. (1994). The ETS domain protein pointed-P2 is a target of MAP kinase in the *sevenless* signal transduction pathway. Nature 370, 386-389.

Buescher, M., Yeo, S.L., Udolph, G., Zavortink, M., Yang, X., Tear, G. and Chia, W. (1998). Binary sibling neuronal cell fate decisions in the *Drosophila* embryonic central nervous system are nonstochastic and require *inscuteable*-mediated asymmetry of ganglion mother cells. Genes Dev. 12, 1858-1870.

Campbell, G., Göring, H., Lin, T., Spana, E., Andersson, S., Doe, C.Q. and Tomlinson, A. (1994). RK2, a glial-specific homeodomain protein required for

embryonic nerve cord condensation and viability in *Drosophila*. Development 120, 2957-2966.

Campos-Ortega, J.A. (1995). Genetic mechanisms of early neurogenesis in *Drosophila melanogaster*. Mol Neurobiol. 10, 75-89.

Campos-Ortega, J.A. and Hartenstein, V. (1997). Central nervous system. In the embryonic development of *Drosophila* melanogaster. 2nd Ed. pp. 233-269. Berlin Heidelberg New York: Springer-Verlag.

Castrillon, D.H., Gonzy, P., Alexander, S., Rawson, R., Eberhart, C.G., Viswanathan, S., Dinardo, S. and Wasserman, S.A. (1993). Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: characterization of male-sterile mutants generated by single P element mutagenesis. Genetics 135, 489-505.

Chu-LaGraff, Q., Schmid, A., Leidel, J., Bronner, G., Jackle, H. and Doe, C.Q. (1995). *huckebein* specifies aspects of CNS precursor identity required for motoneuron axon pathfinding. Neuron 15, 1041-51.

Collins, T., Stone, J.R. and Williams, A.J. (2001). All in the family: the BTB/POZ, KRAB, and SCAN domains. Mol Cell Biol. 21, 3609-15.

Crews, D. (1993). The organizational concept and vertebrates without sex chromosomes. Brain Behav Evol. 42, 202-214.

Crews, S.T. (1998). Control of cell lineage-specific development and transcription by bHLH-PAS proteins. Genes Dev. 12, 607-20.

Crews, S.T., Thomas, J.B. and Goodman, C.S. (1988). The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the *per* gene product. Cell 52, 143-151.

Cui, X . and Doe, C.Q. (1992). *ming* is expressed in neuroblast sublineages and regulates gene expression in the Drosophila central nervous system. Development 116, 943-952.

David, G., Alland, L., Hong, S.H., Wong, C.W., DePinho, R.A. and Dejean, A. (1998). Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein. Oncogene 16, 2549-56.

Desai, C.J., Krueger, N.X., Saito, H. and Zinn, K. (1997). Competition and cooperation among receptor tyrosine phosphatases control motoneuron growth cone guidance in *Drosophila*. Development 124, 1941-1952.

DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W. and Guild, G.M. (1991). The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. Genetics 129, 385-397.

Doe, C.Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. Development 116, 855-63.

Doe, C.Q. and Skeath, J.B. (1996). Neurogenesis in the insect central nervous system. Curr Opin Neurobiol. 6, 18-24.

Doe, C.Q. Chu-LaGraff, Q., Wright, D.M. and Scott, M.P. (1991). The *prospero* gene specifies cell fates in the Drosophila central nervous system. Cell 65, 451-464.

Doe, C.Q., Fuerstenberg, S. and Peng, C.Y. (1998). Neural stem cells: from fly to vertebrates. J Neurobiol. 36, 111-27.

Dong, S., Zhu, J., Reid, A., Strutt, P., Guidez, F., Zhong, H.J., Wang, Z.Y., Licht, J., Waxman, S., Chomienne, C., et al. (1996). Amino-terminal protein-protein interaction motif (POZ-domain) is responsible for activities of the promyelocytic leukemia zinc finger-retinoic acid receptor-alpha fusion protein. Proc Natl Acad Sci USA. 93, 3624-9.

Dormand, E.L and Brand, A.H. (1998). Runt determines cell fates in the *Drosophila* embryonic CNS. Development 125, 1659-1667.

Espinas, M.L., Jimenez-Garcia, E., Vaquero, A., Canudas, S., Bernues, J. and Azorin, F. (1999). The N-terminal POZ domain of GAGA mediates the formation of oligomers that bind DNA with high affinity and specificity. J Biol Chem. 274, 16461-9.

Farkas, G., Gausz, J., Galloni, M., Reuter, G., Gyurkovics, H. and Karch, F. (1994). The *Trithorax-like* gene encodes the *Drosophila* GAGA factor. Nature 37, 806-8.

Featherstone, D.E. and Broadie, K. (2000). Surprises from *Drosophila*: genetic mechanisms of synaptic development and plasticity. Brain Res Bull. 53, 501-511.

Finley, K.D., Taylor, B.J., Milstein, M. and McKeown, M. (1997). *dissatisfaction*, a gene involved in sex-specific behavior and neural development of *Drosophila melanogaster*. Proc Natl Acad Sci USA 94, 913-918.

FlyBase (1999). The FlyBase database of the Drosophila genome projects and community literature. *Nucleic Acids Research* 27:85-88. http://flybase.bio.indiana.edu/).

Freeman, M., Klämbt, C., Goodman, C.S. and Rubin, G.M. (1992). The *argos* gene encodes a diffusible factor that regulates cell fate decision in the *Drosophila* eye. Cell 69, 963-975.

Fritz, J.L. and VanBerkum, M.F.A. (2000). Calmodulin and Son of sevenless dependent signaling pathways regulate midline crossing of axons in the *Drosophila* CNS. Development 127, 1991-2000.

Fujita, S.C., Zipursky, S.L., Benzer, S., Ferrus, A., and Shotwell, S.W. (1982). Monoclonal antibodies against the *Drosophila* nervous system. Proc Natl Acad Sci USA 79, 7929-7933.

Gailey, D.A. and Hall, J.C. (1989). Behavior and cytogenetics of *fruitless* in *Drosophila melanogaster*: different courtship defects caused by separate closely linked lesions. Genetics 121, 773-785.

Gailey, D.A., Taylor, B.J. and Hall, J.C. (1991). Elements of the *fruitless* locus regulate development of the muscle of Lawrence, a male-specific structure in the abdomen of *Drosophila melanogaster* adults. Development 113, 879-890.

Gaines, P., Tompkins, L., Woodard, C.T. and Carlson, J.R. (2000). *quick-to-court*, a *Drosophila* mutant with elevated levels of sexual behavior, is defective in a predicted coiled-coil protein. Genetics 154, 1627-37.

Giesen, K., Hummel, T., Stollewerk, A., Harrison, S., Travers, A. and Klämbt, C. (1997). Glial development in the *Drosophila* CNS requires concomitant activation of glial and repression of neuronal differentiation genes. Development 124, 2307-2311.

Giniger, E., Tietje, K., Jan, L.Y. and Jan, Y.N. (1994). *lola* encodes a putative transcription factor required for axon growth and guidance in *Drosophila*. Development 120, 1385-1398.

Goodman, C.S. (1996). Mechanisms and molecules that control growth cone guidance. Annu Rev Neurosci. 19, 341-77.

Goodman, C.S. and Doe, C.Q. (1993). Embryonic development of the *Drosophila* Central Nerve System. In Development of *Drosophila melanogaster*, (ed. M. Bate and A. Martinez-Arias). pp. 1131-1206. Cold Spring Harbor Laboratory Press.

Goodman, C.S. and Shatz, C.J. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. Cell 72 Suppl, 77-98.

Goodwin, S.F., Taylor, B.J., Villella, A., Foss, M., Ryner, L.C., Baker, B.S. and Hall, J.C. (2000). Aberrant splicing and altered spatial expression patterns in *fruitless* mutants of *Drosophila melanogaster*. Genetics 154, 725-745.

Granderath, S. and Klämbt, C. (1999). Glia development in the embryonic CNS of Drosophila. Curr Opin Neurobiol. 9, 531-536.

Granderath, S., Bunse, I. and Klämbt, C. (2000). *gcm* and *pointed* synergistically control glial transcription of the Drosophila gene *loco*. Mech Dev. 91, 197-208.

Greenspan, R.J. and Ferveur, J.-F. (2000). Courtship in *Drosophila*. Ann Rev Genet. 34, 205-32.

Grenningloh, G., Rehm, E.J., and Goodman, C.S. (1991). Genetic analysis of growth cone guidance in *Drosophila*: *fasciclin II* functions as a neuronal recognition molecule. Cell 67, 45-57.

Grignani, F., De Matteis, S., Nervi, C., Tomassoni, L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F.F., Zamir, I., Seiser, C., Grignani, F., Lazar, M.A., Minucci, S. and Pelicci, P.G. (1998). Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukemia. Nature 391, 815-8.

Guo, M., Jan, L.Y. and Jan, Y.N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron 17, 27-41.

Guthrie, S. (1999).Axon guidance: starting and stopping with slit. Curr Biol. 9, R432-435.

Hall, J.C. (1994). The mating of a fly. Science 264, 1702-1714.

Halter, D.A., Urban, J., Rickert, C., Ner, S.S., Ito, K., Travers, A.A. and Technau, G.M. (1995). The homeobox gene *repo* is required for the differentiation and maintaince of glia function in the embryonic nervous system of *Drosophila melanogaster*. Development 121, 317-332.

Harris, R., Sabatelli, L.M. and Seeger, M.A. (1996). Guidance cues at the *Drosophila* CNS midline: identification and characterization of two *Drosophila* Netrin/UNC-6 homologs. Neuron 17, 217-228.

Heinrichs, V., Ryner, L.C. and Baker, B.S. (1998). Regulation of sex-specific selection of *fruitless* 5' splice sites by transformer and transformer-2. Mol Cell Biol. 18, 450-458.

Hidalgo, A and Brand, A.H. (1997). Targeted neuronal ablation: the role of pioneer neurons in guidance and fasciculation in the CNS of *Drosophila*. Development 124, 3253-3262.

Hidalgo, A. and Booth, G.E. (2000). Glia dictate pioneer axon trajectories in the *Drosophila* embryonic CNS. Development 127, 393-402.

Hidalgo, A., Urban, J. and Brand, A.H. (1995). Targeted ablation of glia disrupts axon tract formation in the Drosophila CNS. Development 121, 3703-3712.

Hiramoto, M., Hiromi, Y., Giniger, E. and Hotta, Y. (2000). The *Drosophila* Netrin receptor Frazzled guides axons by controlling Netrin distribution. Nature 406, 886-889.

Hong, S.H., David, G., Wong, C.W., Dejean, A. and Privalsky, M.L. (1997). SMRT corepressor interacts with PLZF and with the PML-retinoic acid receptor alpha (RARalpha) and PLZF-RARalpha oncoproteins associated with acute promyelocytic leukemia. Proc Natl Acad Sci USA. 94, 9028-33.

Horowitz, H. and Berg, C.A. (1996). The *Drosophila pipsqueak* gene encodes a nuclear BTB-domain-containing protein required early in oogenesis. Development 122, 1859-1871.

Hosoya, T., Takizawa, K., Nitta, K. and Hotta, Y. (1995). *glial cells missing*: a binary switch between neuronal and glial determination in *Drosophila*. Cell 82, 1025-1036.

Hu, S., Fambrough, D., Atashi, J.R., Goodman, C.S. and Crews, S.T. (1995). The *Drosophila abrupt* gene encodes a BTB-zinc finger regulatory protein that controls the specificity of neuromuscular connections. Genes Dev. 9, 2936-2948.

Hummel, T., Krukkert, K., Roos, J., Davis, G., and Klämbt, C. (2000). *Drosophila* futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. Neuron 26, 357-370.

Hummel, T., Schimmelpfeng, K., and Klämbt, C. (1999). Commissure formation in the embryonic CNS of *Drosophila*. II. Function of the different midline cells. Development 26, 771-779.

Ito, H., Fujitani, K., Usui, K., Shimizu-Nishikawa, K., Tanaka, S., and Yamamoto, D. (1996). Sexual orientation in *Drosophila* is altered by the *satori* mutation in the sex determination gene *fruitless* that encodes a Zinc finger protein with a BTB domain. Proc Natl Acad Sci USA 93, 9687-9692.

Ito, K., Urban, J. and Technau, G.M. (1995). Distribution, classification and development of Drosophila glial cells during late embryogenesis. Roux's Arch Dev Biol. 204, 284-307.

Iwata, T., Toyoda, F., Yamamoto, K. and Kikuyama, S. (2000). Hormonal control of urodele reproductive behavior. Comp Biochem Physiol B Biochem Mol Biol. 126, 221-229.

Jacobs, J.R. (2000). The midline glia of *Drosophila*: a molecular genetic model for the developmental function of glia. Prog Neurobiol. 62, 475-508.

Jones, B.W., Fetter, R.D., Tear, G. and Goodman, C.S. (1995). *glial cells missing*: a genetic switch that controls glial versus neuronal fate. Cell 82,1013-1023.

Kambadur, R., Koizumi, K., Stivers, C., Nagle, J., Poole, S.J. and Odenwald, W.F. (1998). Regulation of POU genes by *castor* and *hunchback* establishes layered compartments in the *Drosophila* CNS. Genes Dev. 12, 246-60.

Kaplan, J. and Calame, K. (1997). The ZiN/POZ domain of ZF5 is required for both transcriptional activation and repression. Nucleic Acids Res. 25, 1108-16.

Katsani, K.R., Hajibagheri, M.A. and Verrijzer, C.P. (1999). Co-operative DNA binding by GAGA transcription factor requires the conserved BTB/POZ domain and reorganizes promoter topology. EMBO J. 18, 698-708.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S., Culotti, J.G. and Tessier-Lavigne, M. (1996). Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. Cell 87, 175-85.

Kidd, T., Bland, K.S. and Goodman, C.S. (1999). Slit is the midline repellent for the robo receptor in *Drosophila*. Cell 96, 785-794.

Kidd, T., Brose, K., Mitchell, K.J., Fetter, R.D., Tessier-Lavigne, M., Goodman, C.S. and Tear, G. (1998a). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell 92, 205-215.

Kidd, T., Russell, C., Goodman, C.S. and Tear, G. (1998b). Dosage sensitive and complementary functions of *roundabout* and *commissureless* control axon crossing of the CNS midline. Neuron 20, 25-33.

Kinrade, E.F.V., Brates, T., Tear, G. and Hidalgo, A. (2001). Roundabout signaling, cell contact and trophic support confine longitudinal glia and axons in the *Drosophila* CNS. Development 126, 207-216.

Klaes, A., Menne, T., Stollewerk, A., Scholz, H., and Klämbt, C. (1994). The ETS transcription factors encoded by the *Drosophila* gene pointed direct glial cell differentiation in the embryonic CNS. Cell 78, 149-160.

Klämbt, C. (1993). The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. Development 117, 163-76.

Klämbt, C., Hummel, T., Granderath, S. and Schimmelpfeng, K. (2001). Glial cell development in *Drosophila*. Int J Dev Neurosci. 19, 373-378.

Kolodziej, P.A., Timpe, L.C., Mitchell, K.J., Fried, S.R., Goodman, C.S., Jan, L.Y. and Jan, Y.N. (1996). *frazzled* encodes a *Drosophila* member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. Cell 87, 197-204.

Kopp, A., Duncan, I. and Carroll, S.B. (2000). Genetic control and evolution of sexually dimorphic characters in *Drosophila*. Nature 408, 553-9.

Kornack, D.R. (2000). Neurogenesis and the evolution of cortical diversity: mode, tempo, and partitioning during development and persistence in adulthood. Brain Behav Evol. 55, 336-344.

Kosman, D., Ip, Y.T., Levine, M. and Arora, K. (1991). Establishment of the mesoderm-neuroectoderm boundary in the *Drosophila* embryo. Science 254, 118-122.

Kramer, S.G., Kidd, T., Simpson, J.H. and Goodman, C.S. (2001). Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration. Science 292, 737-740.

Kraut, R. and Campos-Ortega, J.A. (1996). *inscuteable*, a neural precursor gene of *Drosophila*, encodes a candidate for a cytoskeleton adopter protein. Dev Biol. 174, 65-81.

Kraut, R., Chia, W., Jan, L.Y., Jan, Y.N. and Knoblich, J.A. (1996). Role of inscuteable in orienting asymmetric cell divisions in *Drosophila*. Nature 383, 50-5.

Krueger, N.X., Van Vactor, D., Wan, H.I., Gelbart, W.M., Goodman, C.S. and Saito, H. (1996). The transmembrane tyrosine phosphatase DLAR controls motor axon guidance in *Drosophila*. Cell 84, 611-22.

Lai, Z-.C. and Li, Y. (1999). Tramtrack69 is positively and autonomously required for *Drosophila* photoreceptor development. Genetics 152, 299-305.

Lamka, M.L and Lipshitz, H.D. (1999). Role of the amnioserosa in germ band retraction of the *Drosophila* melanogaster embryo. Dev Biol 214, 102-112.

Lee, G. and Hall, J.C. (2001) Abnormalities of male-specific FRU protein and serotonin expression in the CNS of fruitless mutants in *Drosophila*. J Neurosci. 21,513-26.

Lee, G., Foss, M., Goodwin, S.F., Carlo, T., Taylor, B.J. and Hall, J.C. (2000). Spatial, temporal, and sexually dimorphic expression patterns of the *fruitless* gene in the *Drosophila* CNS. J Neurobiol. 43, 404-26.

Lee, G., Villella, A., Taylor, B.J. and Hall, J.C. (2001). New reproductive anomalies in *fruitless*-mutant *Drosophila* males: extreme lengthening of mating durations and infertility correlated with defective serotonergic innervation of reproductive organs. J Neurobiol. 47, 121-149.

Lemke, G. (2001). Glial control of neuronal development. Annu Rev Neurosci. 24, 87-105.

Leonardo, E.D., Hinck, L., Masu, M., Keino-Masu, K., Ackerman, S.L. and Tessier-Lavigne, M. (1997). Vertebrate homologues of C. elegans UNC-5 are candidate netrin receptors. Nature 386, 833-838.

Li, P., Yang, X., Wasser, M., Cai, Y. and Chia, W. (1997a). Inscuteable and Staufen mediate asymmetric localization and segregation of *prospero* RNA during *Drosophila* neuroblast cell divisions. Cell 90, 437-447.

Li, X., Lopez-Guisa, J.M., Ninan, N., Weiner, E.J., Rauscher, F.J. 3rd. and Marmorstain, R. (1997b). Overexpression, purification, characterization, and crystallization of the BTB/POZ domain from the PLZF oncoprotein. J Biol Chem. 272, 27324-27329

Li, X., Peng, H., Schultz, D.C., Lopez-Guisa, J.M., Rauscher, F.J. 3rd. and Marmorstain, R. (1999). Structure-function studies of the BTB/POZ transcriptional repression domain from the promyelocytic leukemia zinc finger oncoprotein. Cancer Res. 59, 5275-5282.

Lin, R.J., Nagy, L., Inoue, S., Shao, W., Miller, W.H. Jr. and Evans, R.M. (1998). Role of the histone deacetylase complex in acute promyelocytic leukemia. Nature 391, 811-4.

Lin, D.M. and Goodman, C.S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron 13, 507-523.

Lin, D.M., Fetter, R.D., Kopczynski, C., Grenningloh, G., and Goodman, C.S. (1994). Genetic analysis of Fasciclin II in *Drosophila*: defasciculation, refasciculation, and altered fasciculation. Neuron 13, 1055-69.

Madden, K., Crowner, D. and Giniger, E. (1999). LOLA has the properties of a master regulator of axon-target interaction for SNb motor axons of *Drosophila*. Dev Biol. 213, 301-13.

Martin-Bermudo, M.D., Carmena, A. and Jimenez, F. (1995). Neurogenic genes control gene expression at the transcriptional level in early neurogenesis and in mesectoderm specification. Development 121, 219-24.

Melnick, A., Ahmad, K.F., Arai, S., Polinger, A., Ball, H., Borden, K.L., Carlile, G.W., Prive, G.G. and Licht, J.D. (2000). In-depth mutational analysis of the promyelocytic leukemia zinc finger BTB/POZ domain reveals motifs and residues required for biological and transcriptional functions. Mol Cell Biol. 20, 6550-67.

Mitchell, K.J., Doyle, J.L., Serafini, T., Kennedy, T.E., Tessier-Lavigne, M., Goodman, C.S. and Dickson, B.J. (1996). Genetic analysis of Netrin genes in *Drosophila*: Netrins guide CNS commissural axons and peripheral motor axons. Neuron 17, 203-215.

Morel, V. and Schweisguth, F. (2000). Repression by *suppressor of hairless* and activation by Notch are required to define a single row of *single-minded* expressing cells in the *Drosophila* embryo. Genes Dev. 14, 377-388.

Morisato, D. and Anderson, K.V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. Annu Rev Genet. 29, 371-399.

Mueller, B.K. (1999). Growth cone guidance: first steps towards a deeper understanding. Annu Rev Neurosci. 22, 351-88.

Nambu, J.R., Lewis, J.O., Wharton, K.A. Jr. and Crews, S.T. (1991). The *Drosophila single-minded* gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. Cell 67, 1157-1167.

Nelson, R.J. (1995). Sex differences in behavior: Sex determination and differentiation. In an introduction to behavioral endocrinology. pp81-120. Sinauer Associates, Inc.

Nordeen, E.J., Nordeen, K.W., Sengelaub, D.R. and Arnold, A.P. (1985). Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus. Science 229, 671-3.

O'Neill, E.M., Rebay, I., Tjian, R. and Rubin, G.M. (1994). The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. Cell 78, 137-147

Orgad, S., Rosenfeld, G., Greenspan, R.J. and Segal, D. (2000). *courtless*, the *Drosophila* UBC7 homolog, is involved in male courtship behavior and spermatogenesis. Genetics 155, 1267-1280.

Patel, N.H. (1994). Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. In *Drosophila melanogaster*: Practical uses in cell and molecular biology, (ed. L.S.B. Goldstein and E.A. Fyrberg). Pp. 446-487. Academic Press.

Patel, N.H., Condron, B.G. and Zinn, K. (1994). Pair-rule expression patterns of *even-skipped* are found in both short and long germ beetles. Nature 367, 429-434.

Patel, N.H., Martin-Blanco, E., Coleman, K.G., Poole, S.J., Ellis, M.C., Kornberg, T.B. and Goodman, C.S. (1989). Expression of *engrailed* proteins in arthropods, annelids, and chordates. Cell 58, 955-968.

Phoenix, C.H., Goy, R.W., Gerall, A.A. and Young, W.C. (1959). Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology 65, 369-382.

Rajagopalan, S., Nicolas, E., Vivancos, V., Berger, J. and Dickson, B.J. (2000a). Crossing the midline: roles and regulation of Robo receptors. Neuron 28, 767-777.

Rajagopalan, S., Vivancos, V., Nicolas, E. and Dickson, B.J. (2000b). Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the *Drosophila* CNS. Cell 103, 1033-1045.

Read, D., Butte, M.J., Dernburg, A.F., Frasch, M. and Kornberg, T.B. (2000). Functional studies of the BTB domain in the *Drosophila* GAGA and Mod(mdg4) proteins. Nucleic Acids Res. 28, 3864-70.

Restifo, L. L. and Hauglum, W. (1998). Parallel molecular genetic pathways operate during CNS metamorphosis in Drosophila. Mol Cell Neurosci. 11, 134-48.

Restifo, L. L. and Merrill, V. K. (1994). Two *Drosophila* regulatory genes, *Deformed* and the *Broad-Complex*, share common functions in development of adult CNS, head, and salivary glands. Dev Biol. 162, 465-485

Restifo, L. L. and White, K. (1991). Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of the central nervous system in *Drosophila*. Dev Biol. 148, 174-94.

Ridley, M. (1995). Animal behavior: an Introduction to Behavioral mechanisms, Development and Ecology, Second Edition (Boston:Blackwell Scientific Publications).

Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Benz, W.K. and Engels, W.R. (1988). A stable genomic source of P element transposase in *Drosophila melanogaster*. Genetics 118, 461-470.

Rothberg, J.M., Hartley, D.A., Walther, Z. and Artavanis-Tsakonas, S. (1988). *slit*: an EGF-homologous locus of *D. melanogaster* involved in the development of the embryonic central nervous system. Cell 55, 1047-1059.

Rothberg, J.M., Jacobs, J.R., Goodman, C.S. and Artavanis-Tsakonas, S. (1990). *slit*: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains. Genes Dev. 4, 2169-2187.

Rubin, G.M., Yandell, M.D., Wortman, J.R., et al. (2000). Comparative genomics of the eukaryotes. Science 287, 2204-15.

Rubin, G.M. and Spradling, A.C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. Science 218, 348-353.

Rusch J and Van Vactor D. (2000). New Roundabouts send axons into the Fas lane. Neuron 28, 637-640.

Ryner, L.C., Goodwin, S.F., Castrillon, D.H., Anand, A., Villella, A., Baker, B.S., Hall, J.C., Taylor, B.J., and Wasserman, S.A. (1996). Control of male sexual

behavior and sexual orientation in *Drosophila* by the *fruitless* gene. Cell 87, 1079-1089.

Sandstrom, D. J, Bayer, C.A., Fristrom, J.W. and Restifo, L.L. (1997). *Broad-Complex* transcription factors regulate muscle attachment in *Drosophila*. Dev Biol. 181, 168-185.

Schmid, A., Chiba, A. and Doe, C.Q. (1999). Clonal analysis of *Drosophila* embryonic neuroblasts: neuronal cell types, axon projections and muscle targets. Development 126, 4653-4689.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J. and Technau, G.M. (1997). The embryonic central nervous system lineages of Drosophila melanogaster. II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. Dev Biol. 189, 186-204.

Schmidt-Ott, U. and Technau, G.M. (1992). Expression of *en* and *wg* in the embryonic head and brain of *Drosophila* indicates a refolded band of seven segment remnants. Development 116, 111-125.

Scholz, H., Sadlowski, E., Klaes, A. and Klämbt, C. (1997). Control of midline glia development in the embryonic *Drosophila* CNS. Mech Dev. 64, 137-151.

Schutt, C. and Nothiger, R. (2000). Structure, function and evolution of sex-determining systems in Dipteran insects. Development 127, 667-677.

Seeger, M., Tear, G., Ferres-Marco, D. and Goodman, C.S. (1993). Mutations affecting growth cone guidance in *Drosophila*: genes necessary for guidance toward or away from the midline. Neuron 10, 409-429.

Shen, C.-P., Jan, L.Y. and Jan, Y.N. (1997). Miranda is required for the asymmetric localization of Prospero during mitosis in *Drosophila*. Cell 90, 449-458.

Shepherd, D. (2000). Glial dependent survival of neurons in *Drosophila*. Bioessays 22, 407-409.

Simpson, J.H., Bland, K.S., Fetter, R.D. and Goodman, C.S. (2000a). Short-range and long-range guidance by Slit and its Robo receptors: a combinatorial code of Robo receptors controls lateral position. Cell 103, 1019-1032.

Simpson, J.H., Kidd, T., Bland, K.S. and Goodman, C.S. (2000b). Short-range and long-range guidance by slit and its Robo receptors. Robo and Robo2 play distinct roles in midline guidance. Neuron 28, 753-66.

Skeath, J.B. (1999). At the nexus between pattern formation and cell-type specification: the generation of individual neuroblast fates in the *Drosophila* embryonic central nervous system. Bioessays 21, 922-31.

Skeath, J.B. and Carroll, S.B. (1992). Regulation of proneural gene expression and cell fate during neuroblast segregation in the *Drosophila* embryo. Development 114, 939-46.

Skeath, J.B. and Carroll, S.B. (1994). The *achaete-scute* complex: generation of cellular pattern and fate within the *Drosophila* nervous system. FASEB J. 8, 714-21.

Skeath, J.B. and Doe, C.Q. (1998). Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fates in the *Drosophila* CNS. Development 125, 1857-1865.

Smith, D.B. (1993). Purification of gluthatione-S-transferase fusion proteins. Methods Mol Cell Biol. 4, 220-229.

Sonnenfeld, M., Ward, M., Nystrom, G., Mosher, J., Stahl, S. and Crews, S. (1997). The *Drosophila tango* gene encodes a bHLH-PAS protein that is orthologous to mammalian Arnt and controls CNS midline and tracheal development. Development 124, 4571-4582.

Spana, E.P. and Doe, C.Q. (1995). The *prospero* transcription factor is symmetrically localized to the cell cortex during neuroblast mitosis in *Drosophila*. Development 121, 3187-3195.

Spana, E.P. and Doe, C.Q. (1996). Numb antagonizes Notch signaling to specify sibling neuron cell fates. Neuron 17, 21-26.

Spana, E.P., Kopczynski, C., Goodman, C.S., and Doe, C.Q. (1995). Asymmetric localization of numb autonomously determines sibling neuron identity in the *Drosophila* CNS. Development 121, 3489-3494.

Spradling, A.C. and Rubin, G.M. (1982). Transposition of cloned *P* elements into *Drosophila* germ line chromosomes. Science 218, 341-347.

Spradling, A.C., Stern, D., Beaton, A., Rhem, E.J., Laverty, T., Mozden, N., Misra, S. and Rubin, G.M. (1999). The Berkeley *Drosophila* genome project gene disruption project. Single P-element insertions mutating 25% of vital *Drosophila* genes. Genetics 153, 135-177.

Stoker, A.W. (2001) Receptor tyrosine phosphatases in axon growth and guidance. Curr Opin Neurobiol. 11, 95-102.

Sullivan, W., Ashburner, M., and Hawley, S.R. (2000). In *Drosophila Protocols*. Chapter 24. CSHL press.

Sun, Q., Bahri, S., Schmid, A., Chia, W. and Zinn, K. (2000). Receptor tyrosine phosphatases regulate axon guidance across the midline of the *Drosophila* embryo. Development 127, 801-812.

Sun, Q., Schindelholz, B., Knirr, M., Schmid, A. and Zinn, K. (2001). Complex genetic interactions among four receptor tyrosine phosphatases regulate axon guidance in *Drosophila*. Mol Cell Neurosci. 17, 274-91.

Tear, G. (1999). Neuronal guidance. A genetic perspective. Trends Genet. 15, 113-118.

Tear, G., Harris, R., Sutaria, S., Kilomanski, K., Goodman, C.S. and Seeger, M.A. (1996). Commissureless controls growth cone guidance across the CNS midline in Drosophila and encodes a novel membrane protein. Neuron 16, 501-514.

Tessier-Lavigne, M. and Goodman, C.S. (1996). The molecular biology of axon guidance. Science 274, 1123-1133.

Thomas, J.B. (1998). Crossing the midline. Curr Biol. 8, R102-R104.

Truman, J.W., Taylor, B.J., and Awad, T.A. (1992). Formation of the adult nervous system. In Development of *Drosophila melanogaster*, (ed. M. Bate and A. Martinez-Arias). pp. 1245-1275. Cold Spring Harbor Laboratory Press.

Udolph, G., Rath, P. and Chia, W. (2001). A requirement for Notch in the genesis of a subset of glial cells in the *Drosophila* embryonic central nervous system which arise through asymmetric divisions. Development 128, 1457-1466.

Usui-Aoki, K., Ito, H., Ui-Tei, K., Takahashi, K., Lukascovich, T., Awano, W., Hankata, H., Piao, Z.F., Nilsson, E.E., Tomida, J-Y. and Yamamoto, D. (2000). Formation of the male-specific muscle in female *Drosophila* by ectopic *fruitless* expression. Nature Cell Biology 2: 500-506.

Van Vactor, D. and Flanagan, J.G. (1999). The middle and the end: slit brings guidance and branching together in axon pathway selection. Neuron 22, 649-52.

Venter, J.C., Adams, M.D., Myers, E.W., et al., (2001). The sequence of the human genome. Science 291, 1304-1351.

Villella, A., Gailey, D.A., Berwald, B., Ohshima, S., Barnes, P.T. and Hall, J.C. (1997). Extended reproductive roles of the *fruitless* gene in *Drosophila melanogaster* revealed by behavioral analysis of new *fru* mutants. Genetics 147, 1107-1130.

Vincent, S., Vonesch, J-L. and Giangrande, A. (1996). Glide directs glial fate commitment and cell fate switch between neurons and glia. Development 122, 131-139.

Wen, Y., Nguyen, D., Li, Y. and Lai, Z.C. (2000). The N-terminal BTB/POZ domain and C-terminal sequences are essential for Tramtrack69 to specify cell fate in the developing *Drosophila* eye. Genetics 156, 195-203.

Xiong, W.C. and Montell, C. (1995). Defective glia induce neuronal apoptosis in the repo visual system of *Drosophila*. Neuron 14, 581-590.

Xiong, W.-C., Okano, H., Patel, N.P. and Montell, C. (1994). *repo* encodes a glial-specific homeo domain protein required in the *Drosophila* nervous system. Genes Dev. 8, 981-994.

Xue, F. and Cooley, L. (1993). *kelch* encodes a component of intercellular bridges in *Drosophila* egg chambers. Cell 72, 681-693.

Yamamoto, D., Fujitani, K., Usui, K., Ito, H., and Nakano, Y. (1998). From behavior to development: genes for sexual behavior define the neuronal sexual switch in *Drosophila*. Mech Dev. 73, 135-46.

Yang, X., Bahri, S., Klein, T. and Chia, W. (1997). Klumpfuss, a putative *Drosophila* zinc finger transcription factor, acts to differentiate between the identities of two secondary precursor cells within one neuroblast lineage. Genes Dev. 11, 1396-1408.

Ye, B.H., Lista, F., Lo Coco, F., Knowles, D.M., Offit, K., Chaganti, R.S. and Dalla-Favera, R. (1993). Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large-cell lymphoma. Science 262, 747-50.

Yeo, S.L., Lloyd, A., Kozak, K., Dinh, A., Dick, T., Yang, X., Sakonju, S. and Chia W. (1995). On the functional overlap between two *Drosophila* POU homeo domain genes and the cell fate specification of a CNS neural precursor. Genes Dev. 9, 1223-1236.

Yip, M.L., Lamka, M.L. and Lipshitz, H.D. (1997). Control of germ-band retraction in *Drosophila* by the zinc-finger protein HINDSIGHT. Development 124, 2129-41.

Zak, N.B., Wides, R.J., Schejter, E.D., Raz, E. and Shilo, B.Z. (1990). Localization of the DER/flb protein in embryos: implications on the faint little ball lethal phenotype. Development 109, 865-874.

Zollman, S., Godt, D., Prive, G.G., Couderc, J.L. and Laski, F.A. (1994). The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in *Drosophila*. Proc Natl Acad Sci USA. 91, 10717-21.