# *robo2* and *robo3* interact with *eagle* to regulate serotonergic neuron differentiation

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## Summary

The function of the central nervous system (CNS) depends crucially 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 neuron 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 neuron differentiation and function independently of their ligand, *slit*. 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 neuron differentiation and is transduced by the transcription factor Eg.

Key words: Serotonin, Axon guidance, Midline, Eagle, Robo, Transporter

## Introduction

Serotonin (5-hydroxytryptamine or 5-HT) 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 neuron 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 neuron differentiation (Hendricks et al., 2003). Despite the importance of serotonergic neuron function in the CNS, the mechanisms controlling serotonergic neuron differentiation remain largely unknown. We have used *Drosophila* to investigate the development of the serotonergic neuron system, which has been highly conserved throughout evolution (Jacobs and Azmitia, 1992). *Drosophila* is useful as a model system for investigating serotonergic neuron development because 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 neuron function and one of the earliest steps in serotonergic neuron differentiation. A highly conserved serotonin transporter, *SerT*, 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 (this study) and grasshopper (Condron, 1999), as well as synapse formation in mouse (Bruning et al., 1997).

In grasshopper, cuts that sever serotonergic neuron contact with the midline lead to a loss of SerT expression, suggesting that the midline plays a crucial 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 neuron differentiation. In mouse and rat, specification of serotonergic neurons also requires FGF signaling (Ye et al., 1998). Axon guidance molecules are also likely to play an important role in serotonergic neuron development, as these 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 neuronal axon guidance and differentiation come from data showing that a loss of function

in genes of the serotonin biosynthetic pathway impairs axon pathfinding and alters branching patterns in the periphery (Budnik et al., 1989).

We use SerT activity, measured by reuptake of serotonin, as a marker of serotonergic neuron differentiation. SerT serves as an early and easily testable step in serotonergic neuron differentiation, because it precedes the onset of serotonin synthesis. 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* (*lea* – FlyBase) and *robo3* (*robo2/3*), but not *robo*, are required for serotonergic neuron 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 neuron 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 subset of serotonergic neurons according to the severity of the mutation (Lundell and Hirsh, 1998). 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* loss-of-function 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[Δe39]*) (Tear et al., 1996); *slit* null (*slit[2]*) (Kidd et al., 1999); *robo* null (*robo[GA285]*) and 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 and 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 Fig. 5 were taken with an Olympus 40× or 20× 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 Fig. 5 were imaged with a Nikon Eclipse E800 microscope (100× 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, 2×2 binning and with an exposure time of 500 mseconds. 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 *SerT* in situ hybridization, a previously reported cDNA was used (Demchyshyn et al., 1994). DNA was cut with *Xba*I, 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, 10× 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 neuron differentiation in the fruit fly is also related to midline crossing, we assayed *SerT* mRNA and SerT enzymatic activity at various embryonic stages of development. *SerT* 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 neuron differentiation, and is expressed in relatively few cells in the CNS (Higashijima et al., 1996; Lundell and Hirsh, 1998). Fig. 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
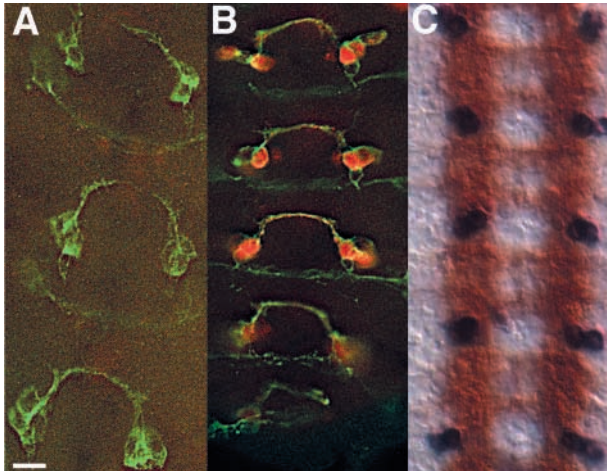
**Fig. 1.** The onset of SerT expression is correlated with axon guidance across the midline. (A,B) Staining for serotonin uptake in red and β-galactosidase in green (UAS-tau-*lacZ* driven by *eg*Gal4). *eg*Gal4 expression of *lacZ* is seen in all four progeny of NB 7-3, which includes the serotonergic neurons, two other neurons and 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 (11:20-13:00 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: 10 μm.

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 *SerT* 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, *SerT* 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, *SerT* 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 neuron 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 and *robo2* gain of function (Fig. 2), as well as *spitz* (*spi* – FlyBase) and *single-minded* (*sim*) (data not shown). Specific overexpression of Robo or Robo2 in the serotonergic
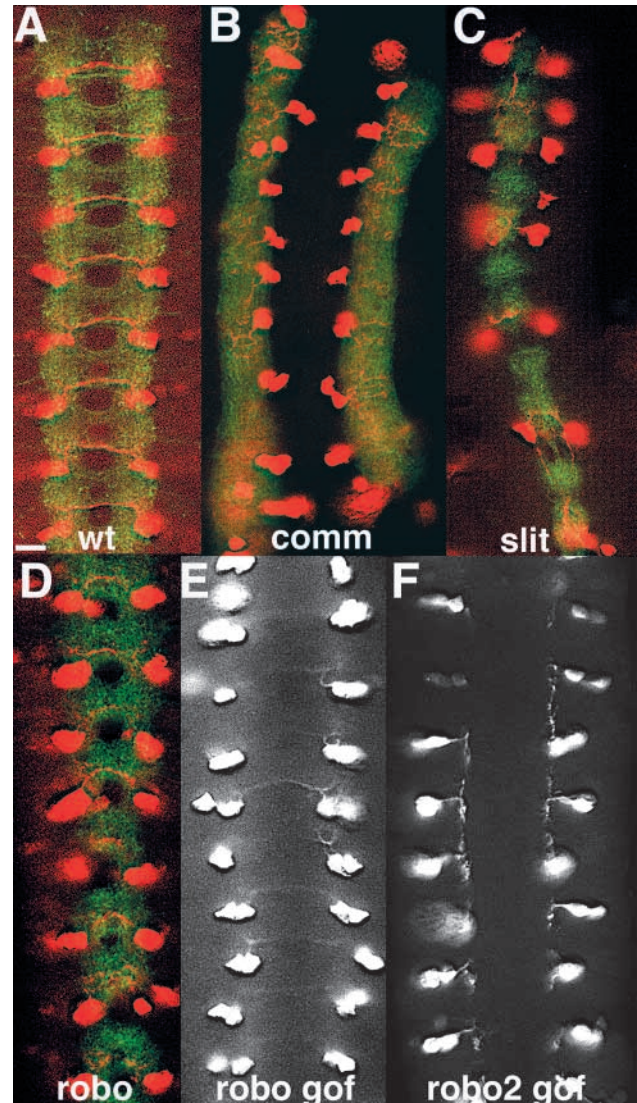


**Fig. 2.** Initial screen of midline axon guidance mutants did not reveal a loss of SerT expression. (A-F) stage 16 ventral nerve cords are stained for serotonin uptake in red (A-D) or in white (E,F). (A-D) show the axon scaffold stained with mAb BP102 in green. (A) The wild-type CNS is organized into 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*$^{\Delta e39}$, (C) s*lit*$^2$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 in *robo2*, but not *robo*, prevents the crossing of the midline by serotonergic neurons. Despite defects in serotonergic neuronal axon guidance seen in these embryos, SerT activity remains normal. Scale bar: 10 μm.

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 mutants for *robo* and *slit* (*sli* – FlyBase; the ligand for *robo*) were also analyzed.

In a *comm* loss-of-function mutant, all axons of the CNS fail to cross the midline, as seen in Fig. 2B with the axonal marker BP102 (green). Overexpression of Robo2, but not Robo, prevents serotonergic axons from crossing the midline (Fig. 2E,F). Conversely, *robo* loss of function causes ectopic midline crossing of most CNS axons and a *slit* loss of function 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* loss-of-function mutant, we observe disorganized and inappropriate serotonergic neuron branching towards 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. By 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 gain-of-function mutants, for example, is that midline crossing is not crucial 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 overexpressing 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 transcription factor, *engrailed* (green, Fig. 3A,B). In a *robo2* gain-of-function 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 gain-of-function
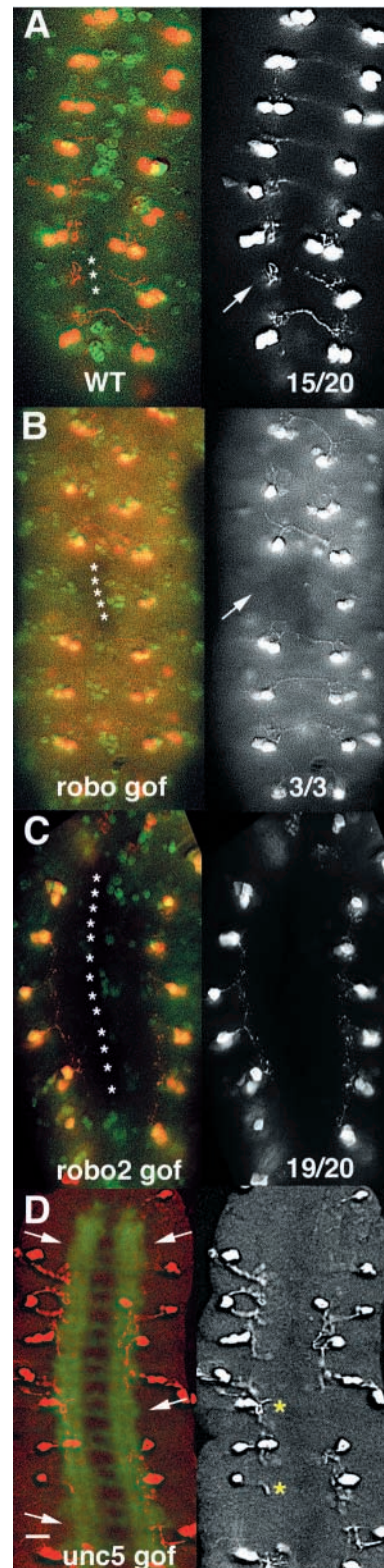


**Fig. 3.** Physical or genetic disruption of midline contact affects SerT activity in wild type, Robo gain-of-function and Unc5 gain-of-function (but not Robo2 gain-of-function) mutants. (A-C) Staining of ventral nerve cords for serotonin uptake (red in left panel and white in right) and for *engrailed* (green in left panel). A cut (line of stars) 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 out of 20 wild-type preparations, SerT activity was lost from the cell bodies on the cut side (arrow). (B) Similar cuts in embryos overexpressing Robo in serotonergic neurons (Robo gain of function; UAS-*robo* with *eagle*Gal4) also showed a loss of SerT activity in the midline-minus side (arrow; three out of three). (C) However, when Robo2 was overexpressed (*eagle*Gal4), only one out of 20 preparations showed a loss of SerT activity after a midline cut. (D) Expression of Unc5 with *eagle*Gal4 specifically prevents the crossing of the midline by serotonergic neurons 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 yellow stars (right panel) indicate axons that extends towards the midline despite expression of Unc5. Scale bar: 10 μm.

VNCs, because serotonergic axons project across the midline in these mutants but remain ipsilateral in a Robo2 gain of function. 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 midline crossing by serotonergic neurons 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, see Fig. 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 gain of function 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 gain-of-function and *robo2* gain-of-function mutants, where (in both cases) axons are prevented from crossing the midline but only Unc5 gain of function disrupts SerT expression (compare Fig. 3D with Fig. 2F). Thus, overexpression 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. Overexpression 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 probably 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 *SerT* 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 *SerT* mRNA or activity at stage 16 (Fig. 4A,D; see 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; see 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 neuron 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 neuron differentiation is *slit* independent.
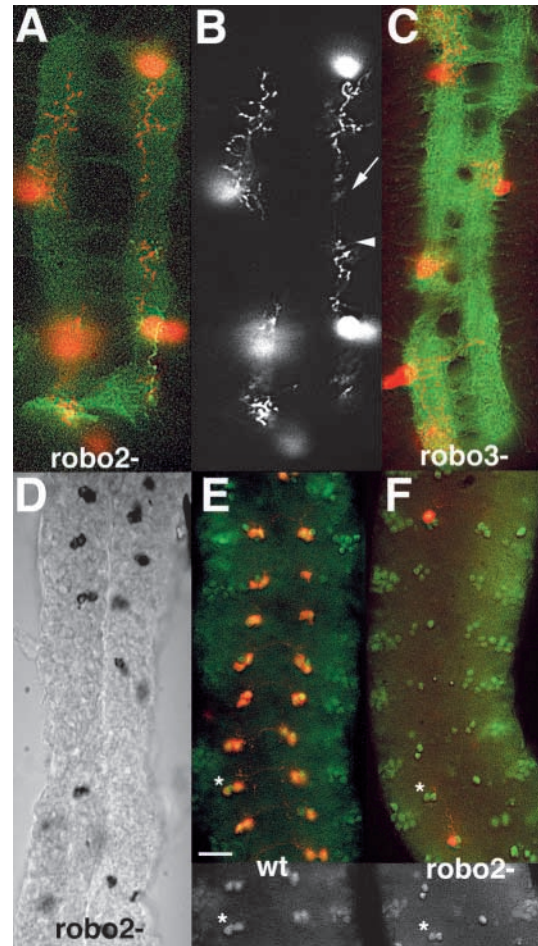


**Fig. 4.** *robo2* and *robo3* are required for normal SerT expression. (A-C) Stage 16 ventral nerve cords from *robo2*[x123] (A,B) and *robo3*[1] (C) loss-of-function mutant embryos stained for serotonin uptake in red (A,C) and white (B). The slightly disorganized axon tracts of the mutant CNS are labeled in green with mAb BP102. *robo2* and *robo3* loss-of-function 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: 20 μm for D-F; 10 μm for A-C.

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.

Because *robo2* gain of function permits maintenance of SerT activity following a midline cut, it seems likely that Robo2, at least, 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 *sim* do not cause defects in serotonergic neuron 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 neuron 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 neuron 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 (see 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 examine the relationship between *robo2/3* and *eg* further, *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 ~30% of VNC hemisegments (Fig. 5B, see Fig. 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, as both *eg* hypomorphs and *robo2* loss-of-function mutants show a loss of SerT activity in only a percentage of neurons (see Fig. 7), a *robo2* loss of function/*eg* hypomorph double mutant line was analyzed for any increases in phenotype severity. There is not an additive effect of *robo2* loss of function and *eg* hypomorph in the double mutant (see Fig. 7). These data provide further evidence that Robo2 functionally cooperates with Eg, because 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 mediolateral position of the serotonergic cell bodies. This is most markedly seen in the *robo2* loss-of-function mutant hemisegment (Fig. 5C), where the cell bodies are positioned close to the midline, and in the Robo2 gain-of-function hemisegment (Fig. 5D), where the cell bodies are shifted laterally. A similar positional shift is observed in the Unc5 gain-of-function (Fig. 3D) and *comm* mutants (Fig. 2B).
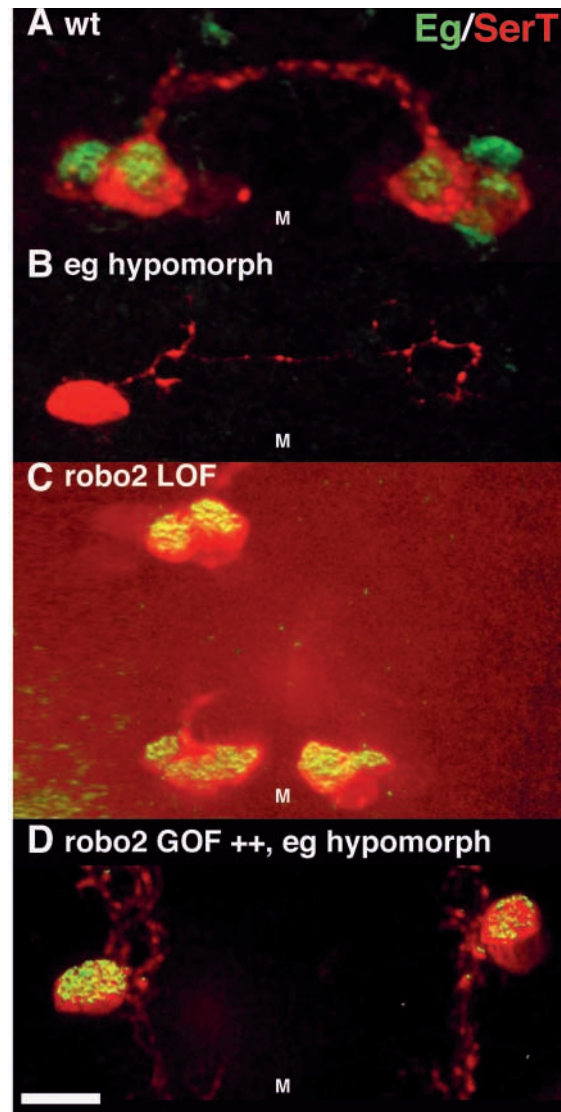


**Fig. 5.** *robo2* mutants lose *eagle* expression. Stage 16 wild-type (A), $eg^{Mz360}$ hypomorph (B), *robo2* loss-of-function (C) and (EP2582/EP2582; $eg^{Mz360}/eg^{Mz360}$) (D) nerve cords. Serotonin uptake, red; Eg, green; yellow, co-localization. M indicates the position of the ventral midline.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

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

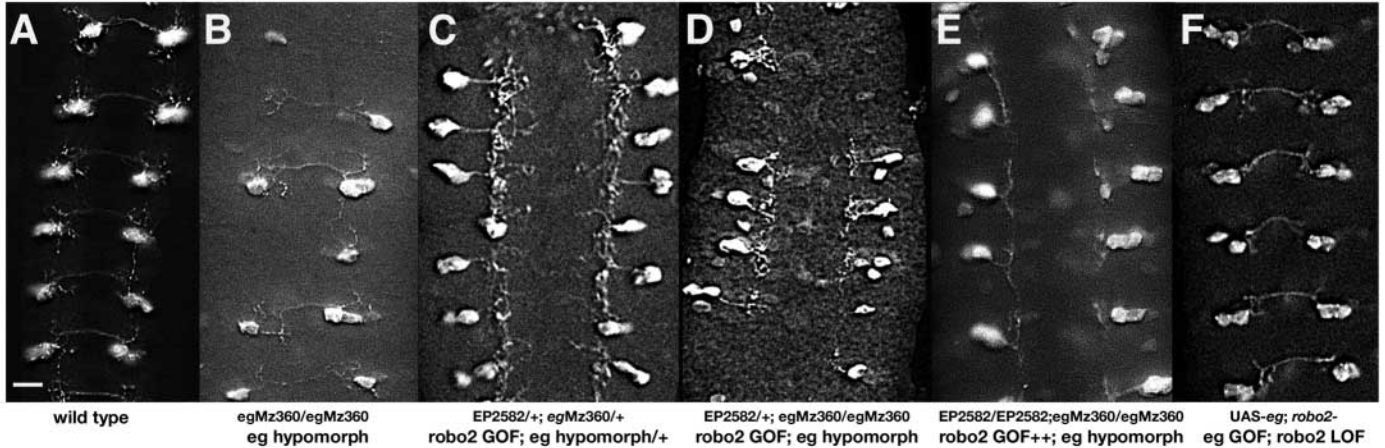To investigate the interaction between Robo2 and Eg,

**Fig. 6.** Overexpression of Robo2 rescues an *eagle* hypomorphic mutation and overexpression 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 ~30% loss of SerT-positive cells as well as axon guidance defects. (C) Expression of one copy of Robo2 (EP2582, Robo gain of function) 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 gain of function++) 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 with Fig. 4B). Scale bar: 10 µm.

combinations of mutations at both loci were examined. Comparison of SerT expression in VNCs with varying levels of Robo2 and Eg reveals a dose-sensitive interaction between the two genes (Fig. 6B-E). Overexpression of one copy of EP2582 (Robo2) with one copy of the *eg*Gal4 driver prevents the midline crossing of serotonergic neurons but does not disrupt SerT expression (Fig. 6C). If one copy of EP2582 (Robo2 gain of function) 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 gain of function++) 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 overexpression 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 gain of function (Fig. 5D, Fig. 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). These results can be seen quantitatively in Fig. 7.

Further evidence linking *robo2* and *eg* comes from converse rescue experiments showing that overexpression 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 (compare Fig. 6F with Fig. 4B). As Eg is expressed in only a small subset of neurons,
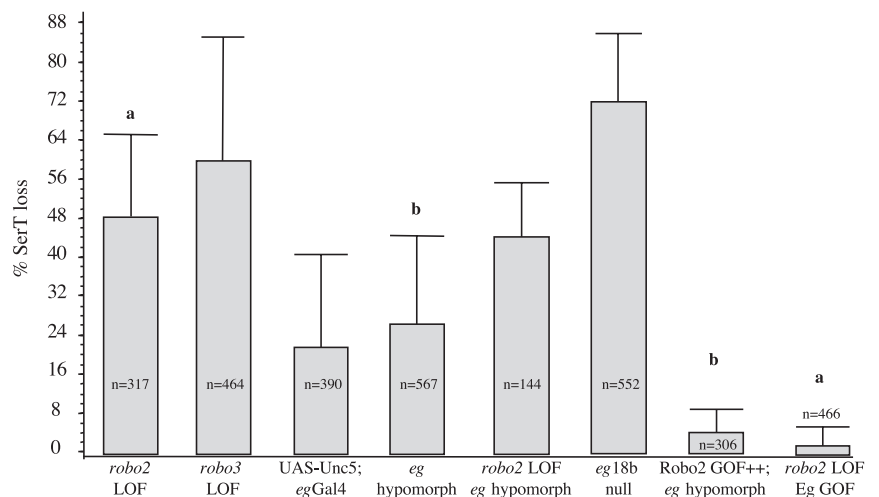


**Fig. 7.** Average loss of SerT activity between genotypes. Error bars represent the standard deviation of the mean. Genotypes were scored for the percent of hemisegments negative for SerT activity/total hemisegments per VNC. n represents the number of total hemisegments scored. At least 10 cords were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

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 overexpressed 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 neuron differentiation, as a loss of *robo2/3* function causes a loss of SerT expression in ~50% of neurons. A *robo2/3* loss of function closely resembles an *eg* mutant phenotype. Finally, our data show a dose-sensitive relationship between Robo2/3, Eg and SerT expression, suggesting that they function in the same genetic pathway to control serotonergic neuron 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 neuron 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 neuron 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 crucial 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.C., M. Levin, E.M.U. and B.G.C., unpublished).

One problem with interpreting the role for the midline is the lack of an abnormal serotonergic phenotype in mutants for the master regulatory gene *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 neuron differentiation, it is likely to be more complicated than a simple switch acting to induce differentiation.

### *robo2* and *robo3* play a role in serotonergic neuron 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 neuron differentiation. A positive role for Robo2 is further supported by our results showing that overexpression 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 indirect 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 neuron differentiation at this time, several lines of evidence suggest a more direct role. Our data shows that overexpression of Robo2 not only spares SerT loss following a midline cut but also rescues an *eg* hypomorph, and furthermore, that an Eg gain of function rescues a *robo2* loss of function; 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 neuron branching does not correspond simply to any Fas2 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, as *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 neuron 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.C. and B.G.C., unpublished) (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 to be due to a lengthy persistence of *lacZ* rather than continued expression of *eg*, as *eg* mRNA is not detectable by in situ hybridization after stage 14 (Higashijima et al., 1996). Most probably, *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 neuron 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 and midline crossing, where all three Robo receptors have separable functions (Godenschwege et al., 2002; Rajagopalan et al., 2000a; Simpson et al., 2000b). Furthermore, 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 neuron differentiation. Additionally, a loss of *slit*, the ligand for all three Robo receptors, does not perturb SerT expression, indicating that either another ligand exists or the function of Robo2/3 in serotonergic neuron 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 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 neuron 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 overexpression of Robo2/3 results in precocious serotonergic neuron differentiation, indicating a requirement for other signals.

### *robo2* functions with the transcription factor *eagle* to regulate serotonergic neuron differentiation

In both a *robo2* and a *robo3* loss-of-function mutant, expression of the zinc-finger transcription factor *eg* is lost in the same cells that lose SerT expression. Additionally, overexpression of Robo2 rescues the loss of SerT observed in an *eg* hypomorph in a dose-sensitive manner. Finally, Eg gain of function 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 neuron 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 neuron 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* loss-of-function mutants only a percentage of neurons lose SerT expression (and serotonin synthesis), indicating the presence of a redundant mechanism for serotonergic neuron 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 differentiate properly. 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 ~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 properties (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 neuron 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 neuron 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 overexpressed, 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 probably reveal how Robo2/3 regulate not only Eg but eventually serotonergic neuron differentiation as well.

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