Molecular Mechanisms of Serotonergic Differentiation in the Developing Fruit Fly Central Nervous System

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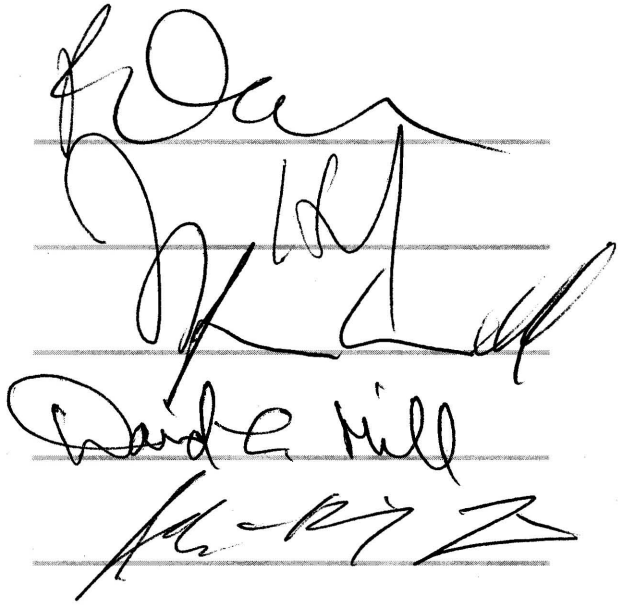

ABSTRACT

Serotonin is an important neurotransmitter that has diverse functions throughout the CNS. However, little is known about the development of the serotonergic neurons. The fruit fly, *Drosophila melanogaster*, is useful as a model system to study serotonergic development, since there are relatively few serotonergic neurons in the ventral nerve cord and they are easily identifiable. The following studies identify (1) a novel role for the axon guidance receptors *robo2* and *robo3* and (2) a role for FGF signaling and the Ets transcription factor *pointed* in *Drosophila* serotonergic differentiation. Specifically, data suggest that *robo2* and *robo3* act as positive regulators of differentiation, while *pointed* and FGF signaling seems to function negatively. Expression of the serotonin transporter (SerT) is used as a primary marker of differentiation, but serotonin synthesis is also examined. Further, a genetic interaction is shown between the transcription factor *eagle* and *robo2/3* as well as between *eagle* and *pointed*. A review of currently known regulators of serotonergic development is presented and a model integrating past and current results is discussed. This work was completed under the advisement of Barry Condron, PhD, in the Department of Biology.

DEDICATION

I would like to dedicate this work to my family and friends, who have not only tolerated my madness but also encouraged me at every step along the way.

GENERAL INTRODUCTION

Serotonergic Differentiation in *Drosophila*

RELEVANCE OF SEROTONERGIC FUNCTION

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter with diverse functions throughout the body. Originally identified as a substance important for vascular constriction in the periphery (Rapport, 1948), serotonin is probably now most widely known for its role in regulating central nervous system (CNS) function. Serotonin influences many physiological and behavioral states, and dysfunction of the serotonergic system is implicated in the pathology of several psychiatric disorders. As a result, medications that alter synaptic levels of serotonin have become popular therapeutic tools.

Physiological and behavioral functions of serotonin in the CNS

The serotonergic system modulates behavior in both invertebrate and vertebrate species, and is a highly conserved neurotransmitter system (Jacobs and Azmitia, 1992). In the nematode *Caenorhabditis elegans* (*C. elegans*), serotonin regulates locomotion, feeding, and egg-laying behavior (Hardaker et al., 2001; Horvitz et al., 1982; Segalat et al., 1995), while in the fruit fly *Drosophila melanogaster* (*D. melanogaster*), serotonin has been implicated in processes of learning and memory as well as sensory processing (Carew, 1996; Nichols et al., 2002). Mammalian systems have yielded insights into the role of serotonin in behaviors such as sleep, mating, and mood (Lucki, 1998). In addition to

mediating these behaviors, serotonin influences the physiological function of cardiovascular, enteric, and thermoregulatory systems, among others (Jacobs and Azmitia, 1992; Lucki, 1998). For a more complete review of the behaviors modulated by serotonin, see (Lucki, 1998).

Role of serotonin in neurological and psychiatric disorders

Given that serotonin plays an important role in so many aspects of CNS function, it is not surprising that dysfunction of the serotonergic system correlates with a number of neurological dysfunctions. In mice, genetically or pharmacologically induced aberrations in serotonergic signaling can lead to increased anxiety, aggression, and vulnerability to drugs of abuse (Gingrich, 2002; Nelson and Chiavegatto, 2001). In humans, serotonergic dysfunction has been linked to psychiatric disorders such as anorexia, autism, depression, anxiety, and schizophrenia, and also seems to contribute to suicidal behavior (Cook and Leventhal, 1996; Lucki, 1998; Ohara et al., 1999; van Heeringen, 2003). Considering the wide range of disorders influenced by serotonin, it is not surprising that a class of drugs known as the selective serotonin reuptake inhibitors (SSRIs), designed to prevent reuptake of serotonin by the serotonin transporter (SerT), are among the most widely prescribed psychiatric therapies to date.

Additionally, evidence suggests that a polymorphism in the promoter region of the human SerT gene may contribute to the likelihood of developing certain psychiatric disorders. The two most prevalent alleles are termed 'short' and

'long' according to the length of repeat elements upstream of the SerT transcriptional start site (Heils et al., 1996). Other less common alleles, including an 'extra long' allele have also been identified (Lesch et al., 1996). The short allele exhibits lower transcriptional efficiency (Lesch et al., 1996) and is associated with anxiety, autism, and predisposition for depression as well as seasonal affective disorder (Caspi et al., 2003; Klauck et al., 1997; Lesch et al., 1996; Rosenthal et al., 1998). Further allelic differences in SerT expression have been reported in response to glucocorticoid administration (Glatz et al., 2003). Although many studies suggest an importance for SerT function in human behavior, little is known about the transcriptional regulation of SerT.

Developmental role for serotonin

Several lines of evidence indicate that serotonin may also play an important role during the early development of the CNS. In *Drosophila*, both the 5-HT_2 receptor and the serotonin biosynthetic enzymes tryoptophan hydroxylase (Tph) and Dopa decarboxylase (Ddc) are transiently expressed during early embryonic segmentation (Colas et al., 1995). Serotonin influences neural crest cell and craniofacial development during rodent morphogenesis as well (Choi et al., 1997; Moiseiwitsch, 2000). Further, serotonin appears important for neuronal differentiation and synapse formation throughout the developing CNS. Interestingly, non-serotonergic processes of the developing telencephalon and diencephalon briefly express 5-HT receptors, SerT, and a vesicular monoamine transporter (VMAT) (Lebrand et al., 1998). Serotonin depletion by

pharmacological blockage of serotonin synthesis causes altered neurogenesis and differentiation of cells innervated by serotonergic processes (Whitaker-Azmitia et al., 1996); while increased levels of serotonin, as seen in mice lacking the serotonin degradation enzyme monoamine oxidase A (MAOA), disrupts axon guidance as well as cortical patterning in non-serotonergic neurons (Luo et al., 2003; Upton et al., 1999). For a complete review of serotonin in CNS development, see (van Kesteren and Spencer, 2003).

Additionally, serotonin seems to autoregulate the growth of serotonergic neurons themselves. In *Drosophila* Ddc mutants incapable of synthesizing serotonin, a dramatic increase in the arborization of peripheral serotonergic neurons is observed (Budnik et al., 1989). A similar autoregulatory function for serotonin is suggested by studies in developing rats, as well as in cell culture experiments (Whitaker-Azmitia et al., 1996). Although it remains unclear how serotonin affects the formation of circuitry in the CNS, a number of studies have indicated that disturbances in early serotonin function may lead to later behavioral abnormalities. For example, disruption of 5-HT_{1A} receptor expression in the developing mouse forebrain causes expression of anxiety-like behaviors in the adult (Gross et al., 2002). A similar adult phenotype results when serotonergic differentiation is disrupted (Hendricks et al., 2003). Finally, there is some evidence that developmental disturbances in serotonin synthesis may contribute to autism in humans (Chugani et al., 1999). In summary, the diversity of serotonergic influence on both the behavior and development of the CNS,

illustrate the importance of achieving a better understanding of the processes of serotonergic neuronal specification and maturation.

EARLY SPECIFICATION OF SEROTONERGIC NEURONS

Neuroblast specification

Initial studies of serotonergic development in *Drosophila* largely recapitulated earlier findings in the grasshopper, and revealed numerous similarities between the two species. As in the grasshopper, serotonergic neurons in the *Drosophila* ventral nerve cord (VNC) are organized in a bilaterally symmetrical pattern, with two cells observed in most hemisegments. Some thoracic hemisegments have three serotonergic neurons, while the last abdominal segment has only one (Valles and White, 1988). However, all serotonergic axons extend across the midline along the posterior commissure (Lundell and Hirsh, 1994; Taghert and Goodman, 1984). Additionally, serotonergic neurons in each hemisegment arise from stereotyped divisions of their neural precursor cell, neuroblast (NB) 7-3, designated by its position and time of delamination from the neuroepithelium (Doe, 1992; Doe and Goodman, 1985). The origin of the serotonergic neurons has been traced to NB 7-3 predominantly by live observation and cell ablation experiments in the grasshopper (Taghert and Goodman, 1984) and use of molecular markers in the fly (Lundell et al., 1996).

Generally, the formation and specification of NBs requires proper expression of proneural genes as well as spatial patterning genes, including those of the segment polarity and homeobox families, reviewed in (Doe and Skeath, 1996).

Specification of the unique NB 7-3 identity depends on interactions between the secreted segment polarity proteins Wingless (Wg) and Hedgehog (Hh), and homeobox transcription factors Engrailed (En) and Gooseberry-distal (Gsb-d) in the neuroectoderm (Matsuzaki and Saigo, 1996; McDonald and Doe, 1997).

Loss of these genes causes phenotypes ranging from severe loss of NB 7-3 seen in *en* mutants to mild loss in *hh* and *wg* mutants, or duplicated NB 7-3s in *gsb-d* mutants (Matsuzaki and Saigo, 1996). It has been proposed that during NB 7-3 specification Wg and Hh function redundantly, because in contrast to the relatively mild individual mutant phenotypes, NB 7-3 almost never forms in the double *wg hh* mutant (Matsuzaki and Saigo, 1996). Wg and Hh may, however, play an additional role later in serotonergic development, as a loss of *hh* or *wg* causes a partial loss of serotonergic neurons (Patel et al., 1989). Other members of the *wg* signaling pathway, including *patched* and *gooseberry*, alter serotonergic neuronal formation as well, although in these mutants an abnormal increase in serotonergic neurons is observed (Patel et al., 1989). Further investigation is required to elucidate the specific roles of Wg signaling members in later serotonergic development.

Finally, *en* is known to function not only in the neuroectoderm during specification, but also throughout the NB 7-3 lineage, where it is necessary for the proper specification of the serotonergic neurons (Lundell et al., 1996). Nonetheless, evidence indicates that the combined action of *en*, *wg*, and *hh* lead to the expression of the transcription factor *huckebein* (*hkb*), in both the NB and its progeny (McDonald and Doe, 1997). Huckebein is required for normal

serotonergic development, and seems to function early in the lineage just after NB formation (Lundell et al., 1996). NB 7-3 uniquely expresses both *en* and *hkb*, thus enabling specific detection of this NB (Doe, 1992; Lundell et al., 1996).

Lineage development

Recent evidence suggests that all NBs in the VNC undergo a similar gene expression program over time as they begin to divide and form their daughter cells, the ganglion mother cells (GMCs) (Brody and Odenwald, 2002; Isshiki et al., 2001; Novotny et al., 2002). This program begins with expression of *hunchback* (*hb*), followed by *Krüppel* (*Kr*), and finally *pdm* in NB 7-3 (Isshiki et al., 2001). Each stage of expression designates a particular GMC, and additionally characterizes the future progeny of that GMC. NB 7-3 produces three GMCs, each dividing once to produce either two neurons or one neuron and one programmed cell death. The final progeny of NB 7-3 total four post-mitotic neurons: three contralaterally projecting interneurons, including the serotonergic neurons (also known as EW1 and EW2) and a neuron expressing the neurotransmitter corazonin (EW3), in addtion to one ipsilaterally projecting motoneuron (also known as GW) (Isshiki et al., 2001; Novotny et al., 2002).

Unlike grasshopper, the two serotonergic neurons in each *Drosophila* hemisegment arise from different GMCs (Isshiki et al., 2001; Novotny et al., 2002). At present, it remains unclear whether functional differences exist between the two serotonergic neurons, although they are known to display slightly different molecular markers. The first-born serotonergic neuron (EW1)

arises from a division of the first GMC (GMC-1) and expresses both *hb* and *Kr*, while the second-born (EW2) is a product of the second GMC (GMC-2) and expresses *Kr* but not *hb*, and can be further identified by expression of the transcription factor *zfh-2* (Isshiki et al., 2001). *hb* and *Kr* are both necessary and sufficient for specification of the EW1 and EW2 serotonergic neurons respectively (Isshiki et al., 2001; Novotny et al., 2002). Although EW2 arises from GMC-2 and initially expresses only *Kr* (Isshiki et al., 2001), evidence indicates that *pdm* expression is activated in this neuron at later stages (Lundell and Hirsh, 1998). This is not entirely surprising given that *Kr* is known to activate *pdm* expression (Isshiki et al., 2001). Thus, both *pdm* and *zfh-2* function as specific markers of EW2, the most lateral of the two serotonergic neurons in each hemisegment. Between the serotonergic neurons, Hb further serves as a unique marker of EW1. Hb, Kr, and Pdm are all nuclear transcription factors that function throughout the developing embryo, and although some of their transcriptional targets have been identified in other tissues, it remains unclear how they specifically mediate serotonergic specification.

Recent studies indicate that programmed cell death occurs to one of the progeny from both GMC-2 and GMC-3 and that this is due to the asymmetrical segregation of the membrane associated protein Numb during cell division (Lundell et al., 2003). Numb is required for the normal formation of EW2, and acts by inhibiting a Notch induced apoptotic signal (Lundell et al., 2003). Additionally, although NB 7-3 and its progeny GMCs follow the stereotypical expression pattern of Hb → Kr → Pdm seen throughout the VNC, many other

genes are also required for specification of the serotonergic lineage. These genes are further useful individually, or in combination, as molecular markers for NB 7-3 and its progeny. Some of these include the orphan steroid hormone receptors *seven-up* and *eagle* (Doe, 1992; Higashijima et al., 1996). Unlike the other genes described thus far, *eagle* (*eg*) seems to be most unique in its expression, as it is detected in the lineage of NB 7-3 and only three other NBs in the VNC (Higashijima et al., 1996). To date, only some of these factors have been examined for their role in serotonergic development, and their interactions with each other remain almost totally uncharacterized.

TERMINAL DIFFERENTIATION

The fully differentiated serotonergic neuron requires expression of many genes to establish functional maturity, including the serotonin transporter (SerT) and the serotonin biosynthetic enzymes tryptophan hydroxylase (Tph) and Dopa decarboxylase (Ddc). Of these, the regulation of Ddc has been most widely characterized in *Drosophila*, as it is also required for biosynthesis of the neurotransmitter dopamine. Ddc expression and serotonin synthesis are first detected in the VNC at embryonic stage 17 (Lundell and Hirsh, 1994). In contrast, SerT expression begins at late embryonic stage 15, and thus serves as an earlier marker for serotonergic differentiation than serotonin synthesis (Novotny et al., 2002). *Drosophila* SerT (*dSerT*), like its mammalian homologs, is responsive to both cocaine and fluoxetine (Demchyshyn et al., 1994).

Most of the factors described above, including *hb*, *Kr*, *hkb*, and *en*, seem to play a master regulatory role in the development of the serotonergic lineage, rather than a specific role in terminal differentiation. A loss of one of these factors induces a range of phenotypes, from an absence of specific serotonergic neurons to a loss of other serotonergic markers such as *eg* (Lundell et al., 1996). Further, it seems unlikely that the lineage genes exclusively control terminal differentiation, since this process does not begin until much later in development. These early lineage markers are, however, normally expressed not only in the progenitors of the serotonergic neurons but also in the neurons themselves, and may play a role in maintaining serotonergic identity.

The zinc-finger transcription factor *eg* is particularly interesting because although it is expressed throughout the NB lineage, it appears to function more specifically in serotonergic differentiation (Higashijima et al., 1996; Lundell and Hirsh, 1998). A loss of *eg* does not disrupt the NB lineage but causes a loss of both SerT (see Chapters 1 and 2) as well as Ddc expression in a percentage of neurons depending on the severity of the mutation (Lundell and Hirsh, 1998). In the serotonergic neurons, *eg* function is not simply restricted to activation of terminal differentiation genes, as it is also required for normal *en* expression (Lundell and Hirsh, 1998). Thus although *en* is required for *eg* expression during lineage development, *en* becomes dependent on *eg* during differentiation. Eg is also required for normal *zfh-2* expression in EW2, and it has been proposed that Zfh-2 functions redundantly for Eg in this neuron (Lundell and Hirsh, 1998).

The LIM-homeodomain transcription factor *islet* is one of the only genes identified thus far that appears to have a truly restricted function in serotonergic terminal differentiation. Loss of *islet* causes a loss of SerT expression (J.A.C. and B.G.C., unpublished observations) as well as serotonin synthesis (Thor and Thomas, 1997). Islet is not required for neuronal specification and is detected in postmitotic serotonergic and dopaminergic neurons but not their progenitors (Thor and Thomas, 1997). Although *islet* plays a critical role in serotonergic differentiation, evidence suggests that it functions as part of a combinatorial code of transcription factors that together specify the serotonergic phenotype. Pan-neural expression of Islet, for example, does not induce formation of ectopic serotonergic neurons (Thor and Thomas, 1997). Similarly, ectopic expression of Hkb or En does not yield ectopic neurons, while pan-neural expression of Eg produces only a few ectopic cells (Dittrich et al., 1997; Lundell et al., 1996). Closer analysis revealed that the Eg-induced ectopic serotonergic neurons where cells that also expressed *hkb* (Dittrich et al., 1997).

Several other genes likely function in serotonergic differentiation as well. In addition to the POU transcription factor *pdm*, two other POU transcription factors have been detected in serotonergic neurons: *drifter* and I-POU, a possible inhibitory factor that may compete with *drifter* for DNA binding (Anderson et al., 1995; Treacy et al., 1991). Both *drifter* and *zfh-2*, a specific marker of EW2, may regulate expression of Ddc (Johnson and Hirsh, 1990; Lundell and Hirsh, 1992). Future genetic analyses should determine whether they are required for expression of Ddc and possibly other genes during serotonergic differentiation.

Normal serotonergic differentiation also requires proper axon guidance, as serotonergic axons must cross the ventral midline in order to appropriately branch in the contralateral neuropil. Like all commissural axons, serotonergic axons respond to midline chemoattractants and repulsants, altering their growth trajectory in response to axon guidance receptor signaling (Georgiou and Tear, 2002)(also see Chapter 1). A link between axon guidance and differentiation is further suggested by observations of aberrant serotonergic processes in Islet or Eg mutant VNCs (Dittrich et al., 1997; Thor and Thomas, 1997). At this point, it remains unknown how these transcription factors might regulate guidance decisions.

Finally, although proper axon guidance is ultimately important for serotonergic axons to reach their innervation targets, it may also be important for directing axons to extrinsic differentiation signals along their route. Developing commissural neurons in the vertebrate require an *en passant* growth factor secreted by the floorplate, an intermediate target along their trajectory (Wang and Tessier-Lavigne, 1999). In the grasshopper, a fibroblast growth factor (FGF) signal at the midline seems to play a role in regulating the onset and early maintenance of SerT expression (Condron, 1999). Possible extrinsic signals required during serotonergic development in *Drosophila* remain unknown.

The goals of the following studies were to determine (1) if the midline and/or FGF signaling play an important role in *Drosophila* serotonergic differentiation as has been suggested in the grasshopper, and (2) to examine the role of axon guidance in differentiation. Through the course of these studies, several novel

regulators of differentiation have been identified, including the Roundabout axon guidance receptors *robo2* and *robo3* (Chapter 1). Additionally, a role for FGF/tyrosine kinase signaling and the Ets transcription factor *pointed* in serotonergic differentiation has been found (Chapter 2). Both Robo2 and Pointed are shown to genetically interact with Eg, although Robo2 shows a positive regulatory relationship to both Eg and serotonergic differentiation while Pointed appears to function antagonistically. The following chapters describe the function of these newly identified regulators in serotonergic development and examine their relationships to the transcription factor Eg.

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CHAPTER ONE

robo2 and *robo3* Interact with *eagle* to Regulate Serotonergic Differentiation

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Data from midline mutant analysis (Figure 2) was contributed by both Heather Rieff and John Chen, and midline cut experiments (Figure 3) were performed by John Chen. Ellen Uri performed some of the work with *robo2* and *robo3* mutants, contributing to experiments shown in Figure 4 and more specifically to those shown in Figure 4 (E-F).

SUMMARY

The function of the central nervous system (CNS) depends critically upon the correct differentiation of neurons and formation of axonal connections. Some aspects of neuronal differentiation are known to occur as axonal connections are forming. Although serotonin is a highly conserved neurotransmitter that is important for many CNS functions, little is known about the process of serotonergic differentiation. We show that in *Drosophila*, expression of the serotonin transporter (SerT) is both temporally and physically related to midline crossing. Additionally, we show that the axon guidance molecules *roundabout2* and *roundabout3* (*robo2/3*) are necessary for serotonergic differentiation and function independently of their ligand, *slit*. A loss of *robo2* or *robo3* causes a loss of SerT expression in about half of neurons, and resembles the phenotype seen in mutants for the transcription factor *eagle* (*eg*). Finally, we show a direct relationship between *robo2/3* and *eg*: *robo2/3* mutants lose Eg expression in serotonergic neurons, and *robo2* and *eg* interact genetically to regulate SerT expression. We propose that post-midline expression of Robo2/3 is part of a signal that regulates serotonergic differentiation and is transduced by the transcription factor Eg.

INTRODUCTION

Serotonin is a neurotransmitter that modulates virtually every neural circuit in the brain, and plays an important role in many behavioral and physiological functions of the CNS (Jacobs and Azmitia, 1992). Dysfunction of the serotonergic system is thought to be involved in several neurological disorders, including depression and autism. Additionally, evidence suggests that some disorders, such as autism, may have a developmental component (Chugani et al., 1999). Recent experiments in mice further suggest that anxiety-like and aggressive behaviors result from defects of serotonergic differentiation (Hendricks et al., 2003). Despite the importance of serotonergic function in the CNS, the mechanisms controlling serotonergic differentiation remain largely unknown. We use *Drosophila* to investigate the development of the serotonergic system, which has been highly conserved throughout evolution (Jacobs and Azmitia, 1992). *Drosophila* is useful as a model system for investigating serotonergic development since the relatively few serotonergic neurons within the ventral nerve cord are easily identifiable (Isshiki et al., 2001).

Serotonergic neurons in the *Drosophila* ventral nerve cord (VNC) are organized in a bilaterally symmetric pattern, with two serotonergic neurons per hemisegment that extend axons across the midline via the posterior commissure and branch in the contralateral neuropil (Valles and White, 1988). The specification of serotonergic neurons has been well described in the fly through use of molecular markers (Broadus and Doe, 1995; Doe, 1992; Isshiki et al., 2001; Lundell et al., 1996; Lundell and Hirsh, 1998), however, the process of later

development remains relatively uncharacterized. Reuptake of released serotonin by the serotonin transporter (SerT) is an essential component of serotonergic function and one of the earliest steps in serotonergic differentiation. A highly conserved serotonin transporter, *dSerT*, is specifically expressed in fly serotonergic neurons and is blocked by both fluoxetine (Prozac) and cocaine (Corey et al., 1994; Demchyshyn et al., 1994). Expression of SerT precedes the onset of serotonin synthesis in fly (present study) and grasshopper (Condron, 1999), as well as synapse formation in mouse (Bruning et al., 1997).

In grasshopper, cuts that sever serotonergic contact with the midline lead to a loss of SerT expression, suggesting that the midline plays a critical role in the induction and maintenance of SerT activity (Condron, 1999). However, application of Fibroblast Growth Factor 2 (FGF2) to the nerve cord rescues SerT expression, indicating the presence of a midline-associated FGF-like signal that induces serotonergic differentiation. In mouse and rat, serotonergic neuronal specification requires FGF signaling as well (Ye et al., 1998). Axon guidance molecules are also likely to play an important role in serotonergic development, since serotonergic neurons must cross the ventral midline and branch in the contralateral neuropil in order to achieve their final differentiated state. Additional indications of a relationship between serotonergic axon guidance and differentiation come from data showing that a loss of function (LOF) in genes of the serotonin biosynthetic pathway impair axon pathfinding and alter branching patterns in the periphery (Budnik et al., 1989).

We use SerT activity, measured by reuptake of serotonin, as a marker of serotonergic differentiation. SerT serves as an early and easily testable step in serotonergic differentiation, since it precedes the onset of serotonin synthesis. Here we show that regulation of SerT activity in the fly embryo, as in the grasshopper, is not only temporally but also physically related to midline crossing. Additionally, we show that members of the *roundabout* (*robo*) family of axon guidance receptors, *robo2* and *robo3* (*robo2/3*), but not *robo*, are required for serotonergic differentiation. *robo2* and *robo3* control axon guidance throughout the CNS by regulating midline crossing and determining lateral position along specific longitudinal fascicles (Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b).

Finally, the present study indicates that *robo2/3* function in the same genetic pathway as the zinc-finger transcription factor *eagle* (*eg*), which is required for serotonergic differentiation and is expressed in all serotonergic neurons of the fly VNC (Dittrich et al., 1997; Higashijima et al., 1996; Lundell and Hirsh, 1998). Loss of *eg* function causes a loss of both serotonin synthesis and the biosynthetic enzyme Dopa decarboxylase (Ddc) in a portion of serotonergic neurons according to the severity of the mutation (Lundell and Hirsh, 1998). Here we show that a loss of *eg* also causes a loss of SerT activity. The pattern of SerT and serotonin loss observed in an *eg* mutant closely resembles the phenotype observed in *robo2/3* LOF mutants. A direct relationship between *robo2* and *eg* is suggested by data showing a loss of Eg expression in *robo2/3* mutants and through genetic rescue experiments.

MATERIALS AND METHODS

Fly strains and genetics

The following fly strains were used: Canton S and Oregon R (wild type); *eagle*Gal4 (*Mz360)*, *eagle*lacZ (eg^{289}), and *eagle* null (eg^{18b}) (Lundell and Hirsh, 1998); UAS-tau-lacZ (Callahan and Thomas, 1994); *commissureless* null ($comm^{e39}$) (Tear et al., 1996); *slit* null ($slit^{2}$) (Kidd et al., 1999); *robo* null ($robo^{GA285}$), UAS-*robo* (Kidd et al., 1998a); EP2582, *robo2* null ($robo2^{x123}$, $robo2^{x135}$), *robo3* null ($robo3^{1}$) and UAS-robo3 (Simpson et al., 2000a) (Rajagopalan et al., 2000b); UAS-Unc5d (Keleman and Dickson, 2001); *scabrous*Gal4 provided by P. Adler; and *elav*Gal4 provided by J. Hirsh. eg^{Mz360} was generated in an enhancer-trap screen (Ito, 1995) using the Gal4 P-element construct (Brand and Perrimon, 1993) and functions as both an *eg*Gal4 driver and an *eg* hypomorphic allele. The P-element construct is inserted 5′ of the first exon in the *eg* locus and causes an *eg* phenotype in a homozygous condition that is allelic to other *eg* mutations (Dittrich et al., 1997).

Immunohistochemistry

Embryos were collected into Eppendorf tubes, bleached for 1 minute, washed with salt solution (0.04% NaCl + 0.03% TritonX-100), followed by Schneider's medium (Sigma). Embryos were then either transferred to a Petri dish containing Schneider's medium for dissection or to a second Eppendorf containing Schneider's where they were dounced gently with a DNA pellet disrupter. VNCs isolated from dissection or limited douncing were then

transferred to another Petri dish containing Schneider's medium and affixed to the surface of a glass coverslip. All stage 14 VNCs were dissected manually as they were too fragile to withstand douncing. Stage 15-17 VNCs were isolated on the basis of embryo morphology (Campos-Ortega, 1985) when dissected or BP102 staining and serotonergic axon morphology when dounced.

To visualize serotonin transporter (SerT) activity, serotonin (Sigma) was added to the medium to a final saturating concentration of 10 μM and incubated for 10 minutes. Media was then removed, replaced with fix (4% paraformaldehyde/PBS, freshly made) and incubated for 60 minutes at room temperature. After fixation, coverslips with attached nerve cords could be transferred out of liquid between washings. VNCs were then processed for histochemical analysis as described previously (Condron, 1999). Imaging was performed on an Olympus BX40 microscope and photographs, DIC and fluorescent, taken with a Photometrics SenSys camera. All images except those in Figure 5 were taken with an Olympus 40x or 20x lens. ImageIP software was used to capture the images, and fluorescent images were subjected to one frame deconvolution (95% removal, 75% gain) using VayTek Hazebuster. Fluorescent channels were stacked and layouts/labeling were performed in Adobe Photoshop. Fixed samples shown in Figure 5 were imaged with a Nikon Eclipse E800 microscope (100x oil lens, NA=1.3), Hamamatsu ORCA-ER camera, with a Perkin-Elmer spinning disc confocal unit. One stack of about 100 slices was taken, spaced 0.24 μm, 2x2 binning and with an exposure time of 500 milliseconds. Image reconstructions were performed with Volocity 2.0. The

antibodies used were as follows: 5HT 1:5000 (Immunostar); βgal 1:100 (Clontech); BP102 1:1000, βgal 1:20 (Developmental Studies Hybridoma Bank, University of Iowa); Mouse anti-Eg 1:40 (gift from Chris Doe); secondary antibodies 1:2000 (Jackson Labs).

In situ hybridization

For *dSerT* in situ hybridization, a previously reported cDNA was used (Demchyshyn et al., 1994). DNA was cut with XbaI, and mRNA was made using the Ambion Megascript T3 kit according to manufacturer's instructions. Slide mounted VNCs were hybridized directly with diluted probe with 50% formamide, 10x SSC and 0.5% Tween-20 overnight at 55°C. After washing, digoxigenin-labeled probe was detected with alkaline phosphatase linked antibodies (1:3000, Roche).

RESULTS

Serotonin transporter expression correlates with midline crossing

During development in both grasshopper and fruit fly, serotonergic neurons cross the midline of the CNS before reaching their fully differentiated state and branching in the contralateral neuropil (Condron, 1999; Lundell and Hirsh, 1994). In the grasshopper, serotonergic neurons require midline contact for proper initiation of SerT activity (Condron, 1999). To determine if serotonergic differentiation in the fruit fly is also related to midline crossing, we assayed *dSerT* mRNA and SerT enzymatic activity at various embryonic stages of

development. *dSerT* mRNA was analyzed by in situ hybridization, and SerT activity was detected by incubating staged, live, dissected cords in 10 μM serotonin for 10 minutes prior to fixation and then staining for serotonin. To visualize serotonergic axonal projections, we used the yeast Gal4 system to express tau-lacZ under the *eagle (eg)* promoter. *eg* is a transcription factor required for normal serotonergic differentiation, and is expressed in relatively few cells in the CNS (Higashijima et al., 1996; Lundell and Hirsh, 1998). Figure 1 shows staining for serotonin uptake in red and β-gal in green. In addition to the serotonergic neurons, a pair of *eg* expressing lateral neurons are also clearly visible; these lateral *eg*-positive neurons cross in the anterior commissure, while serotonergic neurons cross in the posterior commissure. However, little is known about the function of these neurons.

The expression of *dSerT* mRNA and SerT activity correlates temporally with axons reaching the contralateral side of the CNS. As seen in Fig. 1A, stage 14 serotonergic axons are just arriving at the midline, at a point of either pre-crossing or mid-crossing. Wild type stage 14 embryos never show SerT activity. By late stage 15, when growth cones have reached the contralateral side, *dSerT* mRNA (Fig. 1C) and activity (Fig. 1B) is first detected. Stage 16 wild-type serotonergic neurons continue to extend axons into the contralateral neuropil and always show SerT activity (data not shown). Serotonin synthesis does not begin until stage 17 (Lundell and Hirsh, 1994; Valles and White, 1988). Thus, *dSerT* mRNA and activity precede serotonin synthesis, and are not observed until midline crossing is complete. Many signals could potentially initiate SerT

expression, including autonomous timing, contact with either the midline or a contralateral signal.

Preliminary screen of midline guidance mutants did not show a disruption of serotonin transporter expression

In order to further investigate the relationship between midline crossing and serotonergic differentiation, a broad screen of mutants causing disruptions in midline structures and/or axon guidance was conducted. SerT activity was analyzed in mutants including *commissureless (comm)*, *robo* gain-of-function (GOF), and *robo2* GOF (Fig.2) as well as *spitz*, and *single-minded* (data not shown). Specific over-expression of Robo or Robo2 in the serotonergic neurons was achieved by driving expression of UAS-*robo* (Kidd et al., 1998a; Kidd et al., 1998b) or EP2582 (Rajagopalan et al., 2000b; Simpson et al., 2000a) under *eg*Gal4 (*Mz360*) (Dittrich et al., 1997). Loss-of-function (LOF) mutants for *robo* and *slit* (the ligand for *robo*) were also analyzed.

In a *comm* LOF mutant, all axons of the CNS fail to cross the midline, as seen in Fig. 2B with the axonal marker BP102 (green). Over-expression of Robo2, but not Robo, prevents serotonergic axons from crossing the midline (Fig. 2E,F). Conversely, *robo* LOF causes ectopic midline crossing of most CNS axons and a *slit* LOF causes all axons to grow along the midline (Fig. 2C,D) (Kidd et al., 1999; Kidd et al., 1998a; Seeger et al., 1993). In a *robo* LOF, we observe disorganized and inappropriate serotonergic branching toward the midline (seen best in

posterior segments of Fig. 2D), rather than the traditional "roundabout" re-crossing phenotype.

Based on studies in grasshopper, we expected mutants that prevent midline crossing to lack SerT activity. In contrast, the midline mutants analyzed in our initial screen (shown in Fig.2) displayed normal stage 16 SerT activity. Later examination of other axon guidance mutants, including *robo2* and *robo3* (see Fig. 4), revealed their role in regulating SerT. However, the negative results obtained from our original screen led us to address the question of midline function differently, by attempting to recapitulate midline cut experiments previously done in the grasshopper. A simple explanation for the lack of serotonergic phenotype in *comm* and Robo2 GOF mutants, for example, is that midline crossing is not critical for differentiation in the fly as it is in the grasshopper. Another possibility, however, is that transient functional contact occurs between serotonergic axonal projections and the midline in these mutants that are sufficient to induce normal differentiation.

Serotonin transporter expression is spared following midline cuts when robo2 is expressed

To test the above hypothesis, asymmetrical cuts were made in stage 16 VNCs that severed axonal contact with the midline. These cuts separated the serotonergic cell bodies on only one side of the CNS from the midline. When such cuts were made in the grasshopper CNS, SerT activity was lost from the serotonergic neurons (Condron, 1999). In the fly, both wild type serotonergic

neurons and neurons over-expressing Robo (with *eg*Gal4) lost SerT activity when physically separated from the midline (Fig. 3A,B). Cells that lose SerT activity maintain expression of the homeobox transciption factor, *engrailed* (green, Fig. 3A,B). In a *robo2* GOF background, however, SerT activity was unaffected by midline cuts (Fig. 3C). One possibility is that axotomy caused a loss of SerT activity in the wild type and Robo GOF VNCs, as serotonergic axons project across the midline in these mutants but remain ipsilateral in a Robo2 GOF. However, in wild type VNCs cut perpendicularly to the midline or in other orientations that sever axons without removing midline contact, SerT activity was not lost (data not shown). In fact, many of the VNCs analyzed throughout this work were severed at one end during dissection and a loss of SerT was not observed.

Genetic disruption of serotonergic midline crossing by expression of the repulsive axon guidance molecule Unc5 also caused a loss of SerT activity, although in only a portion of neurons (Fig. 3D, 7). Unc5 responds negatively to Netrin at the midline and thereby prevents midline crossing (Keleman and Dickson, 2001). Serotonergic neurons were specifically prevented from crossing the midline by expression of Unc5 with the *eg*Gal4 driver. One possible explanation for an incomplete disruption of SerT expression in the Unc5 GOF is an incomplete midline axon guidance phenotype, given that in these mutants some axonal projections are seen extending toward (asterisks in Fig. 3D) and perhaps even touching the midline (data not shown). However, a clear distinction is seen between the Unc5 GOF and *robo2* GOF mutants, where in both

cases axons are prevented from crossing the midline but only Unc5 GOF disrupts SerT expression (compare Fig. 3D and 2F). Thus, over-expression of Robo2 specifically uncouples midline dependence from induction and/or maintenance of SerT activity. This indicates that *robo2* may either directly or indirectly regulate SerT activity. Over-expression of Robo2 (EP2582), however, is not sufficient to induce precocious SerT expression, as stage 14 SerT activity is never seen (data not shown). SerT induction likely requires other signaling factors in addition to Robo2, or relief from some inhibitory mechanism that prevents inappropriate early differentiation.

Normal serotonin transporter expression requires robo2 and robo3

To further investigate the role of *robo2* in regulating SerT expression, both *dSerT* mRNA and SerT activity were analyzed in a *robo2* null background. In a *robo2* loss of function mutant, more than half of the serotonergic neurons fail to express either *dSerT* mRNA or activity at stage 16 (Fig. 4A,D; Fig. 7). Additionally, serotonin synthesis is lost in many cells at stage 17 and in first instar larvae (data not shown). A loss of SerT activity is observed in both null alleles of *robo2* analyzed (*robo2*^{x123} and *robo2*^{x135}). A *robo3* null has the same SerT/serotonin phenotype as loss of *robo2* (Fig. 4C; Fig. 7), but *robo* null mutants show a wild type pattern of SerT activity (Fig. 2D). These data suggest that *robo2* and *robo3*, but not *robo* are required for normal serotonergic differentiation. *robo2* and *robo3* (*robo2/3)* encode similar receptors that modulate axon guidance across the ventral midline and also contribute to lateral positioning of axons in the

developing CNS (Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b). *Slit*, a chemorepellant expressed on the midline, is a ligand for all three Robos (Brose et al., 1999); however, SerT activity is normal in a *slit* mutant (Fig. 2C). This indicates that the function of Robo2/3 in serotonergic differentiation is *slit* independent.

Possibly, the lack of SerT and serotonin synthesis in *robo2/3* loss of function mutants is due to general developmental defects in these nerve cords. To test the state of cellular differentiation in serotonergic neurons lacking SerT activity, *robo2* mutant VNCs were double stained for SerT activity and lacZ expressed under the *eagle* promoter (eg^{289}; Fig. 4E). Cells lacking SerT still stain for lacZ, indicating that loss of *robo2* at least does not disrupt the lineage or early specification of serotonergic neurons in the VNC, when the *eg* promoter is first active.

Since a *robo2* GOF permits maintenance of SerT activity following a midline cut, it seems likely that at least Robo2 functions autonomously in the serotonergic neurons. However, an alternative possibility suggests that they function indirectly by guiding serotonergic growth cones to a currently unknown differentiation signal in the neuropil. Evidence that this might not be the case comes from data showing that mutations in *robo*, *spitz*, and *single-minded* do not cause defects in serotonergic differentiation despite a sometimes severe disruption in CNS organization (data not shown).

robo2 and robo3 are required for normal expression of the transcription factor eagle

Normal serotonergic differentiation in the fly requires the orphan steroid hormone receptor and zinc-finger transcription factor *eagle* (*eg*), which is expressed in the neuroblast that gives rise to the serotonergic neurons and throughout early stages of serotonergic differentiation (Dittrich et al., 1997; Higashijima et al., 1996; Lundell and Hirsh, 1998). Loss of *eg* function results in a loss of SerT expression and activity in a percentage of neurons according to the severity of mutation (Fig. 7, compare the *eg* hypomorphic allele, eg^{MZ360}, to the *eg* null allele, eg^{18b}). Serotonin synthesis is also disrupted in *eg* mutants (Lundell and Hirsh, 1998).

The pattern of SerT loss observed in *eg* mutants is almost identical to that observed in *robo2/3* mutants. To further examine the relationship between *robo2/3* and *eg*, *robo2* and *robo3* mutant nerve cords were stained with an anti-Eg monoclonal antibody. Wild type serotonergic neurons express Eg until stage 17 (Fig. 5A) (Dittrich et al., 1997) although message is lost by stage 14 (Higashijima et al., 1996). In an *eg* hypomorph (eg^{Mz360}), all Eg staining is absent, and a loss of SerT is observed in approximately 30% of VNC hemisegments (Fig. 5B, 7). A loss of Eg expression is also observed in *robo2* (Fig. 5C) and *robo3* mutants (data not shown). Eg expression is lost in 100% of those cells lacking SerT activity but remains in those cells positive for SerT (Fig. 5C; n= 110 and 112 hemisegments for *robo2* and *robo3* mutants respectively). These data suggest that Robo2/3 function in the same genetic pathway as Eg, and that the loss of SerT seen in *robo2/3* mutants is due to a loss of Eg, as Eg is required for SerT expression.

Finally, since both *eg* hypomorphs and *robo2* LOF mutants show a loss of SerT activity in only a percentage of neurons (Fig. 7), a *robo2* LOF/*eg* hypomorph double mutant line was analyzed for any increases in phenotype severity. There is not an additive effect of *robo2* LOF and *eg* hypomorph in the double mutant (Fig. 7). These data provide further evidence that Robo2 functionally cooperates with Eg, since a partial loss of *eg* function does not exacerbate the loss of SerT in a *robo2* mutant.

Intriguingly, we observe an effect of Robo2 expression on the medio-lateral position of the serotonergic cell bodies. This is most markedly seen in the *robo2* LOF mutant hemisegment (Fig. 5C), where the cell bodies are positioned close to the midline, and in the Robo2 GOF hemisegment (Fig. 5D), where the cell bodies are shifted laterally. A similar positional shift is observed in the Unc5 GOF (Fig. 3D) and *comm* mutants (Fig. 2B). Therefore, the midline axon guidance signaling pathways may also play a role in cell body positioning.

Robo2 expression rescues an eagle hypomorphic phenotype and Eagle expression rescues a robo2 loss of function phenotype

To investigate the interaction between Robo2 and Eg, combinations of mutations at both loci were examined. Comparison of SerT expression in VNCs with varying levels of Robo2 and Eg reveals a dosage-sensitive interaction between the two genes (Fig. 6B-E). Over-expression of one copy of EP2582 (Robo2*)* with one copy of the *eg*Gal4 driver prevents serotonergic midline crossing but does not disrupt SerT expression (Fig. 6C). If one copy of EP2582

(Robo2 GOF) is expressed with two *eg*Gal4 drivers, a loss of SerT results (Fig. 6D) because the *eg*Gal4 driver (eg^{Mz360}) is a hypomorphic allele of *eg* constructed from a P-element insertion into the *eg* locus (Dittrich et al., 1997; Lundell and Hirsh, 1998). A loss of SerT appears only in the eg^{Mz360} homozygous condition (Fig. 6B). However, when two copies of EP2582 (Robo2 GOF++) are expressed with two *eg*Gal4 drivers (homozygous eg^{Mz360}), the *eg* hypomorphic phenotype is rescued. This high dose of Robo2 rescues both SerT (Fig. 6E) and Eg (Fig. 5D) expression in an *eg* hypomorph. The function of Robo2 in midline axon guidance is therefore separable from its function in regulating serotonergic cell fate, as a low level of Robo2 over-expression prevents midline crossing but only a high dose rescues SerT in *eg* hypomorphs. Initial consideration of this rescue may seem to contradict previous results showing that *robo2* regulates Eg expression (Fig. 5C). However, the rescue of an *eg* hypomorph by Robo2 GOF (Fig. 5D, 6E) seems to be due to some unique property of the hypomorphic allele, as not only SerT but also Eg expression is restored (further addressed in Discussion).

Further evidence linking *robo2* and *eg* comes from converse rescue experiments showing that over-expression of Eg rescues SerT expression in *robo2* loss of function mutants. Interestingly, this rescue is observed not only as a rescue of SerT expression but also as a rescue of the serotonergic axon guidance defects seen in *robo2* mutants (Fig. 6F compared to Fig.4B). Since Eg is expressed in only a small subset of neurons, it is not likely to act as a general mediator of Robo2 function in the CNS. In fact, the gross defects in CNS structure of *robo2*

mutants remain when Eg is over-expressed in the serotonergic neurons (data not shown).

DISCUSSION

We have shown that SerT expression in the fly, as in the grasshopper (Condron, 1999), is temporally and physically tied to axon guidance across the midline. Our data further indicate that the axon guidance molecules *robo2* and *robo3* (*robo2/3*) positively regulate serotonergic differentiation, since a loss of *robo2/3* function causes a loss of SerT expression in approximately 50% of neurons. A *robo2/3* LOF closely resembles an *eg* mutant phenotype. Finally, our data show a dosage-sensitive relationship between Robo2/3, Eg, and SerT expression, suggesting that they function in the same genetic pathway to control serotonergic differentiation. This interpretation is supported by the fact that loss of *robo2* or *robo3* causes a loss of Eg expression, and by genetic rescue experiments.

Induction of serotonin transporter expression and midline crossing

By visualizing serotonergic axonal projections with tau-lacZ, we determined that SerT expression begins at the end of stage 15, just after growth cones complete midline crossing and reach the contralateral side. This temporal correlation between midline crossing and SerT induction suggests that the midline is important for serotonergic differentiation in the fly, as it is in the grasshopper (Condron, 1999). Further evidence for the importance of the

midline comes from our data showing that in wild type cords, axons physically separated from the midline fail to express SerT. These results recapitulate similar experiments in the grasshopper. Additionally, when the repulsive axon guidance receptor Unc5 is expressed in serotonergic neurons, a partial loss of SerT expression is observed. Although these results suggest a role for the midline in serotonergic differentiation, it remains unclear whether this role is temporally restricted as it is in the grasshopper, and additionally what factors act as the presumptive midline signal. FGF signaling in the grasshopper is critical for SerT induction (Condron, 1999), and plays a role in the differentiation of vertebrate serotonergic neurons (Ye et al., 1998). In the fly, experiments indicate that FGF signaling also appears to be important for SerT regulation (J.A. Couch, M. Levin, E.M. Uri, and B.G. Condron, unpublished observations).

One problem with interpreting the role for the midline is the lack of an abnormal serotonergic phenotype in mutants for the master regulatory gene *single-minded* (*sim*), where midline cells fail to properly differentiate (Nambu et al., 1990; Nambu et al., 1991). It is difficult to speculate about what factors may allow normal differentiation in the absence of normal midline cells, as there are many changes in gene regulation throughout *sim* mutants (Xiao et al., 1996). Although our results suggest a role for the midline in serotonergic differentiation, it is likely to be more complicated than a simple switch acting to induce differentiation.

robo2 and robo3 play a role in serotonergic differentiation

Our data show that a loss of *robo2* or *robo3* causes a loss of SerT expression, suggesting that Robo2/3 function positively to regulate serotonergic differentiation. A positive role for Robo2 is further supported by our results showing that over-expression of Robo2 prevents a loss of SerT in neurons physically separated from the midline. Possibly Robo2 functions downstream of the midline signal required for SerT induction and thus allows differentiation to proceed in the absence of such a signal. An alternative hypothesis suggests that Robo2/3 function indirectly to induce SerT, by guiding serotonergic axons to an unknown signal in the contralateral neuropil. Such en passant signaling occurs in the developing vertebrate CNS where trophic support is required by commissural axons at the floorplate, an intermediate axonal target (Wang and Tessier-Lavigne, 1999). Although we cannot rule out the possibility that Robo2/3 act indirectly to regulate serotonergic differentiation at this time, several lines of evidence suggest a more direct role. Our data shows that over-expression of Robo2 not only spares SerT loss following a midline cut but also rescues an *eg* hypomorph, and further, that an Eg GOF rescues a *robo2* LOF; these results strongly suggests that Robo2 functions autonomously in the serotonergic neurons. Additionally, SerT loss is not seen in other guidance mutants that disrupt midline crossing or cause general disorganization of the CNS. However, it is difficult to clearly resolve the presence of Robo2/3 protein specifically in the serotonergic neurons because of the broad distribution of neuronal processes and

the fact that serotonergic branching does not correspond simply to any Fasciclin II pathway where axons are known to express Robo2/3.

Robo2 and Robo3 appear to act as overall regulators of differentiation rather than specific regulators of SerT, since *robo2/3* mutants not only lose SerT expression (mRNA and reuptake activity) but also have defects in serotonin synthesis later in development. Thus the role of Robo2/3 in serotonergic differentiation parallels that of other genes, including *eg* and the LIM-homeodomain transcription factor *islet*, that cause both a loss of SerT as well as serotonin synthesis when disrupted (J.A. Couch and B.G. Condron, unpublished observations) (Lundell and Hirsh, 1998; Thor and Thomas, 1997). Our data further indicate that *robo2/3* are not required in the formation of the serotonergic neurons from their progenitor neuroblast 7-3. All serotonergic neurons in a *robo2/3* mutant express *eg*-lacZ, even those with a loss of SerT expression (Fig 4E). This may at first appear to contradict our result showing a loss of Eg at stage 16 in these mutants, as *eg* expression must have occurred in order to produce lacZ. We hypothesize that lacZ staining in stage 16 *robo2* mutants is likely due to a lengthy persistence of lacZ rather than continued expression of *eg*, since *eg* mRNA is not detectable by in situ hybridization after stage 14 (Higashijima et al., 1996). Most likely, *eg*-lacZ expression in *robo2/3* mutants occurred in the progenitors of serotonergic neurons when other factors, such as *engrailed* (*en*), are known to control *eg* expression (Dittrich et al., 1997; Matsuzaki and Saigo, 1996). Even in *eg* mutants, all serotonergic neurons continue to express *eg*-lacZ, despite a disruption in SerT, serotonin synthesis, and expression of Ddc (Lundell and

Hirsh, 1998). Thus, a *robo2/3* mutant, like an *eg* mutant, does not affect the early specification of serotonergic neurons including early *eg* expression, but instead affects later maturation.

Interestingly, we observe an effect of *robo2/3*, but not *robo*, on serotonergic differentiation. Disparities between Robo and Robo2/3 function have been previously observed in the lateral positioning of axons where only Robo2/3 appear to play a role (Rajagopalan et al., 2000b; Simpson et al., 2000a), and in dendritic guidance, synapse formation, as well as midline crossing, where all three Robo receptors have separable functions (Godenschwege et al., 2002; Rajagopalan et al., 2000a; Simpson et al., 2000b). Further, Robo2 and Robo3 show greater homology to each other than to Robo (Rajagopalan et al., 2000b; Simpson et al., 2000b). Robo2/3 have cytoplasmic domains that diverge from Robo, and lack two motifs considered important for Robo signaling (Bashaw et al., 2000; Rajagopalan et al., 2000b; Simpson et al., 2000b). Possibly, Robo2 and Robo3 regulate a Robo-independent signaling cascade that is critical for serotonergic differentiation. Additionally, a loss of *slit*, the ligand for all three Robos, does not perturb SerT expression, indicating that either another ligand exists or the function of Robo2/3 in serotonergic differentiation is ligand independent. In C. elegans, some activities of the Robo homolog SAX-3 are thought to be Slit-independent (Hao et al., 2001).

The transmembrane protein Commissureless (Comm) has been shown to negatively regulate the levels of all three Robo receptors (Kidd et al., 1998b; Rajagopalan et al., 2000a; Tear et al., 1996). After midline crossing, Comm

expression decreases and Robo levels increase in order to prevent inappropriate midline crossing. In serotonergic differentiation, Comm may play a role in regulating Robo2/3, such that levels of both Robo2/3 increase following midline crossing and thereby permit differentiation to proceed. To test this possibility, we expressed Comm using *eg*Gal4 to specifically induce a loss of Robo2/3 in the serotonergic neurons. In our experiments, expression of Comm caused a loss of SerT activity in only a few cells and with low penetrance (data not shown). We believe that this is due to expression of Comm at levels insufficient for total loss of Robo2/3. Alternatively, other regulators of Robo2/3 may exist. However, neither a loss of Comm nor over-expression of Robo2/3 results in precocious serotonergic differentiation, indicating a requirement for other signals.

robo2 functions with the transcription factor eagle to regulate serotonergic differentiation

In both a *robo2* and a *robo3* LOF mutant, expression of the zinc-finger transcription factor *eg* is lost in the same cells that lose SerT expression. Additionally, over-expression of Robo2 rescues the loss of SerT observed in an *eg* hypomorph in a dosage-sensitive manner. Finally, Eg GOF rescues the SerT loss seen in *robo2* loss of function mutants. These results indicate that Robo2/3 function in the same genetic pathway as Eg to control serotonergic differentiation. Although our results suggest that Robo2/3 regulate Eg in stage 16 embryos, other genes such as *en* and *hunchback* (*hb*) also have an established role in regulating Eg during serotonergic differentiation (Dittrich et al., 1997;

Matsuzaki and Saigo, 1996; Novotny et al., 2002). At present, it remains unclear if Robo2/3 cooperate with these genes to regulate Eg expression.

Additionally, in both *robo2* and *robo3* LOF mutants only a percentage of neurons lose SerT expression (and serotonin synthesis), indicating the presence of a redundant mechanism for serotonergic differentiation. The pattern of SerT and serotonin loss in *robo2/3* mutants appears random and differs between nerve cords. At this point, it remains unclear why differentiation is affected in only some cells and not others, or what factors allow remaining cells to maintain normal SerT expression. One possibility is that cells must maintain a threshold level of Eg expression to properly differentiate. This is supported by differences in the degree of SerT loss according to the severity of the mutation in *eg*, as a hypomorphic allele displays a loss of SerT in approximately 30% of hemisegments while a null allele displays closer to 80% loss of SerT. Many studies have also suggested that a combinatorial code of transcription factors act to specify serotonergic identity (Dittrich et al., 1997; Thor and Thomas, 1997). First, loss of function mutations in several genes required for differentiation, including *eg*, *en,* and *hb* show an incomplete loss of SerT phenotype (Lundell et al., 1996; Lundell and Hirsh, 1998; Novotny et al., 2002). Second, if Eg is inappropriately expressed throughout the nervous system, only a few ectopic serotonin positive cells appear. These ectopic serotonergic cells always express the transcription factor *hkb* (Dittrich et al., 1997). Robo2 and Robo3 may also function redundantly. Further studies should indicate the relationship of

Robo2/3 to other genes involved in serotonergic differentiation, and the mechanism by which Robo2/3 regulate Eg expression.

One question that readily follows from our observations is: How does Robo2 influence Eg expression? Robo2 and Robo3 are cell-surface axon guidance receptors, while Eg is a transcription factor. It is likely that other factors interact with both Robo2/3 and Eg to mediate their roles in serotonergic differentiation. Although their relationship remains obscure, data indicate that Robo2 may regulate Eg post-transcriptionally. In a series of real time RT-PCR experiments, no difference in *eg* mRNA levels was detected when EP2582 (UAS-*robo2*) was expressed using *eg*Gal4, *scabrous*Gal4, or *elav*Gal4 (data not shown), suggesting that Robo2 is insufficient to induce ectopic Eg expression. However, when Robo2 is over-expressed, a rescue of Eg protein expression is seen in eg^{Mz360} hypomorphs (Fig. 6E). Through the same series of PCR experiments, we discovered that the eg^{Mz360} allele produces mRNA although no Eg staining is observed. This suggests that the Gal4 insertion responsible for the eg^{Mz360} allele affects Eg protein expression, which in turn causes a disruption in SerT expression. Thus, expression of Robo2 appears to somehow rescue Eg protein expression in an eg^{Mz360} hypomorph sufficiently to rescue SerT activity. At this point, the mechanism of such a post-transcriptional rescue is unclear. Identifying the genetic and intracellular links between Robo2, Robo3, and Eg with more molecular approaches such as RNAi studies will likely reveal how Robo2/3 regulate not only Eg but eventually serotonergic differentiation as well.

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FIGURE LEGENDS

Figure 1. The onset of SerT expression is correlated with axon guidance across the midline. (A-B) Staining for serotonin uptake in red and β-galactosidase in green (UAS-tau-lacZ driven by *eg*Gal4). *eg*Gal4 expression of lacZ is seen in all four progeny of NB 7-3, which includes the serotonergic neurons, and two other neurons, in addition to one pair of lateral neurons that arise from a different lineage. (A) SerT activity was never seen before growth cones crossed the midline in stage 14 ventral nerve cords (10:20-11:20 hours development), but (B) always seen after axons reached the contralateral neuropil at late stage 15 (11:20-13 hours). (C) In situ hybridization for *SerT* mRNA shows robust expression by late stage 15. Staining for the axon scaffold of the ventral nerve cord is shown in brown with mAb BP102. Scale bar in (A) is 10 μm.

Figure 2. Initial screen of midline axon guidance mutants did not reveal a loss of SerT expression. (A-F) stage 16 ventral nerve cords are stained for serotonin uptake in red (A-D) or in white (E,F). (A-D) show the axon scaffold stained with mAb BP102 in green. (A) The wild-type CNS is organized into two longitudinal axon tracts connected by two commissures per segment. Serotonergic axons are seen crossing in the posterior commissure. Loss-of-function mutants (B) *commissureless*^{e39}; (C) *slit*^{2}; and (D) *robo*^{GA285} were identified based on CNS phenotype. In *comm* mutant embryos, the commissures do not form, while in *slit* mutants axons fail to leave the midline and in *robo* mutants serotonergic axons are disorganized as a result of ectopic midline crossing throughout the CNS. (E,

F) *eagle*Gal4 drives expression of (E) *robo (*UAS-*robo*) and (F) *robo2* (EP2582) in the serotonergic neurons. A gain-of-function (GOF) in *robo2*, but not *robo*, prevents serotonergic midline crossing. Despite defects in serotonergic axon guidance seen in these embryos, SerT activity remains normal. Scale bar in (A) is 10 □m.

Figure 3. Physical or genetic disruption of midline contact affects SerT activity in wild type, Robo gain of function (GOF) and Unc5 GOF but not Robo2 GOF mutants. (A-C) Staining of ventral nerve cords for serotonin uptake (red in left panel and white in right) and for *engrailed* (green in left panel). A cut (white line) was made in isolated stage 16 cords asymmetrically down the midline such that one set of cell bodies was separated from the midline. The tissue preparations were allowed to develop for 2 hours prior to fixation. (A) In 15 of 20 wild type preparations, SerT activity was lost from the cell bodies on the cut side (arrow). (B) Similar cuts in embryos over-expressing Robo in serotonergic neurons (Robo GOF; UAS-*robo* with *eagle*Gal4) also showed a loss of SerT activity in the midline-minus side (3 of 3). (C) However, when Robo2 was over-expressed (*eagle*Gal4), only 1 of 20 preparations showed a loss of SerT activity after a midline cut. (D) Expression of Unc5 with *eagle*Gal4 specifically prevents serotonergic midline crossing and also causes a partial loss of SerT activity as seen by staining for serotonin uptake (red/white), but does not affect whole CNS axon guidance (BP102, green). Arrows (left panel) indicate hemisegments where SerT expression is lost and asterisks (right panel) indicate axons that extends towards

the midline despite expression of Unc5 . Scale bar in (D) is 10 μm.

Figure 4. *robo2* and *robo3* are required for normal SerT expression. (A-C) Stage 16 ventral nerve cords from *robo2*^{x123} (A,B) and *robo3*^{1} (C) loss-of-function (LOF) mutant embryos stained for serotonin uptake in red (A,C) and white (B). The slightly disorganized axon tracts of the mutant CNS are labeled in green with mAb BP102. *robo2* and *robo3* LOF nerve cords show a random loss of SerT activity in approximately half of hemisegments. (B) SerT activity is lost in both a single hemisegment (arrow) and an entire segment (arrowhead). (D) In situ hybridization for SerT mRNA in a stage 16 *robo2* mutant indicates a loss of SerT transcription in the same pattern as the loss of SerT activity. Mutant nerve cords were identified by axonal morphology or in the case of (D), irregular width. (E,F) Serotonin uptake is shown in red and *eagle*-lacZ is shown in green in wild type (E) and a *robo2*^{x123} mutant (F). (E) In a wild-type cord, both serotonergic neurons in each hemisegment express *eg* and therefore stain for lacZ. (F) Although *robo2* mutants lack SerT activity in many neurons, lacZ expression remains normal. Serotonergic cell bodies positive for lacZ but not SerT activity are highlighted in the insets below (asterisk). Scale bar in (E) is 20 μm for (D-F) and 10 μm for (A-C).

Figure 5. *robo2* mutants lose *eagle* expression. Stage 16 wild type (A), *eg*^{Mz360} hypomorph (B), *robo2* loss-of-function (C), and (EP2582/EP2582; *eg*^{Mz360}/*eg*^{Mz360}) nerve cords (D). Serotonin uptake, red; Eg, green; yellow, co-localization. (M) indicates the position of the ventral midline. (A) The wild type nerve cord has

two serotonergic neurons per hemisegment, both positive for SerT activity and for the transcription factor Eagle. (B,C) An eg^{Mz360} and a *robo2* mutant show a loss of SerT activity in a percentage of hemisegments. In the eg^{Mz360} (B) CNS, all Eg staining is absent although some SerT remains, while in the *robo2* mutant (C), Eg is lost only in those cells that lack SerT activity. (D) When two copies of *robo2* (EP2582, Robo GOF++) are over-expressed in an eg^{Mz360} (hypomorph) background, both Eg expression and SerT activity are rescued despite the fact that expression of *robo2* prevents axons from crossing the midline. Scale bar in (D) is 5 μm.

Figure 6. Over-expression of Robo2 rescues an *eagle* hypomorphic mutation and over-expression of Eg rescues a *robo2* loss of function.

(A) Wild type stage 16 nerve cord stained for serotonin uptake showing two serotonergic cell bodies per hemisegment with axons extending across the midline. (B) Embryos homozygous for the hypomorphic eg^{Mz360} allele (also an *eagle*Gal4) show an approximately 30% loss of SerT positive cells as well as axon guidance defects. (C) Expression of one copy of Robo2 (EP2582, Robo GOF) with one copy of *eg*Gal4 (heterozygous eg^{Mz360}) prevents axons from crossing the midline but does not disrupt SerT activity. (D) Expression of Robo2 with two copies of the hypomorphic *eg*Gal4 allele (homozygous eg^{Mz360}) disrupts axon guidance, but also causes a loss of SerT as seen in (B). (E) However, expression of two copies of Robo2 (Robo2 GOF++) with two copies of *eg*Gal4 (homozygous eg^{Mz360}) rescues the loss of SerT phenotype. (F) Conversely, expression of Eg

(*eg*Gal4) rescues both SerT activity and the serotonergic axon guidance phenotype observed in *robo2* mutants (compare to Fig. 4B). Scale bar in (A) is 10 μm.

Figure 7. Average loss of SerT activity between genotypes. Error bars represent the standard deviation of the mean. Genotypes were scored for the percent of hemisegments negative for SerT activity / total hemisegments per VNC. (n) represents the number of total hemisegments scored. At least 10 cords were averaged for each genotype. Wild type cords have 0% loss of SerT. (a,b) Significance is $p < 0.001$ (One-way ANOVA, Tukey comparison), showing that SerT activity is rescued by (a) Eg GOF in an *robo2* LOF and (b) Robo2 GOF in an *eg* hypomorph. No significant difference (ns, $p > 0.05$) was found between *robo2* LOF and the double *robo2* LOF; eg^{Mz360} mutants. *robo2* LOF score represents data from both $robo2^{x123}$ and $robo2^{x135}$ alleles, as phenotypes were indistinguishable.

FIGURES

Figure 1

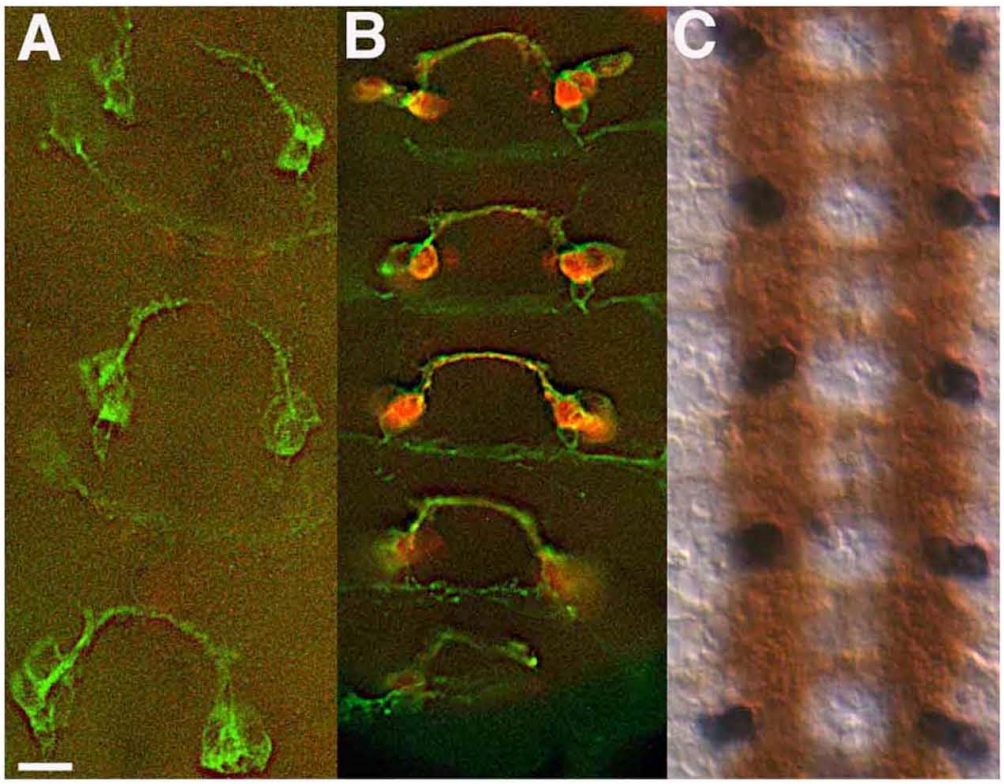


Figure 2

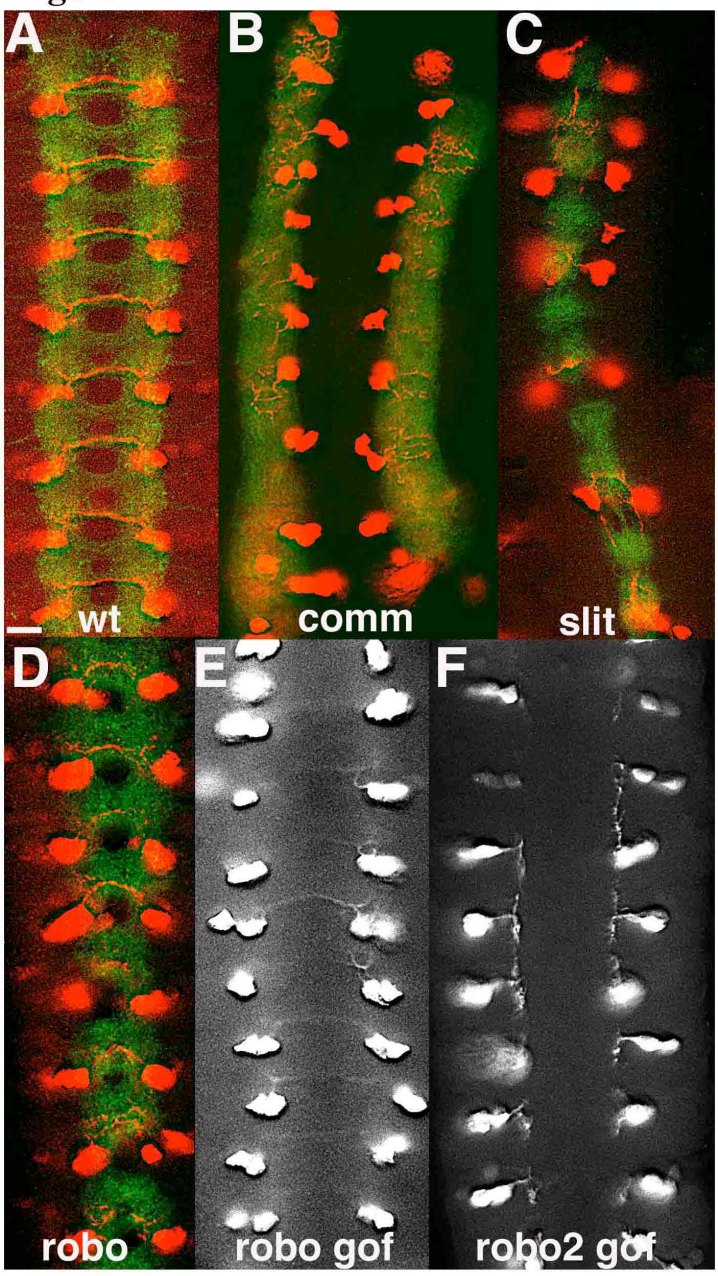


Figure 3

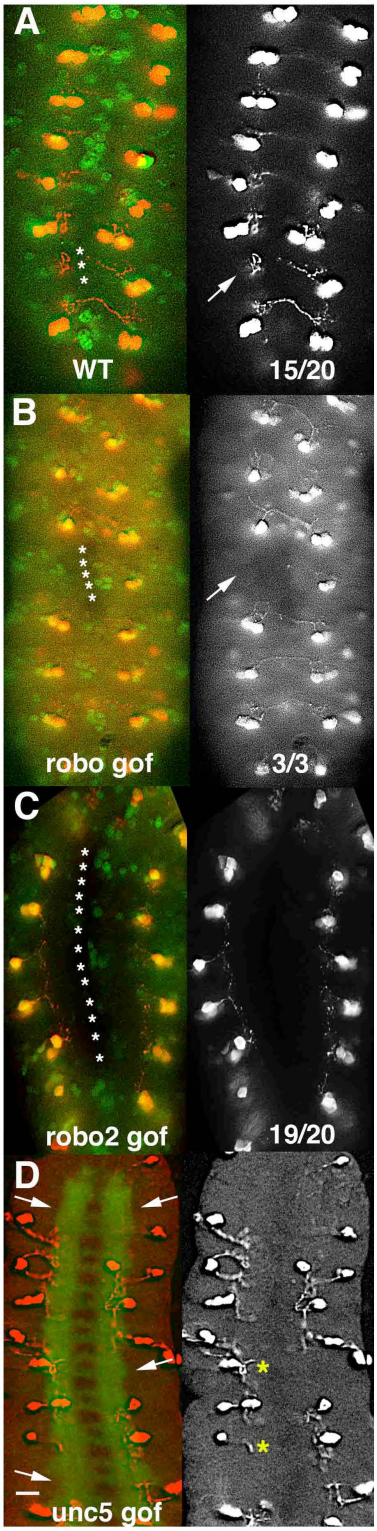


Figure 4

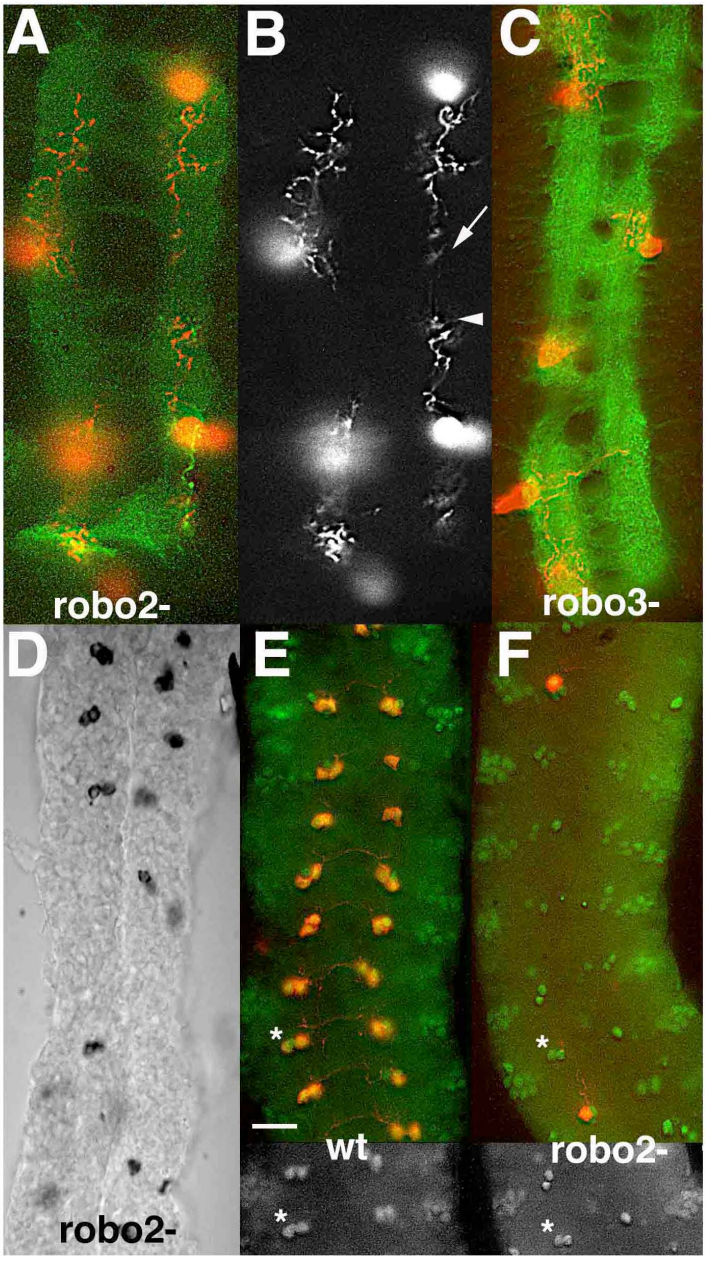


Figure 5

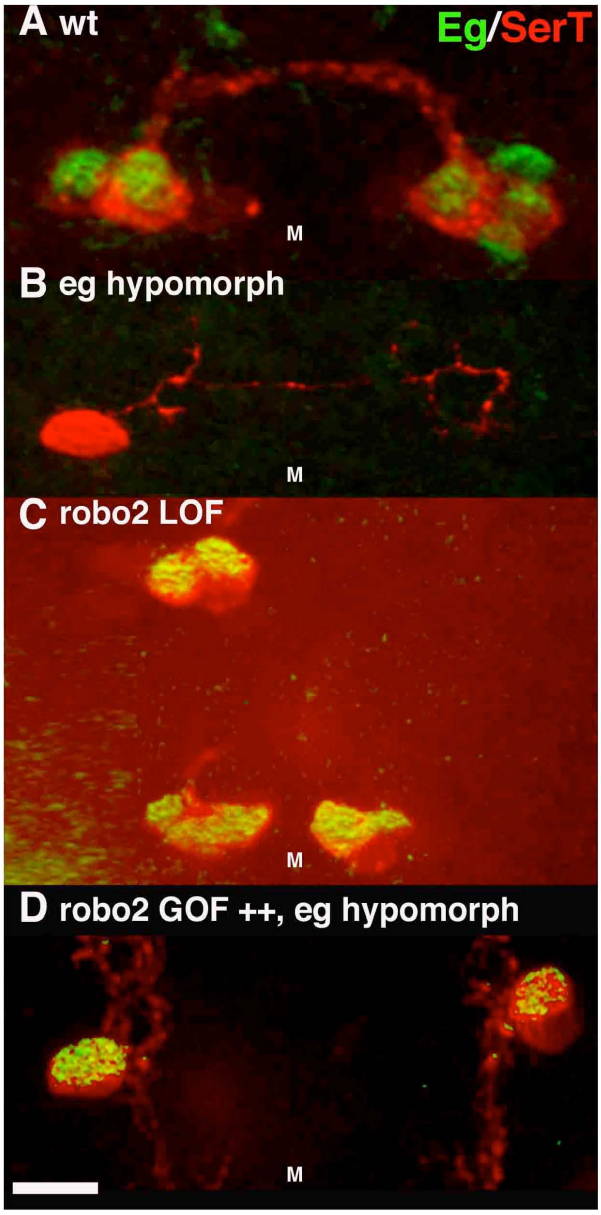

A wt
Eg/SerT
M
B eg hypomorph
M
C robo2 LOF
M
D robo2 GOF ++, eg hypomorph
M

Figure 6

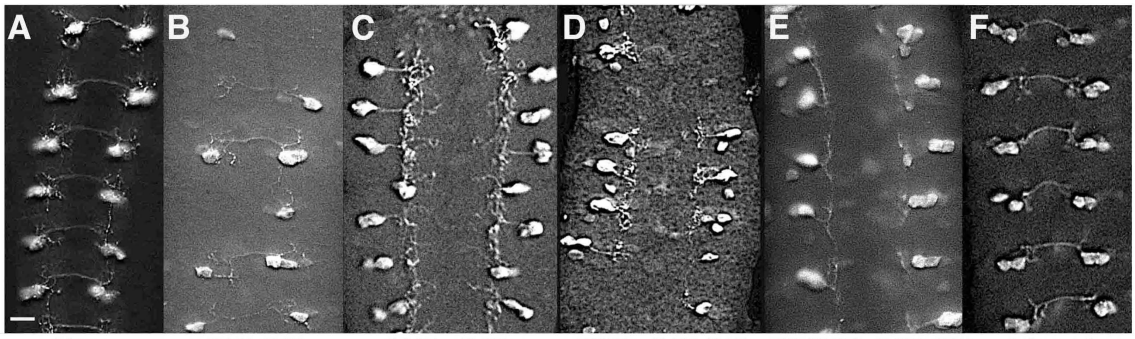
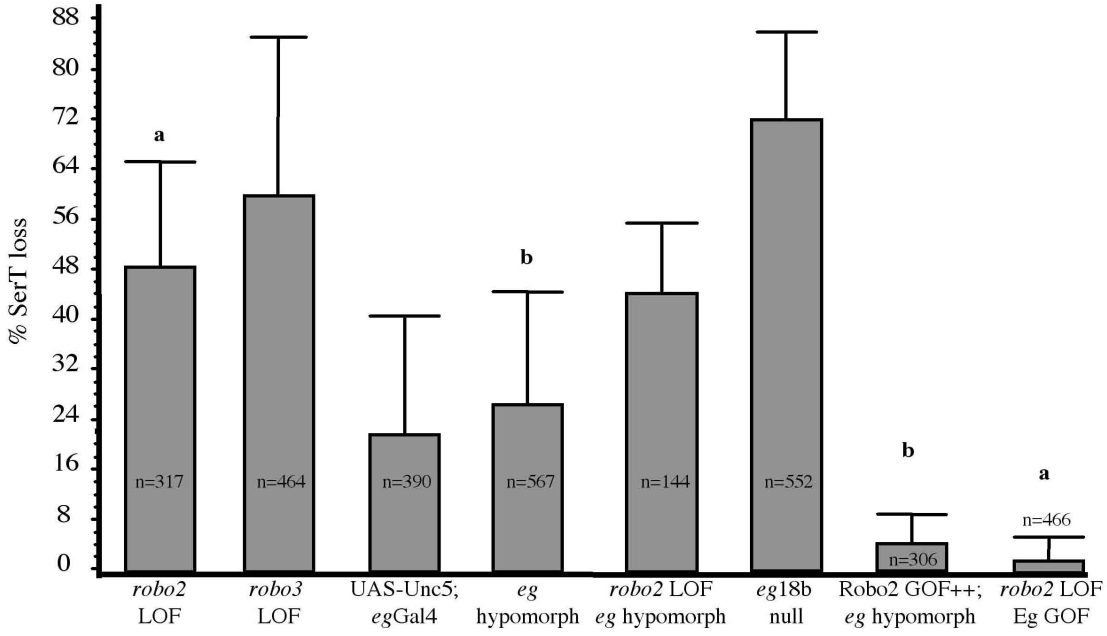

A
B
C
D
E
F
wild type
egMz360/egMz360
eg hypomorph
EP2582/+; egMz360/+
robo2 GOF; eg hypomorph/+
EP2582/+; egMz360/egMz360
robo2 GOF; eg hypomorph
EP2582/EP2582;egMz360/egMz360
robo2 GOF++; eg hypomorph
UAS-eg; robo2-
eg GOF; robo2 LOF

Figure 7


% SerT loss
88
80
72
64
56
48
40
32
24
16
8
0
a
b
b
a
n=317
n=464
n=390
n=567
n=144
n=552
n=306
n=466
robo2 LOF
robo3 LOF
UAS-Unc5; egGal4
eg hypomorph
robo2 LOF eg hypomorph
eg18b null
Robo2 GOF++; eg hypomorph
robo2 LOF Eg GOF

CHAPTER TWO

FGF signaling and the Ets transcription factor *pointed* are involved in *Drosophila* serotonergic differentiation

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Heather Rieff made the initial discovery of Pointed function in serotonergic differentiation and contributed to data shown in Figure 1. Michaela Levin aided in the collection of data for experiments shown in Figures 2, 4 and 5.

ABSTRACT

Serotonin is a neurotransmitter that plays a role in many physiological and behavioral functions of the CNS. However, relatively little is known about the development of the serotonergic neurons. Previous studies in grasshopper showed that a midline-associated FGF signal is required for normal serotonin transporter (SerT) expression. We investigate the role for RTK signaling in *Drosophila* serotonergic differentiation by examining gain and loss of function mutants at different levels of the FGF signaling pathway. Interestingly, we find that the Ets transcription factor *pointed* (*pnt*), which functions downstream of receptor tyrosine kinase signaling in many tissues, plays a role in serotonergic differentiation. Over-expression of an activated Pnt causes a loss of SerT expression in approximately half of serotonergic neurons. Conversely, a loss of *pointed* induces precocious SerT expression. Additionally, we find early SerT expression in mutants for the BTB/POZ-domain transcription factor *tramtrack*, the FGF signaling effector *heartbroken*, the FGF receptor *breathless* and its ligand *branchless*. Our data further suggest that *pnt* functions autonomously during serotonergic development. Finally, we show an interaction between *pnt* and the transcription factor *eagle* (*eg*), a factor known to be critical for normal differentiation. Analysis of double mutants for *pnt* and *eg*, as well as *ttk* and *eg*, suggest that *pnt* and *eg* function antagonistically to regulate SerT expression, while *ttk* functions independently of *eg*. In summary, we propose that the Ets transcription factor *pnt* functions downstream of FGF signaling to negatively regulate serotonergic differentiation.

INTRODUCTION

Serotonin is an important neurotransmitter that plays a role in many functions of the central nervous system (CNS), including locomotion, ingestive behaviors, and sleep (Jacobs and Azmitia, 1992; Lucki, 1998). Additionally, altered levels of serotonin are thought to contribute to psychiatric illnesses such as depression, anxiety, and autism (Flattem and Blakely, 2000; Gingrich, 2002; Klauck et al., 1997). As such, many therapeutic approaches have been designed to target the serotonin transporter (SerT), which reuptakes released serotonin (Mancama and Kerwin, 2003). There is also evidence suggesting that some psychiatric disorders may originate from a developmental dysfunction (Chugani et al., 1999; Gross et al., 2002; Hendricks et al., 2003). Despite the importance of serotonergic function, little is known about the development of serotonergic neurons.

In both invertebrates and vertebrates, some progress has been made in identifying factors critical for the early specification of serotonergic neurons. In *Drosophila*, early patterning genes such as *engrailed*, *hedgehog*, *hunchback*, and *wingless* are required for formation of the neuroblast that gives rise to the serotonergic neurons (Chu-LaGraff and Doe, 1993; Lundell et al., 1996; Matsuzaki and Saigo, 1996; Novotny et al., 2002); and in vertebrates, a combination of signaling molecules including Sonic Hedgehog and Fibroblast Growth Factor (FGF) are required to induce serotonergic precursors (Ye et al., 1998). However, the genetic regulation of terminal serotonergic differentiation remains relatively uncharacterized. Functionally mature serotonergic neurons

must express specific genes including SerT and the serotonin biosynthetic enzymes tryptophan hydroxylase (Tph, Henna) and Dopa decarboxylase (Ddc). It is unlikely that genes required during early specification play a direct role in regulating expression of terminal differentiation genes, as there is a significant time delay between early specification and later maturation, and additionally, genes involved in specification are not specific to serotonergic neurons. Some candidate factors uniquely required for differentiation have been identified. Previous studies in the grasshopper suggest that proper induction and maintenance of SerT expression requires a midline-derived FGF signal (Condron, 1999). Thus, in the grasshopper, FGF appears to function during terminal differentiation rather than in early specification as it does in vertebrates (Ye et al., 1998).

We use *Drosophila* as a model system to further examine the role of FGF signaling in serotonergic differentiation, since the relatively few serotonergic neurons in the ventral nerve cord (VNC) are easily identifiable (Isshiki et al., 2001). Serotonergic neurons in *Drosophila* are bilaterally symmetrical, and organized in groups of two neurons per hemisegment that project axons across the midline into the contralateral neuropil (Valles and White, 1988). We use SerT expression as an early marker of terminal differentiation because it begins before serotonin synthesis in *Drosophila* (Couch et al., in press; Novotny et al., 2002), as well as in grasshopper (Condron, 1999). The *Drosophila* homolog of SerT (*dSerT)*, like its vertebrate counterpart, can be blocked by both fluoxetine and cocaine (Corey et al., 1994; Demchyshyn et al., 1994). Additionally, functional SerT

expression (SerT activity) can be readily detected by uptake of exogenous serotonin prior to the onset of endogenous synthesis (Couch et al., in press).

In the present study, we show evidence that FGF signaling also plays a role in *Drosophila* serotonergic differentiation. *Drosophila* has two known FGF receptors (FGF-Rs): *heartless* (*htl*) (Beiman et al., 1996; Gisselbrecht et al., 1996) and *breathless* (*btl*) (Klambt et al., 1992; Reichman-Fried et al., 1994). The *htl* FGF-R has an important function in mesodermal migration and thus in proper differentiation of mesodermal cells (Beiman et al., 1996; Gisselbrecht et al., 1996), while *btl* plays a critical role in the migration of tracheal cells as well as some CNS glia (Klambt et al., 1992; Reichman-Fried et al., 1994). In the developing tracheal system, *btl* directs cell migration and branching by responding to its ligand, *branchless* (*bnl*), an FGF2 homolog (Ohshiro et al., 2002; Sutherland et al., 1996) . To date, *bnl* is the only FGF identified in *Drosophila*. The function of *btl* in glial migration is still not well understood.

FGF specific signaling in *Drosophila* also depends on the effector protein *heartbroken* (*hbr*, also known as *stumps* and *Dof*), which functions downstream of both *htl* and *btl* but not other tyrosine kinase receptors (RTKs) (Imam et al., 1999; Michelson et al., 1998; Vincent et al., 1998). Ultimately, the result of FGF signaling is often an alteration of gene expression patterns. Ets-domain transcription factors are well-known effectors of RTK signaling in both vertebrates and invertebrates. In *Drosophila*, the Ets-domain transcription factor *pointed* (*pnt*) is a downstream target of signaling from both FGF as well as other RTKs in many developing tissues (Brunner et al., 1994; Gabay et al., 1996; Halfon

et al., 2000; O'Neill et al., 1994; Scholz et al., 1997). Additionally, *pnt* is required for proper differentiation of both midline and longitudinal glia of the CNS (Giesen et al., 1997; Klambt, 1993). The *pnt* gene has two major transcripts, *pointed*-1 (pnt^{P1}) and *pointed*-2 (pnt^{P2}) (Klambt, 1993). Pnt^{P1} is constitutively active, while Pnt^{P2} requires MapK activation for function (O'Neill et al., 1994).

Generally, Ets transcription factors have been shown to regulate the formation of functional connections between motor and sensory neurons, and to play a role in vertebrate as well as invertebrate neuronal specification (Lin et al., 1998; O'Neill et al., 1994; Wasylyk et al., 1998). The Ets transcription factor, Pet-1, is a specific and early marker of serotonergic neurons in both rat and mouse (Hendricks et al., 1999; Pfaar et al., 2002), and has recently been shown to play a critical role in serotonergic differentiation (Hendricks et al., 2003). A loss of Pet-1 causes a failure of normal SerT expression, serotonin synthesis and storage, and additionally, produces anxious and aggressive behavior in mutant mice (Hendricks et al., 2003).

To determine whether FGF signaling through an Ets-transcription factor might play a role in *Drosophila* serotonergic differentiation, we conducted both a gain and loss of function screen for genes involved at different levels of RTK signaling. We show that the Ets transcription factor *pointed* (*pnt)* regulates both SerT expression as well as serotonin synthesis. The BTB/POZ-domain transcription factor *tramtrack* (*ttk*) also appears to regulate SerT expression. *ttk* functions coordinately with *pnt* during development of glia of the VNC and the eye (Giesen et al., 1997; Xu et al., 2000), and *ttk* is thought to be a general

repressor of neuronal differentiation (Badenhorst et al., 2002; Li et al., 1997; Read et al., 1992; Salzberg et al., 1994). In addition, we find a role for *hbr*, *btl*, and *bnl*, suggesting that FGF signaling plays a role in *Drosophila* serotonergic differentiation, and that these genes may function upstream of *pnt*. We also show data supporting an autonomous role for *pnt* in serotonergic neurons. Finally, we show evidence that *pnt* may function antagonistically with the orphan steroid hormone receptor *eagle* (*eg*) which also regulates SerT expression.

RESULTS

Expression of activated Pointed disrupts serotonergic differentiation

Experiments in the grasshopper suggested that FGF signaling is important for the onset and early maintenance of SerT activity (Condron, 1999). In order to determine whether SerT expression is similarly regulated in the fly, we undertook a directed screen of various mutants in tyrosine kinase signaling pathways. Initially, we focused on ectopic expression of either dominant negative (DN) or constitutively active constructs for FGF-R, Epidermal Growth Factor (EGF-R), and downstream effectors specifically in the serotonergic neurons using an *eagle*Gal4 (*eg*Gal4) driver. *eg* is a transcription factor that is expressed in the neuroblast that gives rise to the serotonergic neurons as well as three other neuroblasts in the CNS (Higashijima et al., 1996). However, by stage 13, *eg* is detected only in the serotonergics and one other pair of lateral neurons (Dittrich et al., 1997). In serotonergic neurons, Eg expression occurs from birth until the end of embryonic development (Dittrich et al., 1997).

Expression of a DN or an active form of either the *Drosophila* FGF-R *htl*, or the EGF-R *top* did not cause any obvious disruptions in serotonergic development. However, when an activated form of the Ets domain transcription factor *pointed* (*pnt*) was over-expressed, we observed defects in several aspects of serotonergic differentiation. *pnt* functions downstream of tyrosine kinase signaling in many developing tissues (Brunner et al., 1994; Gabay et al., 1996; O'Neill et al., 1994). Specifically, expression of pnt^{P1} (UAS-Pnt^{P1}) in the serotonergic neurons using *eg*Gal4 causes a loss of both SerT activity (Fig. 1B) and serotonin synthesis (data not shown). A loss of SerT activity is observed in approximately half of the serotonergic neurons (Fig. 1B, Table 1) and is paralleled by defects in serotonergic axon guidance (Fig. 1B). However, when an inactive form of Pnt (UAS-Pnt^{P2}) is expressed, serotonergic development remains unaffected. Results from *in situ* hybridization indicate that Pnt^{P1} inhibits SerT transcriptionally, since over-expression of activated Pnt^{P1} also causes a loss of SerT mRNA (data not shown).

The loss of SerT activity and serotonin synthesis caused by Pnt^{P1} over-expression persists until at least late larval stages, and is observed when Pnt^{P1} is expressed under either the *eg* (early embryonic expression) or *Ddc* (late embryonic expression) promoters. Pnt^{P1} over-expression appears to disrupt serotonergic differentiation but not cell fate, as suggested by normal expression of *engrailed*, a homeobox transcription factor required for early serotonergic cell specification (Fig.1C) (Lundell et al., 1996). Another indication that Pnt^{P1} over-expression does not disrupt cell fate is seen from co-expression of Pnt^{P1} with

UAS-c-*myc*-GFP under *eg*Gal4 (Fig.1D), where neurons without SerT activity not only remain positive for GFP but also extend axons. However, we cannot rule out the possibility that serotonergic differentiation is disrupted by a competing drive toward a glial fate, as ectopic expression of Pnt^{P1} has previously been shown to cause a ectopic expression of glial markers (Klaes et al., 1994).

Loss of function in genes of the FGF signaling pathway induce precocious serotonin transporter activity

Since over-expression of Pnt^{P1} caused a loss of SerT expression, we decided to further examine the role of *pnt* in serotonergic differentiation by analyzing embryos mutant for *pnt*. Wild-type stage 14 VNCs do not show SerT activity (Fig. 2A), since onset of SerT expression does not normally occur until late stage 15 (Couch et al., in press; Novotny et al., 2002). However, a loss of *pnt* causes precocious stage 14 SerT activity (Fig. 2B). Combined with data from Pnt^{P1} over-expression, this result further suggests that *pnt* negatively regulates serotonergic differentiation. Early SerT activity is observed in at least two alleles of *pnt* (pnt^{2} and $pnt^{\Delta 88}$). To test for the possibility that precocious serotonin synthesis, rather than SerT expression, occurred in stage 14 mutants, we also analyzed staining in VNCs not incubated in serotonin. Early serotonin synthesis was not detected. Further, *pnt* mutants display a mild BP102 phenotype by stage 14 (Fig.2B), where the anterior and posterior commissures appear slightly fused (Klambt, 1993). This whole CNS phenotype is the result of failed midline glial migration caused

by a lack of *pnt* and continues to worsen as development proceeds (as in a stage 16 *pnt* mutant, Fig. 4C) (Klambt, 1993).

To identify candidate genes that might function genetically upstream of *pnt* in SerT regulation, we looked for precocious SerT activity in other mutants including *heartbroken* (*hbr,* also known as *stumps* and *Dof)*, *breathless* (*btl*), *branchless* (*bnl*), and *heartless* (*htl*). Stage 14 SerT activity was observed in homozygous loss of function *hbr* mutants ($stumps^{09904b}$, Fig. 2C) as well as heterozygous loss of function *btl* (btl^{LG19}, Fig. 2D) and *bnl* ($bnl^{[P1]}$, Fig. 2E) mutants. Early SerT was not observed, however, in the *htl* loss of function mutant (htl^{AB42}, not shown). Alleles of both *btl* and *bnl* were followed by β-gal staining against a marked balancer chromosome. In most experiments, homozygous mutant nerve cords (without β-gal staining) were not found, most likely due to fragility of the VNC and our inability to dissect them. This was true also for *htl* mutants. In the few *btl* and *bnl* homozygous mutant cords that were found, early SerT activity was also present. Additionally, the homozygous mutant VNCs exhibited a strong BP102 phenotype indicating massive disruption of CNS architecture (not shown). *bnl* has previously been reported as a haplo-insufficient locus for tracheal development (Sutherland et al., 1996). As seen from BP102 staining in Fig 2D-E, the CNS structure appears generally wild type in the heterozygous *btl* or *bnl* VNCs. Thus, SerT expression seems more sensitive to a decreased dose of *btl* or *bnl* than does overall CNS axon guidance. Consistent with our data indicating dosage sensitivity to *btl* and *bnl* in serotonergic differentiation, an

early SerT phenotype was also observed in homozygotes for the hypomorphic alleles *btl*^{dev1}and *bnl*^{06916} (data not shown).

Finally, the *pnt* loss of function results led us to examine other genes with related physiological roles, including the transcription factor *tramtrack* (*ttk*). *pnt* and *ttk* coordinately regulate both glial and eye development (Giesen et al., 1997; Xu et al., 2000). The *ttk* locus encodes two zinc-finger DNA-binding proteins, *ttk*69 and *ttk*88 (Read and Manley, 1992). Loss of *ttk* function causes precocious SerT activity in stage 14 embryos (Fig. 2F) similar to that seen in *pnt* mutants. Early SerT was observed in two different *ttk* loss of function alleles, *ttk*^{1e11} (Fig.2D) and *ttk*^{1} (not shown). As expected, *in situ* hybridization for SerT mRNA in *ttk* mutants showed early SerT transcription as well (data not shown). This result indicates that *ttk*, like *pnt*, acts as a negative regulator of SerT onset. However, SerT activity appears unaffected in embryos over-expressing *ttk*69 or *ttk*88 (UAS-*ttk69* or UAS-*ttk88*, *eg*Gal4). Thus, unlike activated Pnt^{P1}, ectopic Ttk expression alone does not suppress SerT in later stages. At this point, it is not clear whether one or both isoforms of *ttk* are required to maintain normal SerT onset.

Pointed functions in the serotonergic neurons

Although our data suggested that *pnt* plays a role in regulation of SerT expression, the fact that *pnt* expression has thus far been observed only in glial cells of the VNC (Klaes et al., 1994; Klambt, 1993) caused us to question the mechanism of *pnt* function and the relationship of *pnt* to SerT. A similar problem

is confronted in consideration of the role of *ttk* and SerT expression. Possibly, *pnt* functions non-autonomously via cell interactions between serotonergic neurons and glia. In order to investigate this possibility, we first looked for the presence of early SerT in mutants for *glial cells missing* (*gcm*), a master glial differentiation gene that functions upstream of both *pnt* and *ttk* and is required for development of all CNS glia except those at the midline (Giesen et al., 1997). We used the absence of staining for the glial marker *reversed polarity* (*repo*), a well-known downstream target of *gcm*, to identify *gcm* mutants (Akiyama et al., 1996). None of the *gcm* mutants examined showed early SerT activity (n=10, data not shown). This indicates that at least gross disruption of glial differentiation is not responsible for the early SerT phenotype of *pnt* mutants.

Alternatively, loss of function mutations in *pnt* (or *ttk*) might transform glial cells into SerT positive cells. However, SerT positive cells in stage 14 *pnt* mutants express both Eg and another serotonergic marker, Engrailed, throughout the VNC (data not shown). If precocious SerT cells are *eg* positive, it seems unlikely that they are glial, since glial *eg* expression is normally extinguished by stage 12 (Higashijima et al., 1996).

Finally, *pnt* could function autonomously in the serotonergic neurons. Both *pnt* and *ttk* have a role in photoreceptor differentiation (Li et al., 1997), and it is possible that they regulate differentiation in other neurons directly as well. Support for an autonomous role of *pnt* comes the fact that over-expression of Pnt^{P1} in the serotonergic neurons induces a loss of SerT. Data from such ectopic expression experiments, however, can be difficult to interpret, and further

analysis was required to understand the mechanism of *pnt* function in serotonergic development.

For a more precise indication of *pnt* function in the serotonergic neurons, we analyzed lacZ expression driven by the *pnt* promoter (*pnt*-lacZ, pnt^{07825}) and looked for co-localization with serotonin staining from SerT activity. Fig. 3 shows a thin confocal section of one hemisegment in a stage 14 *pnt*-lacZ VNC with clear co-expression of serotonin and β-gal staining (n=12 VNCs). To more directly address the question of autonomous function, we attempted to rescue the early SerT phenotype seen in *pnt* mutants with expression of Pnt^{P1} in the serotonergic neurons. In order to achieve a relatively specific and early expression of Pnt^{P1}, we used the *eg*Gal4 driver. We created two recombinant fly lines to perform the rescue experiment: *pnt* UAS- Pnt^{P1} and *pnt eg*Gal4. These lines were then crossed to each other and mutants identified on the basis of absent β-gal staining from a marked balancer chromosome. When Pnt^{P1} is expressed in a *pnt* mutant background, the early SerT phenotype is rescued in 100% of cords (compare Fig. 4A to Fig. 4B; n=8). Not surprisingly, the loss of SerT phenotype observed when Pnt^{P1} is expressed in stage 16 VNCs remains despite the *pnt* loss of function background (compare Fig.4C to Fig.1B). This is likely due to the high level of Pnt^{P1} expressed under the Gal4-UAS system. The rescue experiment, along with the co-localization data suggest that *pnt* functions autonomously to regulate SerT expression.

Pointed interacts with the transcription factor Eagle to regulate serotonergic differentiation

During the course of our experiments with *pnt*, we observed an unexpected genetic interaction between *pnt* and the zinc-finger transcription factor *eg*, which is known to play a critical role in serotonergic differentiation. Specifically, we noticed a dosage interaction between Pnt^{P1} and a hypomorphic allele of *eg*, wherein an Eagle mutant wing phenotype was enhanced by expression of Pnt^{P1}. The classical "Eagle wing" phenotype is characterized by wings that are held-out and oriented perpendicular to the normal wing axis (Gritzan et al., 2002). Normally, the Eagle wing phenotype is seen only in flies homozygous for a hypomorphic *eg* allele, eg^{MZ360}, an *eg*Gal4 with a P-element insertion in the *eg* locus (see Table 1) (Dittrich et al., 1997). VNCs homozygous for eg^{MZ360} also show an approximately 30% loss of SerT activity (Table 1). A loss of *eg* function (eg^{18b} null) produces mild Eagle wings (wings at a 45° angle) in the heterozygous condition but in the homozygous condition is lethal and causes a severe loss of SerT (approximately 72%, Table 1). Thus, the dosage of Eg not only determines expression of the Eagle wing phenotype but also the degree of SerT loss.

When Pnt^{P1} is strongly expressed with *eg*Gal4 (as in Fig.1), an approximately 50% loss of SerT is observed in stage 16 VNCs and lethality occurs in late pupal stages. When activated Pnt is weakly expressed (UAS-Pnt^{s}; *eg*Gal4) via a different construct at a different insertion site, neither a loss of SerT nor lethality occurs. However, 100% of flies hatching from this cross display an Eagle wing phenotype, indicating an enhancement of the *eg* hypomorphic allele. Expression

of the weak UAS-Pnt^{s} in combination with UAS-activated-Ras (Act.Ras) induces both a loss of SerT as well as late lethality similar to that observed when the strong UAS- Pnt^{P1} is expressed (Table 1). This may result from an increased total amount of activated Pnt following recruitment of endogenous Pnt by Ras.

The genetic interaction observed between *pnt* and *eg* in wing morphology suggested that they might function coordinately to regulate SerT expression. Generally, *pnt* and *eg* appear to have opposite effects on the regulation of SerT expression: a partial loss of SerT results from a loss of *eg* (Fig.5D) (Couch et al., in press) or over-expression of Pnt^{P1} (Fig.1B). To further investigate the relationship between *pnt*, *eg*, and serotonergic differentiation, we assayed SerT activity in mutants with various dosages of *pnt* and *eg*. First, we expressed both Pnt^{P1} and Eg (UAS-Eg) in serotonergic neurons with *eg*Gal4. Expression of Eg does not rescue the loss of SerT caused by Pnt^{P1} over-expression (data not shown), suggesting that *pnt* does not simply function upstream of *eg*.

Additionally, a *pnt eg* hypomorph (*eg*^{MZ360}) double mutant was examined. All double mutants were identified by the absence of staining from a marked balancer. Interestingly, the phenotypes of both a *pnt* loss of function and an *eg*^{MZ360} hypomorph were rescued in the double mutant. Fig. 5A shows the early SerT phenotype typically observed in a *pnt* loss of function mutant, while Fig. 5B shows that the *pnt eg* hypomorph does not express early SerT. A rescue of the *pnt* early SerT phenotype was observed in 100% of double mutant VNCs examined (n= 8). This indicates that the early SerT observed in *pnt* mutants is *eg* dependent. However, a *ttk eg* hypomorph double mutant shows the same early

SerT phenotype as seen in *ttk* mutants alone (compare Fig.5C to Fig.2F; 100% of double mutants show early SerT, n=8). Thus, *pnt* and *eg* seem to function antagonistically to regulate SerT expression, while *ttk* and *eg* appear to be independent of each other.

Analysis of stage 16 VNCs for SerT activity yielded similar results. Fig. 5D shows the loss of SerT typically observed in an *eg* hypomorph. In *pnt eg* hypomorph double mutants, the loss of SerT is rescued (Fig.5E, G). This suggests that the loss of SerT observed in *eg* mutants is due at least in part to the presence of unregulated Pnt. Conversely, a *ttk eg* hypomorph shows a loss of SerT activity similar to that seen in *eg* hypomorphs alone (Fig.5F, G). The loss of SerT in a *ttk eg* double mutant, however, is slightly less severe than in *eg* hypomorphs, presumably due to the relief of some inhibition on SerT by *ttk* loss of function. Finally, we attempted to construct a *pnt eg* null mutant to further examine the relationship between these two genes, but were unable to do so as the combination of a *pnt* loss of function allele and an *eg* null allele in the double heterozygous condition was lethal.

DISCUSSION

This paper describes a role for RTK signaling during early serotonergic differentiation in *Drosophila*. Specifically, we provide evidence that the ETS transcription factor *pnt* negatively and autonomously regulates serotonergic differentiation. We additionally show that the transcription factor *ttk*, like *pnt*, negatively regulates SerT expression, and that the FGF signaling effector *hbr*, the

FGF-R *btl*, its ligand *bnl* may function upstream of *pnt* in regulating serotonergic differentiation. Taken together, our results suggest the presence of a FGF signaling pathway that functions with *pnt* to prevent inappropriate early differentiation. Finally, we show data suggesting an antagonistic relationship between *pnt* and the transcription factor *eg*, which is required for normal serotonergic differentiation.

A role for *pnt* in neuronal differentiation is unexpected, given the well-established role for *pnt* in glial differentiation and previous descriptions of *pnt* expression patterns (Klaes et al., 1994; Klambt, 1993). However, our results showing co-localization of *pnt*-lacZ and SerT (Fig.3), in addition to our rescue experiment (Fig.4), suggest that Pnt may function at least in the serotonergic neurons of the VNC. Previous reports of Pnt expression were derived from whole VNC staining against Pnt antibodies or □-galactosidase expressed under the *pnt* promoter (Klaes et al., 1994). It seems that Pnt expression in a small subset of neurons would have been easily missed using this technique, particularly given the widespread expression of Pnt in all glial cells.

Our results suggest that *pnt* has an early function in preventing inappropriate SerT expression, since *pnt* loss of function mutants show precocious SerT activity. However, some mechanism must inactivate Pnt^{P1} to allow onset of normal differentiation, since ectopic expression of Pnt^{P1} later in development causes a loss of SerT expression as well as serotonin synthesis. Our observation that over-expression of activated Pnt^{P1} causes a loss of SerT expression in only a percentage of neurons (Fig. 1B) suggests the presence of a redundant mechanism for

differentiation. Previous studies have shown that loss of a critical differentiation gene such as *eg*, *hunchback*, *engrailed*, or *huckebein* severely perturbs serotonergic development, but not in all serotonergic neurons (Lundell et al., 1996; Lundell and Hirsh, 1998; Novotny et al., 2002). Additionally, ectopic expression of positive regulatory genes, such as *islet*, does not induce formation of ectopic serotonergic neurons, implying the presence of a combinatorial code for serotonergic differentiation (Thor and Thomas, 1997).

We further show that FGF signaling likely has a negative regulatory role in SerT expression, which conflicts with reports from the grasshopper indicating a positive role for FGF (Condron, 1999). FGF is also required for the early specification of serotonergic precursors in vertebrates (Ye et al., 1998). At this point, the source of the discrepancy between our results and those of previous studies remains unclear. Possibly, there are different and competing FGF signals that together tightly control the timing of differentiation. Although we show a negative role for the FGF *bnl* and the FGF-R *btl* in differentiation, the other known *Drosophila* FGF-R, *htl*, may function in serotonergic differentiation as well, and if so, may function positively. The ligand for Htl is currently unknown (Beiman et al., 1996; Gisselbrecht et al., 1996). Our initial experiments expressing a DN or activated Htl yielded negative results that are difficult to interpret. Redundant mechanisms for differentiation may have compensated for ectopic Htl expression in our experiments and as such, additional factors would have been required to disrupt differentiation. Unfortunately, we were unable to recover enough *htl* mutant stage 16 VNCs to determine if loss of *htl* function

affects later differentiation. In the few stage 16s that were recovered, we observed a loss of SerT activity, but it remains unclear whether this is an indirect effect of *htl* loss in other critical tissues.

Alternatively, FGF signaling might elicit either a positive or a negative differentiation response depending on the time and environment in which it occurs, based on interactions with other signals. Although we observed precocious stage 14 SerT activity in *bnl* heterozygous mutants, we also found a partial loss of SerT in a few stage 16 VNCs. As such, early FGF signaling might prevent inappropriate differentiation by activating Pnt (for example, to inhibit early SerT expression) while later signaling might activate other transcription factors that support normal differentiation. In addition, we do not yet know the source of the FGF *bnl* signal. We did not observe Bnl expression (from *bnl*-lacZ) in serotonergic neurons; however, some unidentified midline cells express *bnl*-lacZ and may be required for Bnl-induced signaling in the serotonergic neurons. Future investigations should examine the expression of both Bnl and Btl in the VNC, as our results indicate that the role for Btl and Bnl in the CNS is only beginning to be elucidated. Recently, a novel function for these genes was identified in dividing neuroblasts, and expression observed in larval brain lobes (Park et al., 2003).

Evidence that Ets transcription factors are involved in serotonergic differentiation comes from recent studies in mice showing that the Ets transcription factor Pet-1 is critical for the normal development of serotonergic neurons. Pet-1 is an early and precise marker of serotonergic neurons in both

mice and rats (Hendricks et al., 1999; Pfaar et al., 2002). A loss of Pet-1 in mice causes a disruption in several aspects of differentiation, including expression of SerT, serotonin and other serotonergic markers (Hendricks et al., 2003). Thus, the Ets transcription factor Pet-1 seems to positively regulate serotonergic differentiation. Our results showing a negative role for *pnt*, also an Ets transcription factor, therefore do not simply correspond to results from the rodent. This may result from differences between species, or may reflect our lack of understanding a more complicated genetic pathway regulating differentiation. For example, more than one Ets transcription factor may be involved in regulating differentiation. Previous reports as well as a recent GenBank BLAST search indicate that the closest *Drosophila* homolog of rat Pet-1 is *Ets65A* (also known as D-Ets-3, 91% identity between rat Pet-1 and an incomplete D-Ets-3 sequence) (Chen et al., 1992; Fyodorov et al., 1998). *Ets65A* is a relatively uncharacterized ETS transcription factor with no known mutants. *pnt* is most similar to vertebrate c-Ets-1 (Hsu and Schulz, 2000; Klambt, 1993). Potentially informative future studies might investigate the role of these genes to determine if they function in serotonergic differentiation.

Our data showing that a loss of *ttk* function causes precocious SerT expression but that ectopic expression of *ttk69* or *ttk88* has no discernable effects suggests that *ttk* may play a permissive rather than an instructive role in differentiation. For example, *ttk* repression of SerT may require another factor present only during early embryonic stages and thus be insufficient to disrupt differentiation when ectopically expressed. In the PNS, *ttk* functions to repress

proneural gene expression, thus preventing ectopic sensilla formation (Badenhorst et al., 2002). Additionally, FGF signaling may modify *ttk* function, as previous reports have shown that *ttk* can be a direct target of MapK activation (Chen et al., 2002). Although *ttk* expression, like *pnt*, has been shown only in glial cells of the VNC (Giesen et al., 1997), there is a possibility that *ttk* functions autonomously in serotonergic development as well. Future experiments looking closely at *ttk* expression in addition to rescue experiments should clarify this issue.

Another gene, *anterior open* (*aop*, also known as *yan*), that functions with *pnt* in other developing tissues (Brunner et al., 1994; Gabay et al., 1996), might also play a role in SerT regulation. Preliminary experiments suggest a serotonergic phenotype is observed in *aop* mutants as well as when activated *aop* is expressed, and future studies should address the possible function of *aop* in serotonergic differentiation as well. Nonetheless, it appears likely that a complex genetic pathway is responsible for normal serotonergic development in *Drosophila*.

Although we show a role for *pnt* in serotonergic differentiation, the molecular mechanism of *pnt* function in serotonergic differentiation remains unclear. The Ets transcription factor Pet-1 has binding sites in promoter regions of SerT, the biosynthetic enzyme tryptophan hydroxylase, as well as other genes that are required in mature rat serotonergic neurons (Hendricks et al., 1999). Possibly, *pnt* functions directly by preventing transcription of SerT. A brief search of *dSerT* for an ETS consensus binding sequence (see Experimental Procedures) found several potential sites for *pnt* function that should be examined in future

experiments. This search also identified a consensus site for *ttk*88 in *dSerT*. However, *pnt* is generally known to function as a transcriptional activator. Different Ets transcription factors, such as *aop*, have better known functions as transcriptional repressors, although evidence suggests that the switch between transcriptional repression and activation may be highly dependent on the cellular context (Mavrothalassitis and Ghysdael, 2000). Another possibility is that *pnt* activates transcription of a gene that then represses SerT expression, or *pnt* may function through many genes.

Finally, we show that *pnt* and *eg* seem to have an antagonistic relationship in regulating SerT. A *pnt eg* double mutant rescues not only the early SerT phenotype of *pnt* mutants but also the loss of SerT phenotype of *eg* mutants. This implies that the early SerT observed in *pnt* mutants is *eg* dependent, and further, that the loss of SerT seen in *eg* mutants is due to some residual *pnt* suppression of SerT. Previously, *eg* has been shown to suppress an activated Ras over-expression phenotype in the eye (Hay et al., 1997), and may similarly interact with FGF signaling during serotonergic differentiation. However, the mechanism for the *eg* – Ras interaction or how this interaction might affect serotonergic differentiation remains unknown. Eg has also been shown to antagonize the activity of AP-1, which interacts with RTK signaling (Gritzan et al., 2002). Further, previous work from our lab has shown that the axon guidance molecules Robo2 and Robo3 genetically interact with Eg to regulate differentiation (Couch et al., in press). Possibly, Eg represents a convergence point of differentiation signals, including those from axon guidance as well as

FGF. A direct interaction between FGF signaling and axon guidance has been previously shown in flies (Garcia-Alonso et al., 2000).

Although *eg* is an established regulator of serotonergic differentiation (Couch et al., in press; Dittrich et al., 1997; Lundell and Hirsh, 1998), the mechanism of *eg* function has not yet been investigated. To identify possible transcriptional targets for Eg, we used the consensus sequence for the related orphan steroid hormone receptor and zinc-finger transcription factor *knirps*, which shares 80% homology with the finger domain of *eg* (Rothe et al., 1989). A search for *knirps* consensus sequences also identified sites in *dSerT*. Interestingly, upstream of *dSerT*, a *knirps* site and an ETS site were found only 52 bases apart. Although this bioinformatics approach does not offer any concrete explanations for *pnt* or *eg* function, it provides interesting possibilities for future consideration. Future molecular and genetic studies are likely to yield interesting information about the function of FGF signaling and *pnt* in serotonergic differentiation.

EXPERIMENTAL PROCEDURES

Drosophila strains

Fly strains used include: UAS-Pnt^{P1}, UAS-Pnt ^{P2}, *pnt*^{2} loss of function, *pnt*$^{\Delta 88}$ amorph, *pnt*^{07825} (*pnt*-lacZ), *ttk*^{1e11} and *ttk*^{1}, *stumps*^{09904b}, *btl*^{dev1} hypomorph, *bnl*^{06916} hypomorph, UAS-*c-myc*-GFP, UAS-DN.EGF, UAS-Act.Top (EGF), UAS-85D.N17 (DN.Ras), and UAS-Ras85D.V12 (Act.Ras) from Bloomington Stock Center (Bloomington, Indiana); UAS-DN.Htl (33-B40; 33-B61), (UAS-Act.Htl), *btl*^{LG19} loss of function, *bnl*$^{[P1]}$ loss of function (Michelson et al., 1998); *eg*^{MZ360} hypomorph

(also an *eg*Gal4) and eg^{18b} null (Lundell and Hirsh, 1998); gcm^{N7-4} amorph (Michele Lamka, University of Virginia); UAS-Pnt^{s} (schwache insertion, Christian Klämbt, Universität Münster, Germany); UAS-ttk69, UAS-ttk88 (Giesen et al., 1997).

Immunohistochemistry and Embryo staging

Embryos were collected from agar egg-laying plates and placed into Eppendorf tubes containing Schneider's insect medium (Sigma), bleached for one minute, and washed with salt solution (0.04% NaCl + 0.03% TritonX-100) followed by Schneider's medium. Dechorionized embryos were then placed into a Petri dish containing Schneider's and isolated by embryonic stage (Campos-Ortega, 1985) prior to dissection. Dissected VNCs were then transferred to a second Petri dish containing Schneider's and affixed to a glass cover slip. SerT activity was visualized by adding serotonin (Sigma) to the medium at a saturating concentration of 10 □M and incubating for 10 minutes (nutating). After serotonin incubation, media was removed and replaced with 4% paraformaldehyde/PBS, freshly made, and incubated for 60 minutes. Following fixation, VNCs remained tightly adhered to the glass cover slip and could thus be transferred between washes during histochemical processing, performed as previously described (Condron, 1999). Antibodies used were: 5HT 1:5000 (Immunostar); □gal 1:40, 4D9 (anti-Engrailed) 1:20, 8D12 (anti-Repo) 1:40, BP102 1:500 (Developmental Studies Hybridoma Bank, University of Iowa); Mouse anti-Eg (gift from Chris Doe).

Photography, Image Processing, and Computational Analysis

An Olympus BX40 microscope and a Photometrics SenSys camera were used for fluorescent imaging. All images except Figure 3 were taken with an Olympus 20x or 40x lens. Image IP was used to capture images that were subjected to one frame deconvolution (95% removal, 75% gain) using VayTek Hazebuster. Adobe Photoshop was used to process fluorescent images. Figure 3 shows a fixed sample imaged with a Nikon Eclipse E800 microscope (100x oil lens, NA=1.3), Hammamatsu ORCA-ER camera and a Perkin-Elmer spinning disk confocal unit. One stack of approximately 100 slices was taken, spaced 0.24 □M, with 2x2 binning and an exposure time of 500 milliseconds. Image reconstruction for Figure 3 was performed with Volocity 2.0.

For analysis of consensus binding sites, *dSerT* was searched using MacVector (Accelrys) for the following sequences: Ets, NSYGGAWRY (Halfon et al., 2000); *ttk88*, AGGGC/TGG (Read and Manley, 1992); *knirps*, ATCTAAATC (Hoch et al., 1992).

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FIGURE LEGENDS

Figure 1

Over-expression of an activated form of the Ets domain transcription factor Pointed causes a loss of serotonin transporter expression. (A, B) Staining for serotonin uptake (white) at stage 16 (A) in a wild-type ventral nerve cord or (B) when an activated Pnt^{P1} is over-expressed in the serotonergics using the *eg*Gal4 driver. (A) The wild type nerve cord has two serotonergic neurons in each hemisegment that are positive for SerT activity and show normal axon guidance across the midline. (B) Over-expression of activated Pnt^{P1} causes a loss of SerT activity in approximately 50% of neurons and disrupts axon guidance. (C) Staining for SerT activity (red) and the homeobox transcription factor *engrailed* (green) shows that expression of activated Pnt^{P1} does not disrupt early serotonergic specification. (D) Co-expression of *c-Myc*-GFP (green) with activated Pnt^{P1} illustrates that those neurons negative for SerT activity (red) are present and extending axons. Scale bar is 5 □M in (A, B) and 10 □M in (C, D).

Figure 2

Mutations in genes of the FGF signaling pathway induce precocious serotonin transporter uptake activity. (A-F) Stage 14 ventral nerve cords stained for serotonin uptake (red) and the general axonal marker BP102 (green). (A) Wild-type stage 14 nerve cords do not show serotonin uptake, as SerT expression does not begin until late stage 15. Precocious serotonin uptake is seen in embryos with a homozygous loss-of-function in (B) the transcription factor *pointed* or (C)

the downstream of FGF effector *heartbroken*, as well as in embryos heterozygous for a loss of function in (C) the FGF receptor *breathless* or (D) the FGF *branchless*. Finally, a homozygous loss of function in (F) the transcription factor *tramtrack* also causes early SerT activity. Scale bar in A is 10 μM.

Figure 3

Expression of *pointed* is observed in serotonergic neurons. A thin confocal section of one hemisegment shows co-localization of staining for serotonin uptake (red) and lacZ (green) expressed under the *pointed* promoter (*pnt*-lacZ). Scale bar is 5 μM.

Figure 4

pointed functions autonomously in the serotonergic neurons. (A-C) Staining for serotonin uptake is seen in red and for the axonal marker BP102 in green. (A) Stage 14 *pointed* mutants show precocious SerT activity. (B, C) Expression of an activated Pointed in serotonergic neurons (UAS- Pnt^{P1}; *eg*Gal4) in a *pointed* mutant background (B) rescues the *pointed* early SerT phenotype. The red level has been enhanced in panel B to emphasize the lack of early SerT. (C) Despite the *pointed* mutant background, the stage 16 loss of SerT phenotype caused by Pnt^{P1} over-expression remains (compare to Figure 1B). Scale bar in A is 10 μM.

Table 1

Pointed interacts with Ras to enhance a mutant phenotype for the transcription factor *eagle*.

Figure 5

pointed interacts with *eagle* to affect SerT expression while *tramtrack* does not. (A-F) Serotonin uptake is shown in red and BP102 in green in (A-C) stage 14 or (D-F) stage 16 ventral nerve cords. (A) The precocious serotonin uptake activity observed in *pointed* mutants is not observed in (B) a *pointed eg* hypomorph (eg^{MZ360}) double mutant. However, the early SerT phenotype seen in *tramtrack* mutants remains in a (C) *tramtrack* eg^{MZ360} double mutant. Conversely, the loss of SerT activity observed in (D) eg^{MZ360} homozyogotes is rescued in (E) a *pointed* eg^{MZ360} double mutant, but remains in (F) a *tramtrack* eg^{MZ360} double mutant. Asterisks (*) in (D) and (F) indicate hemisegments where SerT activity is lost. The average loss of SerT activity between genotypes at stage 16 is shown in the graph below. Error bars represent the standard deviation from the mean. A Kruskal-Wallis Nonparametric ANOVA (Dunn's comparisons) showed a significant difference between eg^{MZ360} homozyogotes and *pnt* eg^{MZ360} double mutants ($p < 0.001$), but not between eg^{MZ360} homozyogotes and *ttk* eg^{MZ360} double mutants. Scale bar in D is 10 □M.

FIGURES

Figure 1

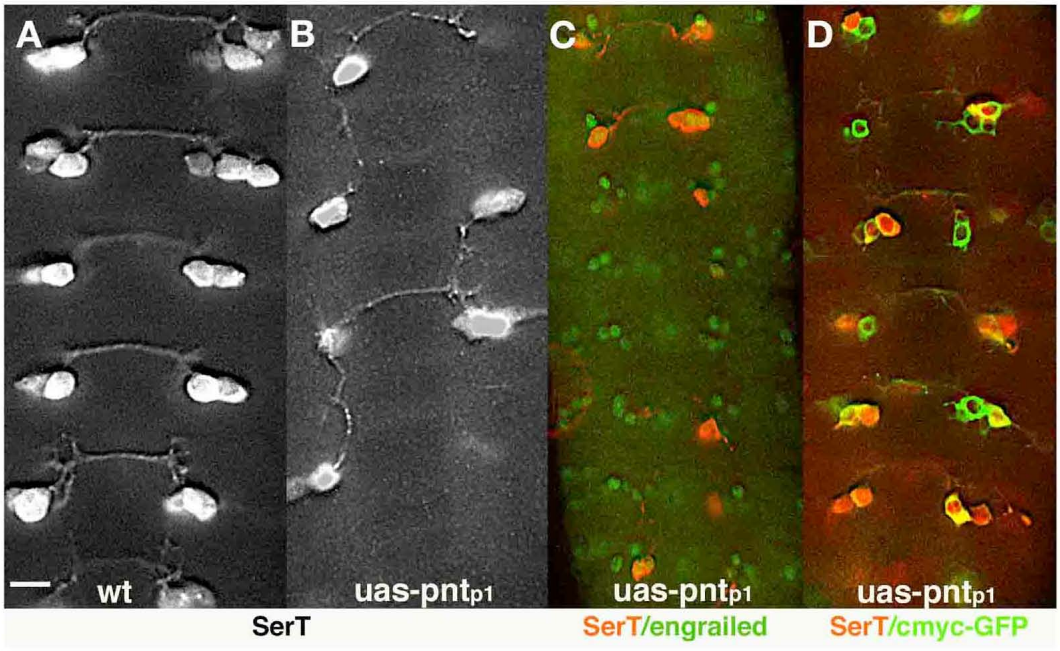


Figure 2

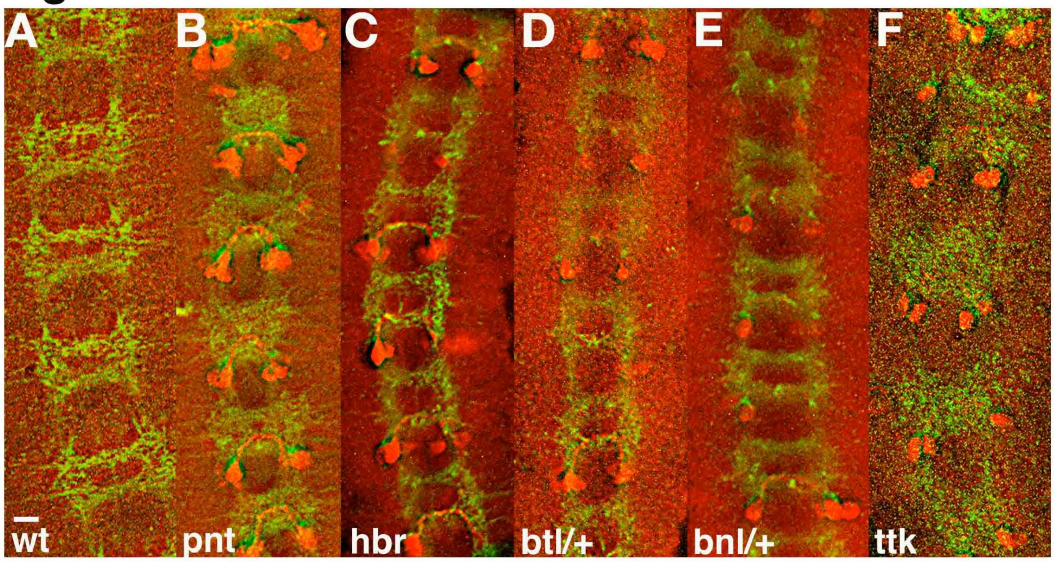


Figure 3

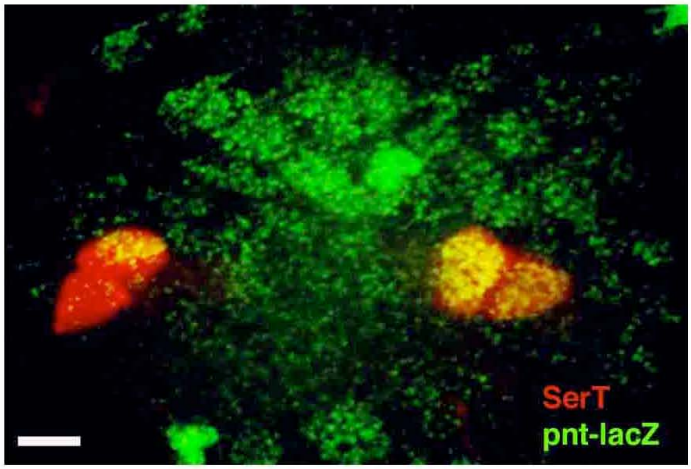


Figure 4

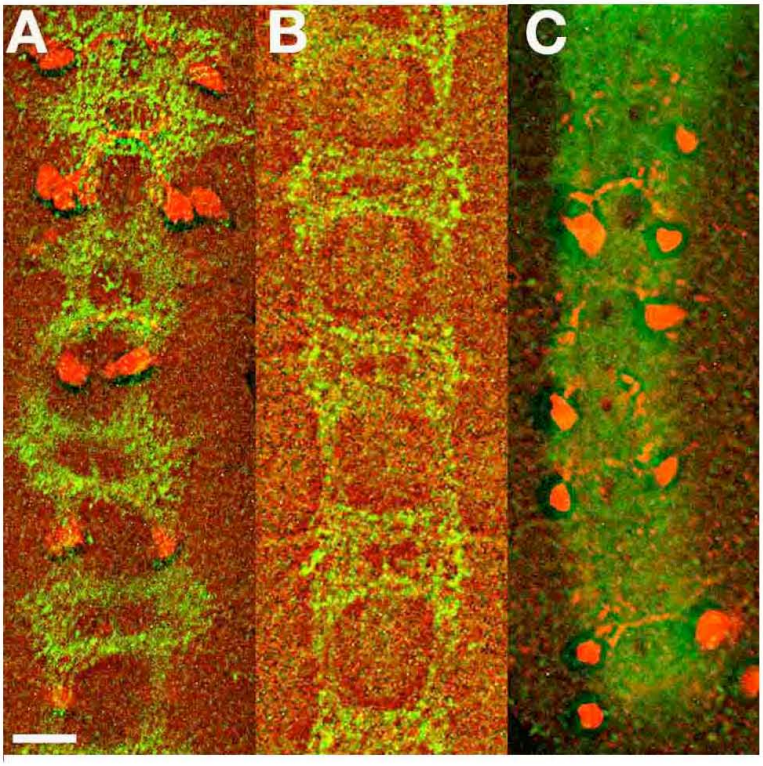


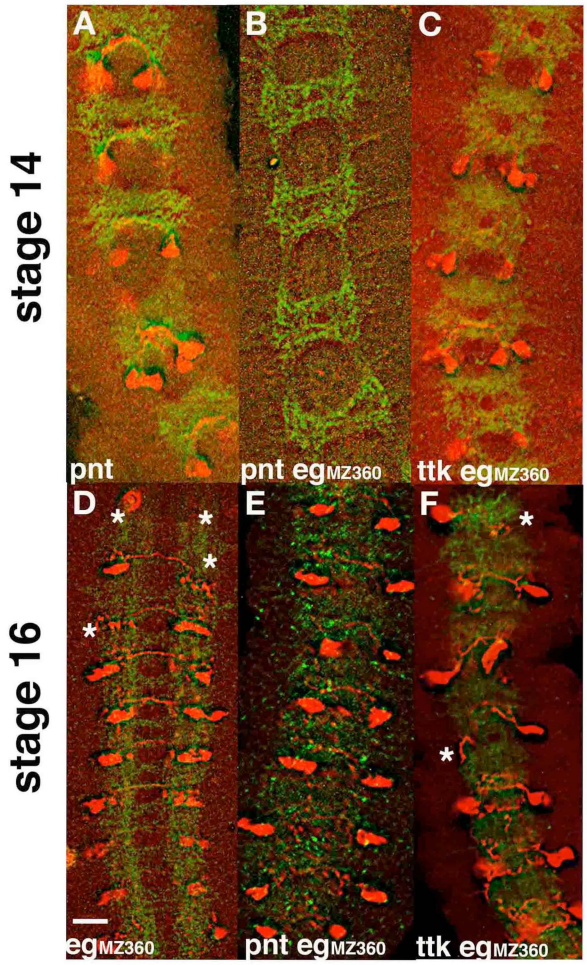
TABLE 1

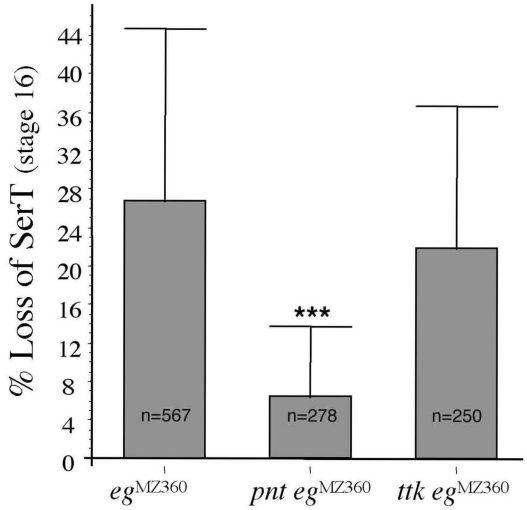
Genotype	**% SerT loss** (mean ± s.d.)	**Eagle wings**	**Lethality**
$eg^{MZ360}/+$	0	No	No
eg^{MZ360}/ eg^{MZ360} (*eg* hypomorph)	28 ± 20	Yes	No
UAS-pnt^{s} (weak expression of activated pnt)	0	Yes	No
UAS-DN.Ras; UAS-pnt^{s}	nd	Yes	No
UAS-pnt^{P1} (strong expression of activated pnt)	49 ± 22*	---	Yes
UAS-Act.Ras; UAS-pnt^{s}	63 ± 32*	---	Yes
eg^{18b}/ eg^{18b} (*eg* null)	72 ± 16*	---	Yes

All UAS constructs expressed with the *eg*-Gal4 driver ($eg^{MZ360}/+$)

* Indicates $p<0.05$ compared to eg^{MZ360}/ eg^{MZ360}, Kruskal-Wallis Nonparametric ANOVA

Figure 5


A
B
C
stage 14
pnt
pnt egMZ360
ttk egMZ360
D
E
F
stage 16
egMZ360
pnt egMZ360
ttk egMZ360


% Loss of SerT (stage 16)
44
40
36
32
28
24
20
16
12
8
4
0
***
n=567
n=278
n=250
egMZ360
pnt egMZ360
ttk egMZ360

GENERAL DISCUSSION

This dissertation has focused on the final steps of serotonergic maturation, as the neurons begin to differentiate and establish their unique, functional identities. Primarily, the expression of the serotonin transporter (SerT) and serotonin synthesis have been used as markers of differentiation. The data presented here identify novel regulators of serotonergic differentiation that are highlighted in Figure 1. The model shown in Figure 1 illustrates the general relationships of these newly identified regulators to serotonergic differentiation and to each other, as surmised predominantly from genetic interactions rather than their precise molecular function. Although these genes have been previously characterized for their roles in the development of other CNS structures, they have not been specifically implicated in the development of the serotonergic neurons until now.

The axon guidance receptors Robo2 and Robo3 appear to play a positive role in serotonergic differentiation, and may function upstream of the transcription factor Eagle. This interpretation is supported by data showing that a loss of function in *robo2* or *robo3* causes a loss of SerT and Eg expression as well as serotonin synthesis in a percentage of neurons. Although the specific molecular function of Robo2 and Robo3 in serotonergic differentiation remains unknown, data showing that Eg expression completely rescues a *robo2* loss of function serotonergic phenotype suggest a direct relationship between Robo2 and Eg.

Additionally, data indicate that a negative regulatory pathway, controlled by FGF signaling and the transcription factors Pointed and Tramtrack, represses serotonergic differentiation until the appropriate developmental stage. A loss of either *pointed* or *tramtrack* causes precocious SerT expression, and over-expression of an activated Pointed causes a loss of SerT in addition to serotonin synthesis. A loss of the FGF signaling effector *heartbroken*, the FGF receptor *breathless*, and its ligand *branchless* additionally induce precocious SerT expression. Thus, Pointed may function downstream of FGF signaling during serotonergic differentiation. At this point, it is unclear how this repression is relieved during the normal maturation process, or what consequences precocious differentiation might have on the serotonergic system or the development of the CNS in general.

MODEL FOR SEROTONERGIC DIFFERENTIATION IN *DROSOPHILA*

A model for serotonergic differentiation shown in Figure 1 integrates data presented here with that of previous studies, and illustrates the currently known regulators of serotonergic differentiation in *Drosophila*. Table 1 serves as a reference for the primary regulators and offers a brief description of their function in serotonergic developement. Previous studies have shown that the transcription factors *engrailed* (*en*), *huckebein* (*hkb*), *hunchback* (*hb*), and *Krüppel* (*Kr*) are critical for early specification of the serotonergic lineage (Isshiki et al., 2001; Lundell et al., 1996). Although these genes are expressed throughout differentiation, it remains unclear what specific role these genes play at later

stages of development since a temporally restricted functional analysis has not yet been done. For example, experiments disrupting *en* or *hkb* function only in the post-mitotic serotonergic neurons, as with RNA interference, may lend insight into the later roles of these genes in serotonergic maturation.

The role of the zinc-finger transcription factor and orphan steroid hormone receptor *eagle* (*eg*) in serotonergic development has been established by several previous studies. Eg expression is regulated by the transcription factors *hkb* and *en*, and is required for both SerT and Ddc expression (Dittrich et al., 1997; Higashijima et al., 1996; Lundell and Hirsh, 1998). The severity of SerT or Ddc loss varies with the severity of the *eg* mutation (Chapter 1)(Lundell and Hirsh, 1998). Eg is also required for expression of *en* and *zfh-2* as serotonergic maturation proceeds (Lundell and Hirsh, 1998). However, the effect of *eg* loss on Tph (Henna) expression or other terminal differentiation genes has not yet been examined. Possibly, Eg serves as a master regulatory switch favoring the onset of serotonergic differentiation. Eg is a good candidate for such a role, given that it is expressed in only a few other neurons in addition to the serotonergic neurons (Higashijima et al., 1996).

However, several lines of evidence suggest that serotonergic differentiation is controlled by a "combinatorial code" including Eg in addition to several other factors. Eg is expressed in NB 7-3 and all of its progeny, far before the onset of differentiation; however, a loss of *eg* does not appear to perturb lineage specification. Further, pan-neural expression of Eg with *scabrous*Gal4 results in only a few ectopic serotonergic neurons, all of which also express *hkb* (Dittrich et

al., 1997). The fact that ectopic serotonergic cells in an Eg over-expression background are restricted to those that express *hkb* rather than *en* has led to the suggestion that *hkb* and *eg* may play a more direct role in differentiation as compared to *en* (Dittrich et al., 1997). Interestingly, expression of Eg in only the post-mitotic neurons with an *elav*Gal4 driver does not produce ectopic cells. Thus Eg may function between lineage specification and terminal differentiation, and may either induce or interact with other factors that allow differentiation to proceed. Evidence that this is the case comes from data presented in previous chapters showing a genetic interaction between Eg and the guidance molecules Robo2 and Robo3. Since the onset of SerT expression seems to be correlated with serotonergic axons crossing the midline, an interaction between Robo2/3 and Eg might contribute to the proper temporal initiation of differentiation. Future studies should investigate the transcriptional targets of Eg in order to identify its specific molecular function in serotonergic maturation.

Another critical requirement for serotonergic differentiation is the LIM-homeodomain transcription factor *islet*. Islet is expressed in the post-mitotic serotonergic neurons, and is required for both SerT expression and serotonin synthesis (J.A.C. and B.G.C., unpublished results) (Thor and Thomas, 1997). Islet is also required for proper differentiation of the dopaminergic neurons (Thor and Thomas, 1997). Although Islet seems to function specifically during differentiation and not during lineage development, it is not sufficient to induce ectopic serotonergic cells when expressed throughout the VNC (Thor and Thomas, 1997). Thus, Islet appears to be a component of the combinatorial code

required for serotonergic differentiation. Taken together, data from previous studies suggest that this code includes *islet*, *eg*, *en*, *hkb* and possibly others. Further analysis of these combinations through various mis-expression experiments may yield interesting insights into the transcriptional control over serotonergic differentiation. Additionally, it is not known what factors activate *islet* expression in the serotonergic neurons.

Finally, several factors are known to be expressed in the serotonergic neurons but remain relatively uncharacterized for their specific function during development. These include the POU transcription factors *pdm*, *drifter*, and *I-POU*, as well as the zinc-finger transcription factor *zfh-2*. Zfh-2 binds a serotonergic specific regulatory element in Ddc (Lundell and Hirsh, 1992), and Drifter is able to activate Ddc transcription in dopaminergic neurons; however, the specific function of these genes in serotonergic differentiation has not yet been examined (Johnson and Hirsh, 1990). Additionally, I-POU may prevent Drifter from activating Ddc transcription, and may function antagonistically in serotonergic differentiation as well (Treacy et al., 1991). Interestingly, Drifter functions upstream of the FGF receptor *breathless* in tracheal development (Anderson et al., 1996), and might also interact with tyrosine kinase signaling in serotonergic neurons. Future genetic study of these genes is clearly required to understand their potential functions in serotonergic differentiation. Further, these genes may interact with previously identified regulators of differentiation. For example, an interaction between LIM and POU transcription factors in

C.elegans has been shown to alter DNA binding affinities of the proteins and may function to coordinate cell fate specification (Xue et al., 1993).

THE LINK BETWEEN AXON GUIDANCE AND DIFFERENTIATION

The Robo family of receptors, which includes Robo2 and Robo3, mediate repulsive axon guidance in response to their ligand, Slit (Kidd et al., 1999; Kidd et al., 1998). Signaling from Robo/Slit functions to prevent inappropriate midline crossing. Robo2/3 also control the lateral positioning of longitundinal fascicles in the CNS (Rajagopalan et al., 2000; Simpson et al., 2000a). Results presented in Chapter 1 indicate that the axon guidance molecules Robo2 and Robo3 play a role in serotonergic differentiation as well. Additionally, data suggest that *robo2/3* functionally interact with the transcription factor Eg to regulate SerT expression (Chapter 1). At this point, it remains unclear how the cell surface receptors *robo2/3* interact with the nuclear localized *eg*.

Recent progress has been made in elucidating the downstream signaling effectors of axon guidance receptors. The tyrosine kinase Ableson (Abl) and its substrate Enabled (Ena) play a role in downstream signaling from Robo. Initial reports indicated that Ena functions to promote Robo signaling by directly binding Robo's cytoplasmic domain; while Abl antagonizes Robo through phosphorylation (Bashaw et al., 2000). However, as the cytoplasmic domains of Robo2 and Robo3 vary considerably from that of Robo (Rajagopalan et al., 2000; Simpson et al., 2000b), it remains unclear whether Robo2/3 function through alternative downstream effectors. A more complicated role for Abl has recently

been suggested, in that it may also positively mediate Robo signaling (Wills et al., 2002). These data further suggest that Abl can function downstream of Robo2 and Robo3 as well, and that Abl does not depend on a specific Robo cytoplasmic domain.

New evidence further suggests that the SH2/SH3 adaptor protein Dreadlocks (Dock) functions with the serine-threonine kinase Pak and the Rac small GTPase to facilitate Robo mediated repulsion through an interaction with cytoskeletal components (Fan et al., 2003). The Rho family of small GTPases, including Rac and Cdc42 have been implicated in many aspects of axon guidance and growth cone motility, and functionally interact with Robo signaling (Dickson, 2001; Fritz and VanBerkum, 2002; Ng et al., 2002). In general, the Rac GTPases typically function to promote axon guidance and motility, while Rho GTPases function to mediate repulsion (Patel and Van Vactor, 2002). However, recent studies indicate that this is not always the case, and suggest a much more complicated model of axon guidance regulation (Fan et al., 2003). Additionally, previous studies have suggested that Calmodulin and the Ras/Rac guanine nucleotide exchange factor Son of sevenless interact with Robo to mediate repulsive signaling at the midline (Fritz and VanBerkum, 2000). Thus, although important players in axon guidance signaling pathways are beginning to be identified, many questions remain. Many of the downstream effectors identified thus far, particularly those of the Rho GTPase family, are likely to function in regulating actin cytoskeletal dynamics (Patel and Van Vactor, 2002). As such, the link between axon guidance receptor signaling and alterations in cellular

transcription programs remains almost completely uncharacterized.

Further, other factors play a role in controlling the expression and activation of Robo family members, in order to effectively restrict their function to the appropriate developmental stage. Commissural axons initially grow towards the midline in response to the chemoattractant Netrin, which is detected by the Netrin receptor *Frazzled* (known as DCC in vertebrates). This initial and critical midline-crossing event is disrupted in mutants with excessive repulsive signaling from Robo family members. One of the most well-known regulators of the Robos is the transmembrane protein Commissureless (Comm), which recruits Robo to the endosome, preventing Robo expression at the cell surface, and thereby preventing inappropriately early Robo signaling (Keleman, 2002)(also see Appendix 1). In addition to the Robo family members, many other axon guidance molecules exist that may modulate midline signaling in the serotonergic neurons. Previous results have shown that in *Xenopus* growth cones, activated Robo interacts with the cytoplasmic domain of the netrin receptor DCC and thereby downregulates attractive guidance cues after midline crossing is complete (Stein and Tessier-Lavigne, 2001). Clearly, many different factors interact with Robo signaling, and may play a role in regulating Robo2 and Robo3 function during serotonergic differentiation.

An interaction between axon guidance and serotonergic differentiation has been previously noted in studies of mutants for the transcription factors *eg* and *islet*, suggesting that the coordination of these processes may be under transcriptional control (Lundell and Hirsh, 1998; Thor et al., 1999). Since the

transcriptional targets of both *eg* and *islet* remain unknown, it is difficult to speculate on how these genes specifically contribute to axon guidance. Other transcription factors known to affect axon guidance include the homeobox protein *even-skipped* and the LIM-homeodomain protein *apterous* (Fujioka et al., 2002; Lundgren et al., 1995). In *C. elegans*, the POU transcription factor UNC-86 regulates expression of the serotonin synthetic enzyme *tph-1* and the vesicular monoamine transporter *cat-1*, but also influences axon guidance in a subset of serotonergic neurons (Sze et al., 2002). Finally, in vertebrates, Ets transcription factors not only regulate differentiation but are also critical for the formation of functionally specific neuronal connections as well as proper branching patterns (Lin et al., 1998; Livet et al., 2002).

VERTEBRATE CORRELATES

Progress has recently been made in identification of factors important for mammalian serotonergic differentiation. Initial specification of the serotonergic lineage requires the combined signaling of FGF4, FGF8, and Sonic Hedgehog (Ye et al., 1998) to activate expression of the transcription factor *Nkx2.2* in serotonergic precursor cells (Briscoe et al., 1999). *Nkx2.2* functions to specify serotonergic neurons early in development, and a loss of *Nkx2.2* causes a loss of serotonergic neurons (Briscoe et al., 1999). A model for vertebrate serotonergic differentiation has been proposed based on loss of function studies and temporal expression patterns, wherein *Nkx2.2* activates the LIM homeodomain transcription factor *Lmx1b* which in turn activates expression of the serotonin

specific Ets transcription factor *Pet-1* (Ding et al., 2003). A loss of *Lmx1b* or *Pet-1* causes a severe disruption of serotonergic differentiation (Ding et al., 2003; Hendricks et al., 2003). *Pet-1* is an early and specific marker of serotonergic neurons in both mice and rats, and has been shown to act as a transcriptional activator of several genes required for serotonergic function, including SerT and Tph (Hendricks et al., 1999). Thus, *Pet-1* appears to act as a terminal differentiation gene that is critical for promoting the mature serotonergic phenotype. Both *Nkx2.2* and *Lmx1b* are required for expression of *Pet-1* in addition to the zinc-finger transcription factor *Gata3*, which plays a role in the differentiation of the caudal subset of serotonergic neurons (Ding et al., 2003; van Doorninck et al., 1999). Another zinc-finger transcription factor, *Gli1*, is an early downstream target of Shh signaling and appears to be important for development of some serotonergic neurons (Matise et al., 1998). Although some of the critical players in vertebrate serotonergic differentiation have been identified, many appear to be important only for a subset of serotonergic neurons. FGF8, for example, is not required for the specification of the caudal neurons (Ye et al., 1998). For a more complete review of vertebrate serotonergic development, see (Hynes and Rosenthal, 1999).

An interesting and potentially productive approach might be to look at the possible serotonergic function of homologous genes in *Drosophila*. A recent GenBank BLAST search indicates that the closest homologs are as follows: *ventral nervous system defective* (*vnd*) for *Nkx2.2*, *lim3* for *Lmx1b*, *cubitus interruptus* (*ci*) for *Gli1*, *dGatac* for *Gata3*, and *Ets65A* for *Pet-1*. Both *vnd* and *lim3* are known to

regulate development of many tissues, including the CNS, while *ci* is a segment polarity gene that functions in the wingless signaling pathway during early embryonic development. Although *vnd* is not known to regulate formation of NB 7-3, *engrailed* is a related homeobox family member and may serve this function in the serotonergic lineage. Interestingly, *ci* is repressed by Engrailed during pattern formation (Alexandre and Vincent, 2003). Lim3 functions with Islet to regulate differentiation and pathway selection of in a distinct class of motor neurons (Thor et al., 1999). Both *dGatac* and *Ets65A* are perhaps the most interesting candidate genes, since they are both expressed in the CNS but have relatively uncharacterized functions (Berkeley *Drosophila* Genome Project, unpublished) (Chen et al., 1992; Lin et al., 1995).

Finally, serotonergic neurons in the vertebrate brainstem are organized in spatially discrete nuclei, and send diffuse axonal projections throughout the CNS. Although serotonergic axons are thought to rely predominantly on pre-existing tracts for pathfinding, it is possible that axon guidance signaling mechanisms additionally play a role in differentiation. Unlike the insect, most serotonergic axons in the vertebrate appear to maintain an ipsilateral trajectory. However, a study of ascending projections from the dorsal raphe has shown that a sizeable percentage of serotonergic projections are in fact contralateral (Jacobs and Azmitia, 1992). During development, neurons in bilateral nuclei extend projections across the midline in order to maintain interconnectedness between the nuclei, and neurons in the caudal NRM nucleus are observed extending processes across the midline (Jacobs and Azmitia, 1992). Additionally, vertebrate

serotonergic neurons must properly migrate through the early developing brain to reach their final positions. As development proceeds, cells further migrate both medially and laterally from the midline to form distinct nuclei. Interestingly, different migration patterns are also associated with differences in the onset of differentiation, and may contribute to differences between the serotonergic neurons observed in the adult brain, such as with co-localization of other neurotransmitters (Jacobs and Azmitia, 1992). A preliminary role for axon guidance molecules in cell body positioning has been observed in *Drosophila* (J.A.C. and B.G.C., unpublished observations) suggesting that these proteins may also play a role in cell migration, although this possibility awaits future study.

FUTURE DIRECTIONS

Many questions regarding the molecular mechanisms of serotonergic differentiation remain. To further understand the specific function of critical regulators such as *eg* and *islet*, their transcriptional targets need to be identified. Knowing the transcriptional targets of these genes would be highly informative not only to elucidate their precise roles in differentiation, but might also unveil potential mechanisms for their relationship to axon guidance. An analysis of promotor binding sites in serotonin specific genes such as SerT, Tph (*Henna*), and Ddc might also prove informative to this end. Similar analysis of the human SerT promoter has identified a number of canonical binding sites for transcription factors such as AP1 and Elk1, a human Ets transcription factor (Flattem and Blakely, 2000).

Additionally, although some genes, such as *seven-up* and *Drifter*, are expressed in the serotonergic neurons during development, their functions have not been directly assessed through genetic analysis. Of those genes with previously characterized functions in the serotonergic lineage, it remains unclear how they specifically interact with each other to control differentiation. Several lines of evidence suggest that specification of the unique serotonergic phenotype requires a combinatorial code of transcription factors (Dittrich et al., 1997; Lundell et al., 1996; Thor and Thomas, 1997). Future genetic analysis combining various loss of function and mis-expression mutants should reveal the nature of this code. Further, some elements of serotonergic regulation are also likely to be missing from the current model. For example, both *eg* and *islet* are required for serotonin synthesis as well as SerT expression, although SerT appears earlier in development. What factors specifically activate SerT expression before serotonin synthesis, and what is the functional advantage of such differential expression? Novel regulators of differentiation could be identified in a large-scale screen that examines SerT or serotonin expression over mapped chromosomal deficiencies.

Another important future direction lies in uncovering the function of the serotonergic system in the whole organism, and assessing how disturbances during development might affect behavior. The fly is a good model in which to pursue such studies, as the relatively simple serotonergic system can be easily manipulated through genetics. Effects of serotonin on behavior in the fly have been previously observed, and might serve as a useful starting point; for example, Eg mutants exhibit decreased locomotor activity (Lundell and Hirsh,

1998). Finally, this review has been limited to serotonergic development in the VNC, and data suggest that serotonergic maturation in the fly brain is controlled by other factors. Mutations in *en*, *hkb*, *eg*, or *islet* do not appear to disrupt differentiation in the brain (Lundell et al., 1996; Lundell and Hirsh, 1998; Thor and Thomas, 1997). Precedence for alternate mechanisms of differentiation among serotonergic neurons comes from *C. elegans*, where UNC-86 controls expression of tph-1 (tryptophan hydroxylase) and cat-1 (the vesicular monoamine transporter) in only a subset of serotonergic neurons (Sze et al., 2002). However, few studies have examined the factors controlling brain serotonergic differentiation.

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FIGURE LEGEND

Model for serotonergic differentiation. Positive regulators of differentiation are indicated in green and negative regulators in red. Those genes with recently identified roles in serotonergic differentiation (described in previous chapters) are highlighted. Factors with uncharacterized but proposed functions in serotonergic differentiation are shown in balck with gray arrows. Notable exclusions from this model include members of the Wingless signaling pathway *patched* and *gooseberry*, as well as the orphan steroid hormone receptor *seven-up*, as it remains unclear how these genes specifically contribute to serotonergic development. Additionally, the list of factors required for proper neuroblast specification is not exhaustive.

FIGURE 1

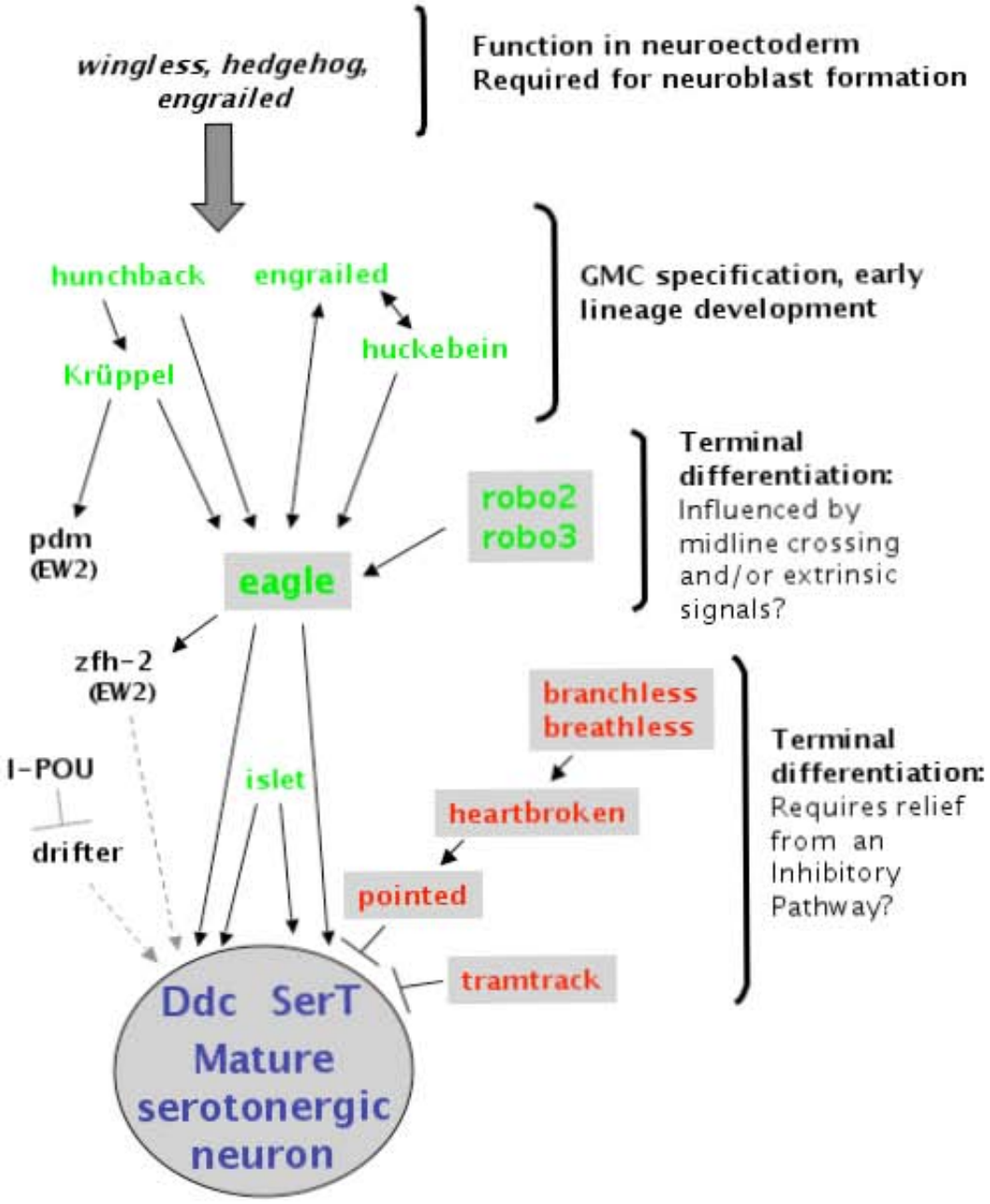
wingless, hedgehog, engrailed
Function in neuroectoderm
Required for neuroblast formation
hunchback
engrailed
huckebein
Krüppel
GMC specification, early lineage development
pdm (EW2)
robo2
robo3
eagle
Terminal differentiation: Influenced by midline crossing and/or extrinsic signals?
zfh-2 (EW2)
branchless
breathless
I-POU
islet
heartbroken
drifter
pointed
tramtrack
Terminal differentiation: Requires relief from an Inhibitory Pathway?
Ddc SerT
Mature serotonergic neuron

TABLE 1

Gene	Abbrev./ Synonyms	Molecular type	Serotonergic function	Vertebrate homolog	Vertebrate function
engrailed	*en*	Segment polarity homeobox transcription factor	NB 7-3 specification, Lineage development and differentiation	Engrailed-1, Engrailed-2	Embryonic brain morphogenesis
wingless	*wg*	Secreted segment polarity growth factor/ Wnt protein	NB 7-3 specification	Wnt family Wnt-1	Embryonic brain morphogenesis
hedgehog	*hh*	Secreted segment polarity protein	NB 7-3 specification	Sonic hedgehog	Embryonic patterning
huckebein	*hkb*	Zinc finger/ gap protein	Lineage development and differentiation	Undetermined	---
hunchback	*hb*	Zinc finger/ segmentation and gap protein	Lineage development (expressed in GMC1 and EW1 only)/differentiation	Undetermined	---
Kruppel	*Kr*	Zinc finger/ gap protein	Lineage development/differentiation	Undetermined	---
pdm	*pdm1:pou19 pdm2:pou28 nubbin*	POU transcription factor	Unknown	Pou2f3, Pou2f1	Unknown
eagle	*eg*	Zinc-finger transcription factor/Orphan steroid hormone receptor	Required for SerT/Ddc expression and correct axon guidance	Rev-erb-β?	Unknown
zfh2	*zfh2*	Zinc finger transcription factor	Known to bind Ddc promoter in 5HT neurons	Zfh-4	Unknown, but expressed in developing midbrain and hindbrain
islet	*isl*	LIM homeodomain transcription factor	Required for SerT expression, 5HT synthesis, and correct axon guidance	Islet	Neuronal specification and survival
drifter	*dfr/Cf1a/vvl*	POU transcription factor	Unknown; binds Ddc promoter in DA neurons	Pou3f2/BRN-2; Pou5f1/Oct-3	Production and migration of neocortical cells; Neuroectoderm formation/ differentiation of ES cells

Gene	Abbrev./ Synonyms	Molecular type	Serotonergic function	Vertebrate homolog	Vertebrate function
I-POU	*lpou/acj6*	POU transcription factor	Unknown; Can inhibit Dfr binding to Ddc	Pou4f1/BRN-3; UNC-86 (*C. elegans*)	Axon guidance/differentiation of sensory ganglia; Serotonergic differentiation (*C.elegans*)
roundabout2	*robo2*	Axon guidance receptor	Required for SerT/serotonin synthesis and Eg expression	Robo2	Axon guidance
pointed	*pnt*	Ets transcription factor	Can inhibit SerT and Ddc expression	c-Ets-1	Proto-oncoprotein, unknown nervous system function
tramtrack	*ttk*	BTB/POZ transcription factor	Prevents precocious SerT expression	Undetermined	---
heartbroken	*hbr/stumps/ Dof*	Downstream of FGF signaling	Prevents precocious SerT expression	Undetermined	---
breathless	*btl*	FGF-R	Prevents precocious SerT expression	FGFR-2	Embryo morphogenesis
branchless	*bnl*	FGF	Prevents precocious SerT expression	FGF20, FGF10	Embryo morphogenesis

Vertebrate Gene	Molecular type	Serotonergic function	*Drosophila* homolog	*Drosophila* function
Nkx2.2	Homeobox transcription factor	Early specification, required for formation of 5HT neurons except in dorsal raphe nuclei, Expression of *Lmx1b*	*ventral nervous system defective (vnd)*	Controls neuronal identities in VNC (ventral to dorsal patterning)
Gli1	Zinc finger transcription factor	Development of some 5HT neurons	*cubittus interruptus*	Segment polarity/ Wingless signaling pathway
Lmx1b	LIM homeodomain transcription factor	Required for serotonergic differentiation (SerT/5HT expression), Upstream of *Pet-1* and *Gata3*	*lim3*	Motor neuron specification and axon guidance
Gata3	Zinc finger transcription factor	Development of caudal 5HT neurons	*dGatac*	Unknown
Pet-1	Ets transcription factor	Expressed in both rostral and caudal 5HT neurons, Required for expression of SerT/Tph/VMAT in most 5HT neurons	*Ets65A*	Unknown

APPENDIX

AXON GUIDANCE: COMM HITHER, ROBO

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In the developing CNS, some axons cross the midline and some do not. The question is how are these guidance decisions made? Generally, attractants and repellants produced by midline cells direct this critical guidance decision. Commissural axons are initially attracted to the midline, but acquire sensitivity to midline repellent cues after crossing. This new sensitivity prevents re-crossing. Axons that never cross the midline, on the other hand, react to midline repellents from the outset. Thus, growth cones cross the midline only in the absence of a repulsive response.

A large-scale screen for axon guidance perturbations identified both *roundabout* (*robo*) and *commissureless* (*comm*) in *Drosophila* almost a decade ago [1]. Since then, genetic studies consistently demonstrate that the relative levels of Robo and Comm control axon behavior at the midline. The Robo receptor, activated by its ligand Slit, initiates the repulsive response that prevents axons from inappropriately crossing the midline [2, 3]. Conversely, Comm encourages midline crossing by downregulating Robo and effectively suppressing Robo-induced midline repulsion [4].

Early analysis of Comm expression patterns indicated that Comm was only expressed in midline glia, suggesting that a transfer of protein to axons was necessary for Comm function [5]. New data, however, reveal that Comm is actually expressed in commissural neurons as well, and functions autonomously to mediate guidance decisions. Using RNA interference techniques, Georgiou and Tear recently suggested an autonomous function for Comm [6]. A

compelling series of transplantation experiments by Dickson and colleagues now confirm this result [7].

But how does Comm physically control Robo levels? Important new papers from the Dickson and Tear labs offer an explanation [7, 8]. Both groups reveal that Robo is regulated through intracellular trafficking and show strong evidence that (1) Comm acts as a sorting protein that directs Robo away from the cell surface and targets Robo for internalization, presumably to endosomes, and (2) the cytoplasmic domain of Comm contains an LPSY motif that is critical for this function. Together, the Tear and Dickson papers offer substantial but unique insights into the mechanism of Comm function.

Dickson and colleagues confirm previous results indicating that Comm is normally observed in intracellular vesicles, and that the cytoplasmic domain of Comm is required for the downregulation of Robo both *in vitro* and *in vivo* [4, 5]. They further show that Comm binds to Robo directly, and that this Comm-Robo interaction allows Comm to regulate the sub-cellular distribution of Robo. Robo is prevented from reaching the plasma membrane and is instead found co-localized with Comm in endosomes. Thus, as axons cross the midline, Comm regulates the surface levels of Robo and thereby prevents premature repulsion from this intermediate target.

Tear and colleagues also show that Comm acts as a sorting protein that recruits Robo for internalization [8]. Ubiquitination of Comm by the ubiquitin ligase DNedd4 is required for proper sorting of Comm away from the cell surface, and additionally, an LPSY motif in the cytoplasmic domain of Comm is

necessary for DNedd4 binding. This motif is required for internalization [7]. Comm is able to bind both DNedd4 and Robo *in vitro*, and this may link Robo to the ubiquitination pathway. An *in vivo* function for DNedd4 in axon guidance was confirmed through over-expression and RNA interference.

A clear model for Robo regulation emerges from the results presented by Dickson and Tear. Prior to arrival at the cell surface, Robo is sequestered by Comm, which either becomes or is already ubiquitinated by DNedd4. The Robo-Comm complex is sent to endosomes for destruction before repulsive signaling can be initiated, and the growth cone maintains its trajectory across the midline. This model is new but not surprising, as Comm was shown previously to play a role in endocytosis of surface proteins during synaptogenesis [9].

Degradation of proteins during development has become a recognized means of regulating critical signaling events. Levels of other guidance molecules, such as the netrin receptor DCC, are also regulated through ubiquitination [10]. Recently, the protein Numb was shown to mediate the internalization and potential ubiquitination of Notch [11]. Numb also plays a role in polarizing the distribution of Notch on the cell surface. Such a polarizing function could also be operating in selectively distributing Robo along axons to impart localized signaling capabilities.

If Comm determines whether or not axons cross the midline, then what turns *comm* on in commissural neurons at the correct time? Dickson and colleagues suggest that control of Comm expression occurs at the cell body. Although this is an appealing idea, it seems also possible that local processing in

the growth cone may be responsible for changes in Comm expression. Important new work has shown that severed growth cones are capable of local protein synthesis, trafficking, and degradation, and respond appropriately to guidance signals including netrin [12, 13].

Growth cones, like dendritic spines, may be equipped with compartmentalized molecular machinery necessary to specifically regulate gene expression in different processes of a single axon. Such local processing would confer an ability to respond rapidly to changes in guidance cues, and would allow secondary axonal branches to develop independently. In fact, filopodia appear to respond individually to midline guidance cues [14]. In an elegant experiment, Murray and Whitington examined midline crossing in both wild type and *robo* backgrounds. Their results indicated that Robo influences the dynamics of neuronal processes rather than directing their initial outgrowth. Unfortunately, these critical cellular descriptions of midline guidance [14] remain disjointed from the molecular explanations.

We now know that Robo levels at the cell surface are controlled by Comm through intracellular trafficking, and that this process is responsible for directing axon behavior at the midline. However, we are still left with many questions. What is the function of Comm in midline cells? How are different members of both the Robo and Comm families regulated by each other, if at all? It will also be interesting to see whether the sorting of Robo by Comm influences growth cone responsiveness to other guidance signals. A physical interaction between DCC and Robo has been shown in vertebrates to silence attraction to netrin and

thus prevent lingering at the midline [15]. Does a similar interaction occur in *Drosophila*, and if so, how does the Fra-Robo complex interact with Comm? In addition, very little is known about how the cell biology of midline axon guidance is related to these molecular interactions. A combination of in vivo growth cone studies coupled with detailed molecular analyses will likely reveal many of the secrets of this complex process.

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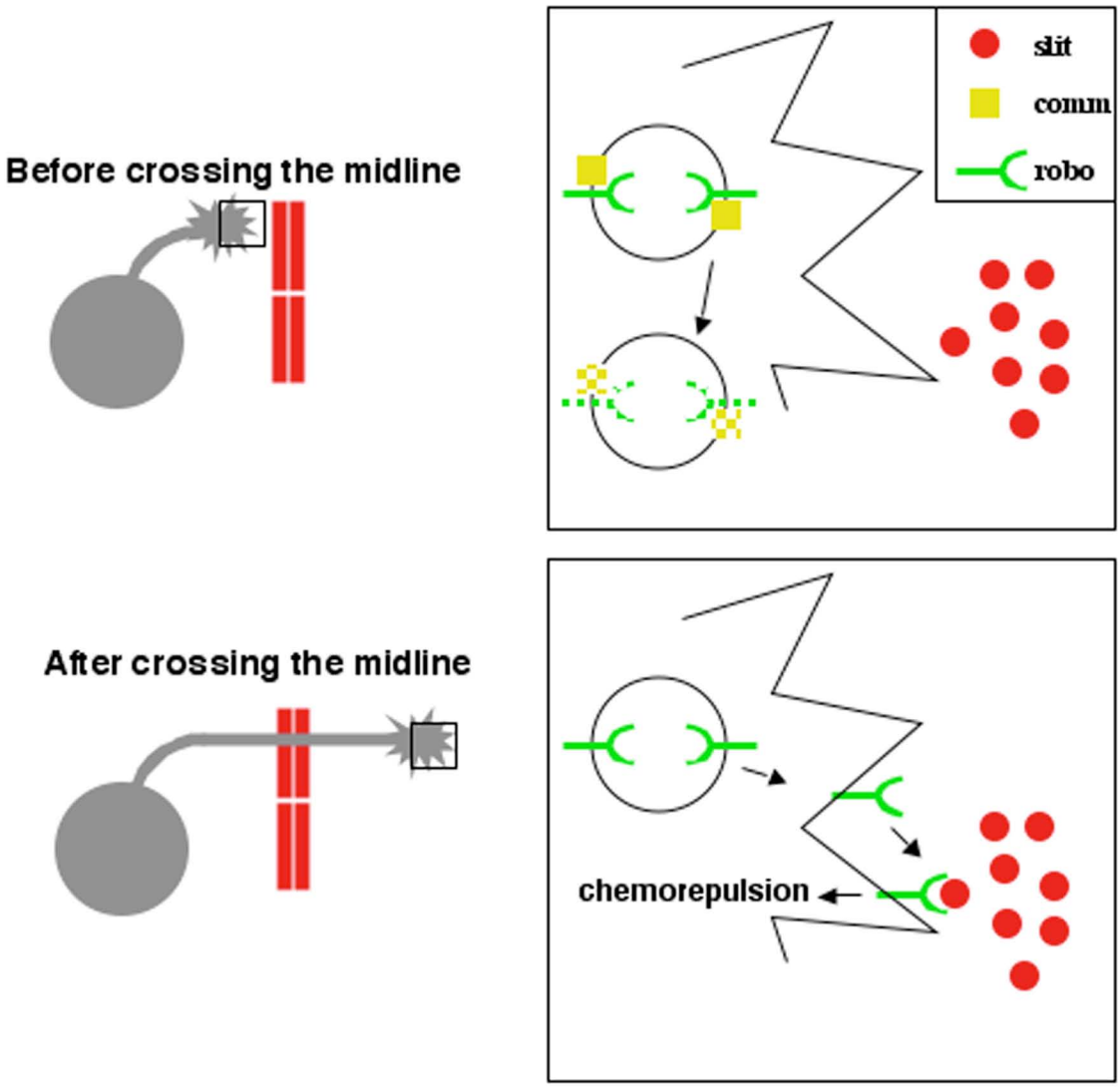
FIGURE LEGEND

Before crossing the midline, Robo is sequestered by Comm, sent to endosomes, and degraded. Thus, Robo does not reach the cell surface and is unable to bind its ligand Slit, allowing axons to continue their trajectories. After crossing the midline, Comm turns off, and Robo is able to initiate repulsive signaling that prevents axons from re-crossing.

FIGURE


Before crossing the midline
slit
comm
robo
After crossing the midline
chemorepulsion