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Lola regulates midline crossing of CNS axons in *Drosophila*

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SUMMARY

The pattern and level of expression of axon guidance proteins must be choreographed with exquisite precision for the nervous system to develop its proper connectivity. Previous work has shown that the transcription factor Lola is required for central nervous system (CNS) axons of *Drosophila* to extend longitudinally. We show here that Lola is simultaneously required to repel these same longitudinal axons away from the midline, and that it acts, in part, by augmenting the expression both of the midline repellant, Slit, and of its axonal receptor, Robo. Lola is thus the examplar of a class of axon guidance molecules that control axon patterning by coordinating the regulation of multiple, independent guidance genes, ensuring that they are co-expressed at the correct time, place and relative level.

Key words: Axon guidance, Transcription regulation, Slit, Robo, Quantitative immunofluorescence, *Drosophila*

INTRODUCTION

In each hemisegment of the developing *Drosophila* ventral nerve cord, the axons of ~20% of the 350 or so embryonic interneurons grow longitudinally on the same side of the midline where their cell bodies lie; they never cross the midline of the CNS (Schmid et al., 1999). In part, this pattern of axon growth reflects the existence of molecular forces that repel axons away from the midline (Klambt et al., 1991; Seeger et al., 1993; Kidd et al., 1998b), and other forces that attract axons to the developing longitudinal axon tracts (Giniger et al., 1993; Giniger, 1998). Midline repulsion alone cannot explain CNS axon pattern, however, as ~70% of interneurons do cross the midline – once – to project in the contralateral longitudinal tract (Schmid et al., 1999). Evidently, the trajectory of a CNS axon reflects a delicate balance of repulsion from and attraction toward the midline, as well as attraction to the longitudinal tracts (Seeger et al., 1993; Hummel et al., 1999a).

In recent years, a great deal has been learned about the extracellular molecules that provide some of these competing signals to CNS axons, and about the growth cone receptors that read and interpret those signals. Thus, the secreted protein Slit is made by cells of the midline glia (Rothberg et al., 1990) and repels susceptible axons away from the midline (Kidd et al., 1999). Growth cones recognize and respond to Slit because they have on their surface a family of receptors related to the protein Roundabout (Robo), a repulsive guidance receptor that binds and is activated by Slit (Kidd et al., 1999; Rajagopalan et al., 2000; Simpson et al., 2000). All CNS neurons express Robo, but its activity is modulated post-translationally by the transmembrane protein Commissureless (Comm) (Tear et al., 1996; Kidd et al., 1998b). Comm apparently removes Robo from the plasma membrane, perhaps by activating its endocytosis (Kidd et al., 1998b; Wolf et al., 1998). Comm thus allows particular axons to cross the midline by rendering them insensitive to Slit.

The complement of proteins on the surface of a growth cone, and thus the trajectory of that axon, depends upon the genetic program which specifies the identity of that neuron (Ghysen et al., 1985; Miller et al., 1992; Nottebohm et al., 1992; Jurata et al., 2000). For example, a combinatorial 'code' of Lim family homeodomain proteins determines particular motoneuron trajectories both in flies (Thor et al., 1999) and in vertebrates (Tsuchida et al., 1994). In this example, the nuclear control of axonal trajectory is intimately intertwined with the very definition of the identities of these neurons. It is not enough, however, for a particular spectrum of proteins to be present on the growth cone of a given neuron. Rather, the precise level (Winberg et al., 1998) and timing (Rose et al., 1997) of the expression of these proteins must also be coordinated exactly (Daston and Koester, 1996; Madden et al., 1999). At the *Drosophila* midline, the net effect of Robo, Slit and Comm depends on their relative levels of expression (Kidd et al., 1998b; Rajagopalan et al., 2000), and of their level of activity relative to that of the midline attractant(s) (Bashaw and Goodman, 1999). Thus, modest overexpression of Comm in a normally ipsilateral neuron can reduce Robo level – and consequently the sensitivity to Slit – to the point where the axon crosses the midline inappropriately (Kidd et al., 1998b). Similarly, simultaneous reduction of both Robo and Slit levels by just 50% suffices to cause widespread midline crossing (Battye et al., 1999; Kidd et al., 1999). Mechanisms must therefore exist that coordinate the programs of guidance gene expression to ensure that all of the many guidance proteins

required for a particular axonal decision are displayed at the right relative level.

One example of a transcription factor required for proper axon patterning is that encoded by the *Drosophila* gene *lola* (*longitudinals lacking*) (Seeger et al., 1993; Giniger et al., 1994; Cavarec et al., 1997). In the absence of Lola function, CNS axons fail to grow longitudinally, even though analysis of a wide variety of molecular markers in *lola* mutant embryos demonstrates that the neurons and their glial substratum cells are born, establish their appropriate identities and differentiate normally (Seeger et al., 1993; Giniger et al., 1994). It has therefore been suggested that *lola* might be responsible for the expression of some cell surface or signaling protein that is required in the growth cone for *lola*-dependent axon guidance decisions. This conjecture received support from detailed phenotypic analysis of a specific *lola*-dependent guidance event, development of the ISNb motonerve. In that system, the phenotypes observed upon varying the expression of a particular *lola* isoform were consistent with the notion that *lola* may regulate the expression of a number of the molecules that together control ISNb axon growth and guidance (Madden et al., 1999), though no specific downstream effectors of *lola* were identified in that study.

Lola encodes a large number of protein isoforms by alternative splicing (Giniger et al., 1994) (S. G., E. A. Greene and E. G., unpublished observations). All but one of the *lola* splice variants bears its own C-terminal exon that encodes a unique Zn-finger or pair of Zn fingers. For one Lola isoform it has been shown that the protein binds DNA *in vitro*, and that it can modulate transcription of a reporter gene that bears a Lola-binding site in its promoter (Cavarec et al., 1997). It appears that Lola can either activate or repress gene expression *in vivo*, depending on the cell type (Cavarec et al., 1997). This dual effect may reflect expression of different *lola* isoforms in different tissues, or else recruitment of different transcriptional co-activators and co-repressors in different contexts (Hong et al., 1997; Huynh and Bardwell, 1998). All Lola isoforms also include an N-terminal dimerization domain known as a BTB domain (or variously as a POZ domain) (Bardwell and Treisman, 1994; Ahmad et al., 1998). BTB domains can mediate both homo- and heterodimerization, but it is not yet known whether Lola forms heterodimers, either between various Lola isoforms or with other, related, BTB-containing proteins. The expression patterns of many *lola* isoforms have not yet been determined, but at least one is expressed preferentially in midline-derived cells (Giniger et al., 1994), while others are preferentially expressed in neurons (Giniger et al., 1994). An antibody that recognizes all Lola variants demonstrates that all CNS cells express substantial amounts of one or more Lola isoforms (Giniger et al., 1994).

We show that, in addition to its requirement to promote longitudinal growth of CNS axons, *lola* is also required to establish the proper pattern of midline crossing: in its absence, many axons cross the midline inappropriately. This phenotype arises, in part, because the levels of both Robo and Slit are reduced in *lola* mutant embryos, as we show by examination of Robo and Slit protein levels. Consistent with this finding, mutations in *lola* interact genetically with both *robo* and *slit* mutations, and ectopic expression of a midline-enriched Lola isoform causes ectopic expression of Slit. We suggest that Lola helps to establish the pattern of longitudinal projection of axons in the fly CNS by inhibiting growth across the midline while simultaneously promoting axon growth longitudinally.

MATERIALS AND METHODS

Drosophila stocks

$lola^{5D2}$, $lola^{1A4}$ (Giniger et al., 1994) and $lola^{cn03089}$ (Verheyen et al., 1996) are transposon insertion alleles; $lola^{ORB40}$, $lola^{ORC16}$, $lola^{0RC46}$, $lola^{ORE76}$ and $lola^{ORE120}$ are EMS-induced alleles. $lola^{5D2}$,$lola^{ORC46}$ and $lola^{ORE76}$ are strong alleles; $lola^{1A4}$, $lola^{cn03089}$, $lola^{ORB40}$ and $lola^{ORC16}$ are hypomorphic; $lola^{ORE120}$ is quite weak (though still lethal). $lola^{cn03089}$ was obtained from Esther Verheyen; all EMS alleles were from Mike Forte and Tom Schwarz. $slit^{IG107}$ is a null allele obtained from Roger Jacobs; $robo^{z14}$ is a strong allele obtained from Tom Kidd and Corey Goodman. Depending on the experiment, embryo genotypes were either inferred from the axonal phenotype, or assayed by staining with anti-Lola antibodies or by segregation of *lacZ*-marked balancer chromosome. *Ap-C-tau-lacZ* (Lundgren et al., 1995) was from John Thomas. *GAL4-112A* provides GAL4 activity at high levels throughout the embryonic neuroectoderm (Fuerstenberg and Giniger, 1998), and was used to express *lola* ectopically by crossing to a line carrying four copies of a UAS_G-*lola 4.7* transgene (Madden et al., 1999).

Embryo staining

Embryo fixation and staining for in situ hybridization, immunocytochemistry and immunofluorescence were performed by standard methods. Fluorescent samples were mounted in Fluorogard (BioRad); peroxidase and alkaline phosphatase samples were mounted in JB4 embedding medium (Polysciences). Antibodies (and their dilutions) were as follows: anti-Fasciclin II (mAb1D4; 1:300), anti-Wrapper (mAb10D3; 1:3), anti-Slit (mAb555.4; 1:50) and anti-Robo (mAb13C9; 1:25) from, respectively, Greg Helt, Jasprien Noordermeer, Tom Kidd and Corey Goodman; and affinity purified anti-Lola (1:100) (Giniger et al., 1994). Incubation of samples for Slit or Robo staining was performed using 0.1% Tween 20 as detergent rather than 0.3% Triton X-100. Rabbit anti-β-galactosidase (1:10,000) and rabbit anti-HRP (1:100) were from Cappel; anti-β-galactosidase was pre-absorbed before use and anti-HRP was affinity purified. Secondary antibodies were from Jackson ImmunoResearch; 'multiple label' grade secondaries were used for all experiments, and were pre-absorbed against fixed, wild-type embryos before use. Fluorescent secondaries were used at 1:250 dilution. Peroxidase histochemistry was performed using biotinylated secondaries (1:1500) and the Vectastain Elite tertiary reagent (Vector Labs) using DAB for color development. Peroxidase- and alkaline phosphatase-detected samples were examined on a Nikon Optiphot microscope and images captured with a CoolSnap CCD camera. Where necessary, focal planes were montaged in Photoshop.

Quantitative immunofluorescence

Embryos for quantitative analysis were derived from a brief (1 hour) embryo collection and staging of each individual was verified by embryo morphology. Confocal microscopy was performed with a Leica TCS confocal system, using a 40× oil-immersion objective without zoom. The microscope was warmed-up for more than 1 hour before collecting data. Several embryos were examined to set gain, offset and laser intensity such that CNS signals neither saturated nor fell into the bottom ~10% of the intensity range, and to verify absence of bleedthrough between channels. Machine settings were not varied after calibration, and calibration embryos were not included in datasets. All embryos in a dataset were stained together in a single tube, mounted on the same slide and imaged in a single confocal session. Linearity and intrinsic offset of the confocal detection system were verified in experiments systematically varying input laser

intensity and by quantitation of a fluorochrome dilution series. Image slices of the entire ventral nerve cord were collected at 1 μm spacing; two scans were averaged for each slice. Comparison of multiple complete image stacks of the same embryo verified that no significant bleaching of the fluorochromes occurred during collection of an image stack. Imaging of wild-type and mutant embryos was interspersed within a session. Occasional embryos were found to have anomalously high or low absolute signal intensities in one or (usually) both channels and were excluded from the analysis.

Confocal data were quantified in NIH Image 1.62. Typically, a summation Z-projection was first performed (as an average projection). The CNS was outlined manually on the projection (avoiding the HRP-labeled ring gland), the reference (anti-HRP) and experimental (anti-Robo) signals were integrated within the outline and the ratio of these signals was calculated. In one experiment, intensity ratios were also determined on CNS outlines section by section and these ratios then averaged; results were the same as those obtained by the usual method. Background was not subtracted from fluorescent signals; this will tend to reduce measured intensity differences.

RESULTS

lola suppresses midline crossing of CNS axons

In characterizing the phenotypes of an allelic series of *lola* mutations, we examined the pattern of anti-Fasciclin II immunoreactivity in embryos homozygous for a hypomorphic allele, *lola^{1A4}*. *1A4* is a P-element insertion in the *lola* promoter region that appears to reduce, but not eliminate, the expression of most or all *lola* isoforms (Giniger et al., 1994; Madden et al., 1999). In wild-type embryos, Fasciclin II-positive axon bundles run longitudinally, parallel to the midline (Fig. 1A). By contrast, in *lola^{1A4}* embryos we observed Fasciclin II-positive axons crossing back and forth across the midline (Fig. 1B). This phenotype was not unique to *lola^{1A4}* but was seen in several independent partial loss-of-function alleles of *lola*, including *lola^{cn03089}*, *lola^{ORB40}* (Fig. 1D-E) and *lola^{ORC16}* (not shown). Moreover, Fasiclin II immunoreactive bundles are also seen to cross the midline in embryos bearing strong/null *lola* alleles (for example *lola^{ORE76}*; Fig. 1F).

We wished to verify at single axon resolution that the phenotype observed with anti-Fasciclin II was indeed due to inappropriate midline crossing by axons that normally remain ipsilateral. We therefore made use of a transgene in which a tau-β-galactosidase fusion protein is expressed under control of a fragment of the *apterous* promoter (*apC-tau-lacZ*) and labels a small set of axons that project strictly ipsilaterally (Lundgren et al., 1995) (Fig. 2A). When this transgene was crossed into a genetic background that lacked *lola*, the fusion protein was expressed in what appear to be the typical Ap$^+$ cells based on their number and positions, but the labeled axons projected across the CNS midline (Fig. 2B). The Ap$^+$ axons then stall when they reach the mediolateral position where the contralateral connective would be found in a wild-type CNS; of course, this connective is largely absent in a *lola* mutant. In contrast to the axons of the Ap$^+$ neurons, axons of the longitudinal pioneer neurons pCC and MP1 were not observed to cross the midline in *lola* mutant embryos. It could be that *lola* is specifically required for midline guidance of follower, but not pioneer, neurons. Alternatively, the ability of pioneer axons to respect the midline in mutant embryos could simply reflect perdurance of maternal *lola* product: we know that maternal *lola* does not decay completely until after the time when longitudinal pioneers begin extending their axons (E. G., unpublished).

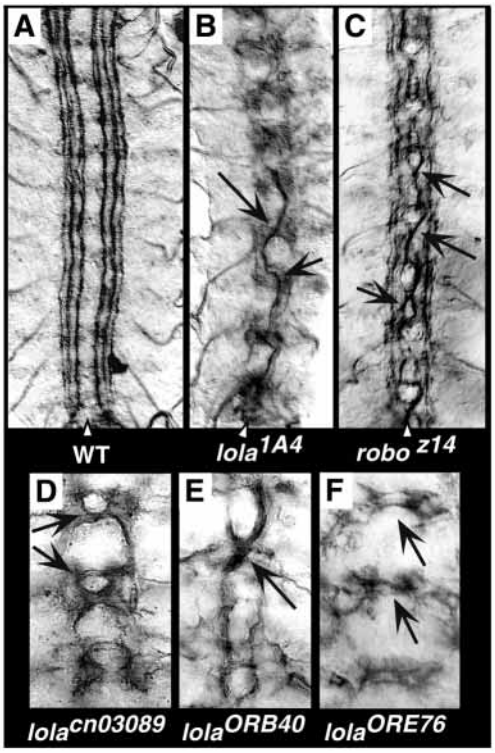

Fig. 1. *lola* limits midline crossing of CNS axons. Wild-type (A) and *lola* mutant (B,D-F) stage 16/17 embryos were fixed, stained with anti-Fasciclin II (mAb1D4) and visualized by peroxidase histochemistry. (C) A *robo^{z14}* embryo of the same stage is shown for comparison. Note multiple crossings of the CNS midline (arrows) by immunoreactive nerve tracts in all mutant panels, but not in the wild type. Midline is indicated by white triangle. (B) *lola^{1A4}*; (C) *robo^{z14}*; (D) *lola^{cn03089}*; (E) *lola^{ORB40}*; (F) *lola^{ORE76}*. (B,D,E) Hypomorphic *lola* alleles; (F) A strong/null *lola* mutant. Note reduction of longitudinal axon tracts in hypomorphic *lola* alleles and nearly complete absence in the strong allele. Anterior is towards the top in all panels.

If midline glial cells die or fail to develop, CNS axons are found to cross the midline inappropriately, presumably as a consequence of the absence of Slit (Klambt et al., 1991; Hummel et al., 1999b). We therefore labeled embryos bearing the strong *lola* allele *ORC46* with an antibody directed against the Wrapper protein, which specifically labels midline glia (Noordermeer et al., 1998). While the precise arrangement of midline glial cells is somewhat disturbed in the mutant embryos, and Wrapper levels appear to be diminished, in all cases we found Wrapper-expressing cells at the CNS midline (Fig. 2C). These cells also expressed a second midline marker, Slit (Fig. 3). Thus, the extra midline crossing of axons in *lola* mutants cannot be ascribed to an absence of midline glial cells. Nonetheless, we cannot rule out the possibility that defects in glial positioning might contribute to some aspects of the *lola* mutant phenotype, for example, the failure of separation of the anterior and posterior commissures (Giniger et al., 1994).

Slit levels are decreased in *lola* mutants

Inappropriate midline crossing is normally prevented by the

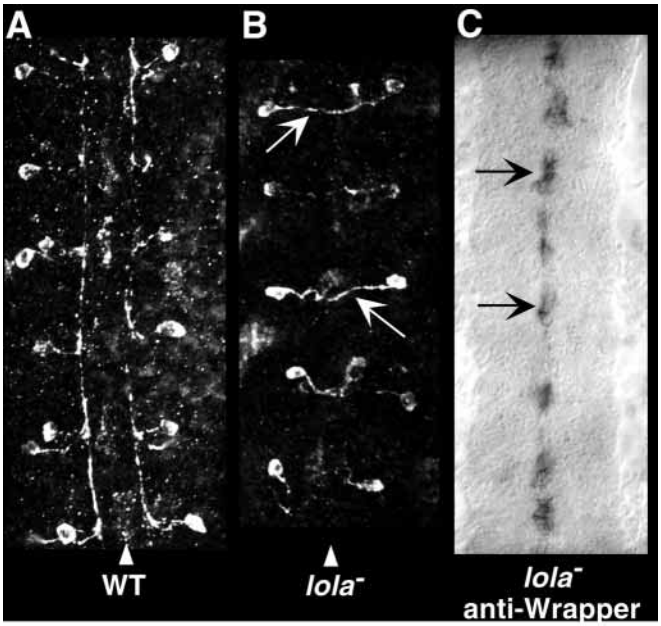

Fig. 2. Cellular analysis of *lola* midline phenotype. Wild-type and mutant embryos were fixed and stained with the indicated antibodies. (A,B) Ventral views of the axonal projections of Apterous-positive interneurons, visualized by anti-β-galactosidase staining of wild-type (A) or $lola^{ORC46/ORE76}$ (B) embryos bearing a *tau-lacZ* fusion gene under control of a fragment of the *apterous* promoter. Note that β-gal positive axons do not cross the midline (triangle) in wild type, but do so in the mutant (arrows). (C) Ventral view of an anti-Wrapper staining of a $lola^{ORC46}$ mutant embryo. Note presence of immunopositive midline glial cells (arrow) in every segment. Anterior is towards the top in all three panels.

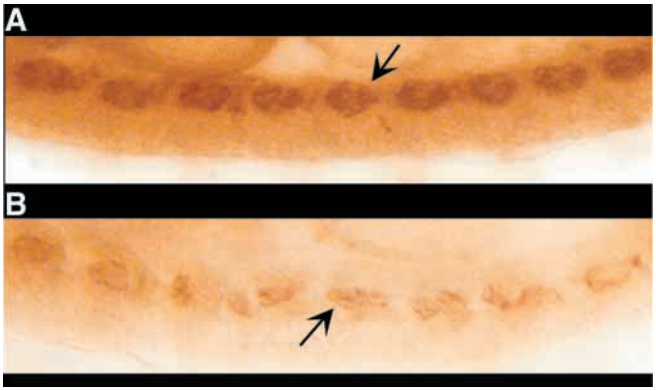

Fig. 3. Slit protein levels are reduced in *lola* mutant embryos. A collection of *lola* mutant embryos and their non-mutant siblings was fixed, stained with anti-Slit and visualized by peroxidase histochemistry. (A) Wild type; (B) $lola^{1A4}$. Identical camera settings were used for both embryos shown. Lateral view of the CNS midline of a late stage 16 embryo is shown in each panel; midline glial cells are prominently stained (arrows). Note that Slit immunoreactivity is present, but at reduced level, in glial cells in each segment in the mutant. Anterior is towards the left.

midline repellant protein, Slit. As one *lola* isoform is highly expressed in midline cells (Giniger et al., 1994) we reasoned that the extra midline crossings in *lola* embryos might arise from an effect on *slit* expression. We therefore stained embryos bearing the hypomorphic allele $lola^{1A4}$ with anti-Slit antibodies. Though still detectable, Slit protein was present at substantially lower levels in the midline glia of *lola* mutant embryos than in their non-mutant siblings in the same embryo collection (Fig. 3, compare wild type in 3A with mutant in 3B). Comparison of the residual Slit staining in this experiment to the signal seen in *slit* heterozygous embryos suggests that Slit levels are likely to be decreased by more than 50% in $lola^{1A4}$ embryos, though this impression was not carefully quantified. Embryos bearing a strong/null *lola* allele ($lola^{5D2}$, not shown) appear to have even less residual Slit than $lola^{1A4}$ embryos, though again, some Slit is still detected in all segments.

Robo levels are decreased in *lola* mutants: A 2-color ratio method for quantitative immunofluorescence

Reduced Slit expression in *lola* mutants could, in principle, account for the excess midline crossing of CNS axons in these individuals, but it does not rule out the possibility that *lola* might also affect expression of the Slit receptor, Robo. This seems particularly likely as some *lola* isoforms are expressed preferentially in CNS neurons, rather than midline glia (Giniger et al., 1994) (S. G., E. A. Greene, P. K. Grant and E. G., unpublished) and because the aberrant midline crossing phenotype in *lola* embryos more closely resembles the phenotype of *robo* (Fig. 1C) than that of *slit*. We therefore examined the levels of Robo immunoreactivity in *lola* mutant embryos. Expression of Robo protein in many other embryonic tissues (Kidd et al., 1998a) ruled out the use of any whole-embryo method (such as western analysis) for quantitative comparison of Robo levels in mutant versus wild type CNS. We therefore developed a sensitive method for quantitative measurement of immunofluorescence intensity in whole-mount embryos.

The method is outlined in Fig. 4A. Embryos were incubated both with anti-Robo and with an antibody against a reference epitope, and we then used confocal microscopy to quantify the ratio of the anti-Robo fluorescence signal relative to the reference signal for mutant and non-mutant embryos in a single embryo collection. By performing a ratio measurement, we avoid many of the artefacts that plague efforts at quantitation of fluorescence microscopy (Pawley, 2000), and the remainder are controlled by use of different combinations of experimental versus reference fluorochromes in parallel experiments and by appropriate calibration of the confocal detection system (see Materials and Methods for further details). For the reference antibody we used anti-HRP, which, like Robo, labels the surface of all *Drosophila* CNS neurons (Jan and Jan, 1982). The HRP antigen is particularly useful as a reference epitope because it is a sugar modification present on a large number of otherwise unrelated neuronal cell-surface proteins, including housekeeping proteins such as the Na, K ATPase (Snow et al., 1987; Tolar and Pallanck, 1998), and so is unlikely to be affected systematically by the *lola* mutation. Indeed, in analysis of a large number of *lola* alleles, we have never observed evidence for alterations in the level or distribution of anti-HRP immunoreactivity (E. G., unpublished).

In multiple independent experiments using two different strong *lola* alleles ($lola^{ORC46}$ and $lola^{ORE76}$), we observed that the level of Robo immunoreactivity relative to the reference signal is reduced in *lola* mutant embryos as compared with

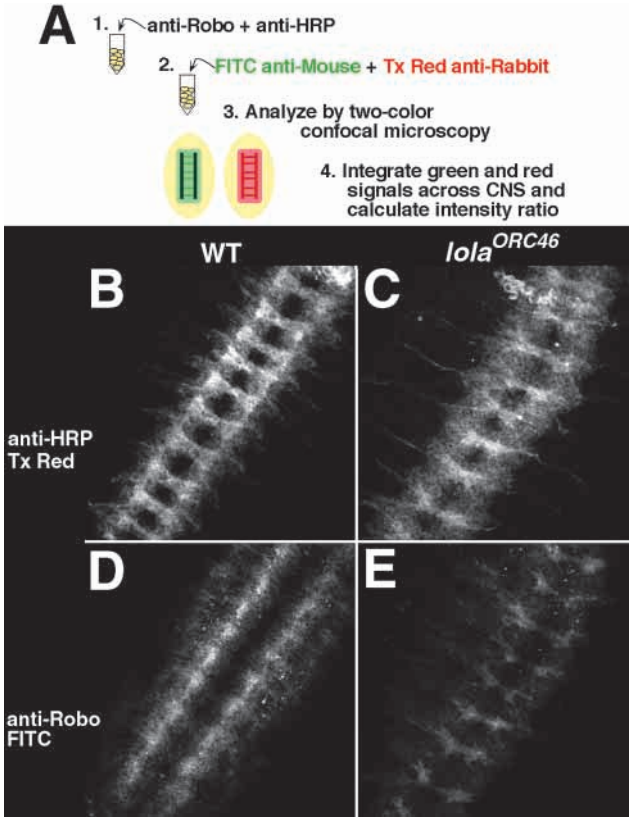

Fig. 4. Robo protein levels are reduced in *lola* mutant embryos. (A) Outline of the two-color ratio method for quantification of immunofluorescence. *lola* mutant embryos and their non-mutant siblings were collected and fixed 10-11 hours after egg-laying (AEL) and doubly stained with mouse anti-Robo and rabbit anti-HRP. Antibodies were detected by incubating with the indicated fluorescent secondary antibodies and using the confocal microscope to collect a stack of image slices across the entire CNS. Integrated fluorescence intensities for each CNS were determined using NIH Image 1.62 to perform an average (i.e. summation) projection of the image stack; manually outlining the CNS in the projected image, and integrating the fluorescence signals for both chromophores within the outline. Fluorescence intensity comparisons were made between embryos from a single embryo collection and staining, with data collected from a single slide in a single confocal session. (B,D) Wild-type embryo; (C,E) homozygous $lola^{ORC46}$ embryo. (B,C) Anti-HRP reference signal (visualized with Texas Red-conjugated secondary antibody); (D,E) Anti-Robo experimental signal (visualized with FITC secondary). For purposes of presentation, the intensities of the wild-type and mutant reference signals have been approximately matched and the cognate Robo signals adjusted accordingly. Quantification of the intensity ratios in this example show the Robo/HRP ratio to be decreased ~40% in the mutant embryo when compared with the wild type.

their wild-type siblings. Fig. 4B-E show the integrated fluorescence signals derived from summing all planes of a confocal *z*-series of the CNS for a wild-type (B,D) and a $lola^{ORC46}$ (C,E) embryo at stage 14, when many *lola*-dependent CNS axons are extending. For purposes of presentation, the intensities have been adjusted to equalize the wild-type and mutant reference signals (Texas Red; B,C) to permit direct comparison of the anti-Robo images (FITC; D,E). The reduced Robo signal in the mutant is clearly apparent.

Table 1. HRP:Robo immunofluorescence intensity ratios in *lola* mutant and non-mutant embryos

Fluorochrome		$lola^+$	$lola^-$	Robo($lola^+$)/
α-HRP	α-Robo	HRP:Robo	HRP:Robo	Robo($lola^-$)
Texas Red	FITC	1.7±0.1*	2.5±0.3	1.5
FITC	Texas Red	1.2±0.1	1.6±0.1	1.3
Cy5	FITC	2.0±0.2	2.7±0.2	1.4
FITC	Cy5	2.3±0.2	2.7±0.3	(1.2)†

Intensity of CNS immunofluorescence was determined as for the experiment in Fig. 4. HRP:Robo immunofluorescence ratio was determined for the indicated combinations of fluorochromes, and used in each case to calculate a normalized ratio of Robo expression level. All experiments shown employed the strong/null allele $lola^{c46}$; similar results were obtained with another strong allele ($lola^{e76}$).

*Fluorescence intensity ratios are presented as mean±s.e.m., derived in each case from a dataset of six to eight embryos.

†Absolute intensity of the Cy5 signal was systematically low in this configuration of the experiment, leading to poor discrimination of signal from background, and consequently an anomolously low value for the calculated reduction of Robo levels in *lola* mutant embryos. Nonetheless, normalized Robo intensity was reduced in $lola^-$ embryos in all experiments.

Similar results were obtained in experiments in which the fluorochromes were swapped on the two secondary antibodies, and experiments in which different combinations of fluorochromes were used (e.g. FITC and Cy5, in both orientations of reference versus experimental fluorochrome). Summing the fluorescence intensity values across the entire CNS for each sample and comparing the ratio of Robo:reference signal for wild-type and mutant embryos revealed that the integrated anti-Robo fluorescence intensity in wild-type CNS was ~30-50% greater than that in *lola* mutants [e.g. 42±20% for one typical experiment employing FITC anti-mouse and Texas Red anti-rabbit secondaries (n=6 embryos each in mutant and wild-type data set)]. The integrated HRP:Robo intensity ratios for four combinations of fluorochromes from one set of experiments are presented in Table 1. Similar results were obtained with later stage embryos (data not shown). Note that Robo expression is not dependent on Slit (Kidd et al., 1999) (D. C. and E. G., unpublished), so it is unlikely that the reduction in Robo level documented above is a secondary consequence of the reduction in Slit expression. By contrast, Kidd et al. (Kidd et al., 1999) have shown that subcellular localization of Robo does depends on Slit, and we note that, in *lola* mutant embryos, Robo protein is relocalized to commissural axon tracts from which it is normally excluded (compare Fig. 4D with 4E). It therefore seems probable that the mislocalization of Robo protein in *lola* mutants is a secondary consequence of the reduction of Slit levels, though other models cannot be excluded.

lola interacts genetically with both *slit* and *robo*

If the effect of *lola* on midline crossing is mediated, in part, by controlling the levels of *slit* and *robo*, we would predict that hypomorphic mutations in *lola* should interact genetically with *slit* and *robo* mutations. To test this, we made use of a very weak *lola* allele, $lola^{ORE120}$. By itself, the effect of $lola^{ORE120}$ on midline crossing is barely detectable: Fasciclin II-immunoreactive axon bundles cross the midline in only 25% of homozygous mutant stage 16/17 embryos, and usually just in one or two segments per affected embryo (Fig. 5A; results quantified in Table 2). By contrast, when we removed one copy

of *slit* from such an embryo (*lola*^{ORE120}*slit*^{IG107}/*lola*^{ORE120}), the aberrant midline crossing phenotype was observed in nearly all mutant embryos (98%), and commonly affected more than four segments/affected embryo (Fig. 5C). Similarly, aberrant midline crossings were observed in 93% of embryos which were *lola*^{ORE120}*robo*^{Z14}/*lola*^{ORE120} (Fig. 5D), with mean of 4.4 crossings/affected embryo. Embryos heterozygous for *slit*^{IG107} (Fig. 5B) or for *robo*^{Z14} displayed only extremely rare midline crossing defects (7% and 9% of embryos, respectively).

Ectopic expression of *lola* induces ectopic expression of *slit*

One of the characterized *lola* isoforms (*lola 4.7*) is expressed at high levels in the developing midline (Giniger et al., 1994), making this isoform a plausible candidate for a regulator of *slit* expression (Rothberg et al., 1990). To test this possibility, we used the *GAL4* system (Fischer et al., 1988) to misexpress *lola 4.7* at high levels throughout the neuroectoderm under the control of an appropriate GAL4 effector line [*GAL4-112A* (Fuerstenberg and Giniger, 1998)] and assayed accumulation of Slit. We found that ectopic expression of *lola 4.7* indeed led to ectopic expression of *slit* mRNA (Fig. 6A,B) and protein (Fig. 6C-F). Curiously, however, ectopic Slit was detected only in midline derivatives, i.e. in the progeny of precursor cells which themselves expressed Slit, and not throughout the domain of *lola* misexpression. Evidently, *lola 4.7* can augment *slit* expression in cells that are competent to express the gene but is not sufficient to completely reprogram the transcriptional repertoire of CNS neurons (Gerber et al., 1997). We did not detect any change in the pattern of Robo expression in this experiment (data not shown). Presumably Robo regulation is downstream of some other Lola isoform.

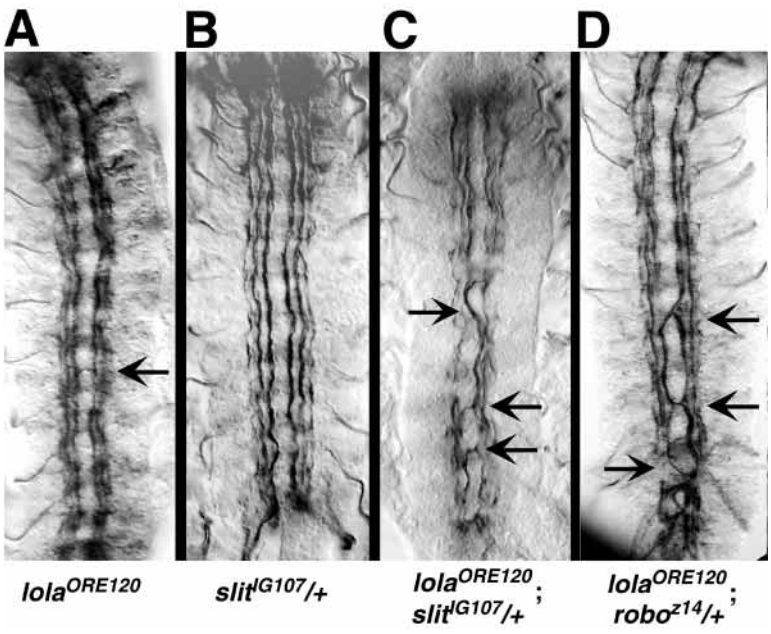

Fig. 5. *lola* interacts genetically with *slit* and *robo*. Embryos that were either (A) *lola*^{ORE120}/*lola*^{ORE120}, (B) *slit*^{IG107}/+, (C) *lola*^{ORE120}*slit*^{IG107}/*lola*^{ORE120} or (D) *lola*^{ORE120}*robo*^{z14}/*lola*^{ORE120} were fixed, stained with anti-Fasciclin II and visualized by peroxidase histochemistry. Midline crossing of immunoreactive nerve bundles (arrows) was extremely rare in *lola*^{ORE120} embryos and in *slit* heterozygous embryos (or *robo* heterozygous, not shown), but very common in embryos that were simultaneously *slit* or *robo* heterozygous and *lola*^{ORE120} homozygous (see Table 2 for quantitation). All panels show ventral views of the CNS of stage 17 embryos; anterior is towards the top.

DISCUSSION

Before the growth cone can grapple with a particular axon guidance decision, the outcome of that decision has first to be specified by the co-expression of a precise constellation of guidance cues, receptors and signaling proteins. On phenotypic grounds, we have speculated previously that the transcription factor Lola may be responsible for coordinating the expression of multiple guidance factors, thereby orchestrating the delicate balance of a particular axon guidance decision (Giniger et al., 1994; Madden et al., 1999). We have now uncovered a requirement for Lola in establishing the pattern of midline crossing of axons in the fly CNS. We have found that *lola* is required to limit midline crossing of CNS axons, and we show directly that proper expression of the midline repellant, Slit, as well as its axonal receptor, Robo, depend on Lola. Thus, in *lola* mutants, the levels of Slit and Robo are reduced; *lola* interacts genetically with mutations in *slit* and *robo*; and ectopic expression of a midline-enriched *lola* isoform leads to ectopic expression of *slit*. In conjunction with previous evidence that Lola also promotes longitudinal growth of these same axons (Seeger et al., 1993; Giniger et al., 1994), therefore, these data show that Lola coordinately specifies the 'attractiveness' of each of the two basic trajectories available to a CNS neuron: to grow longitudinally or across the midline.

These observations add a new level of regulation to our picture of CNS midline crossing. It is clear that the immediate mechanism that instructs a given growth cone whether or not to be repelled by the midline is the regulated exposure of the Slit receptor, Robo, on the surface of that growth cone. However, the overall probability of an axon crossing the midline reflects a balance between repulsion from, and attraction to, the midline, as well as attraction to the longitudinal axon tracts. This balance of guidance forces, in turn, depends upon the precise expression levels of many

Table 2. *lola* interacts genetically with *slit* and *robo*

Genotype	Embryos with ≥1 midline crossing (%)	Crossovers per affected embryo
lola^{120}/*lola*^{120}	25	1.75 (1)*
slit^{1G}/+	7	1.0 (1)
lola^{120}*slit*^{1G}/*lola*^{120}	98	4.8 (5)
robo^{z14}/+	9	1.2 (1)
lola^{120}*robo*^{z14}/*lola*^{120}	93	4.4 (4)

Embryos of the indicated genotypes were prepared and stained with anti-Fasciclin II as for the experiment in Fig. 5. Aberrant midline crossings of Fasciclin II-immunoreactive axon bundles were tabulated in late stage 16 and stage 17 embryos; *n*=40-55 embryos in each sample reported.

*Number of midline crossings per affected embryo is reported as mean (median).

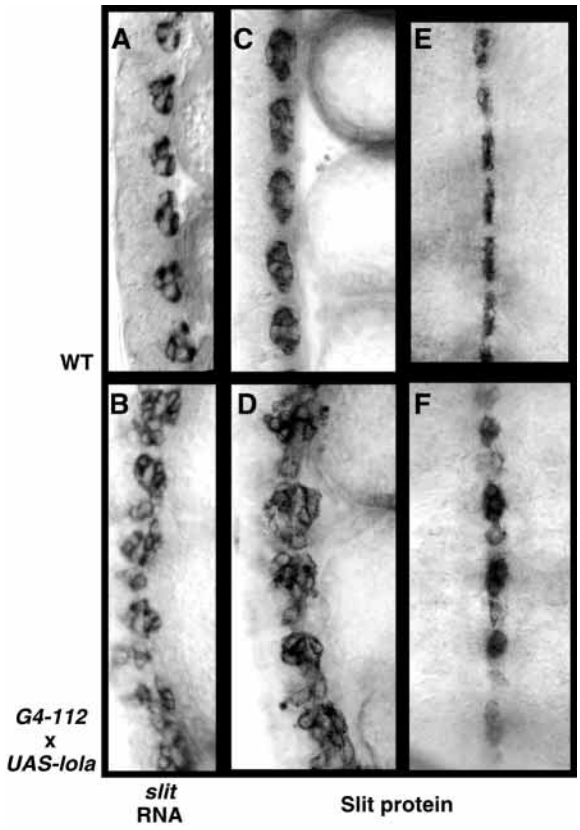

Fig. 6. Ectopic *lola* induces ectopic *slit*. The splice variant *lola 4.7* was placed under control of UAS_G and expressed throughout the developing neuroectoderm by crossing to *GAL4-112A* (Fuerstenberg and Giniger, 1998). *slit* expression was then assayed by in situ hybridization (A,B) or antibody staining (C-F). Ventral (E,F) or sagittal (A-D) views of stage 17 embryos are shown. In wild-type stage 17 embryos (A,C,E), Slit is expressed only in midline glial cells; expression of *lola 4.7* throughout the neuroectoderm causes ectopic expression of *slit* mRNA (B) and protein (D,F), but only in additional midline cells.

cooperating guidance proteins (Kidd et al., 1998b; Winberg et al., 1998; Battye et al., 1999; Sun et al., 2000). Lola apparently contributes to the pattern of midline crossing in part by augmenting Slit and Robo protein levels: in the absence of *lola*, both Slit and Robo are still expressed, but at reduced levels, and excessive midline crossing is observed. Evidently, the distinguishing feature of Lola is not to act as an on/off switch for cell-surface guidance genes; that role is more likely to be provided by regulators of neuronal identity akin to the Lim homeoproteins (Tsuchida et al., 1994; Thor et al., 1999). Rather, it seems that Lola fine-tunes axon patterning by setting the precise expression levels of multiple, cooperating guidance genes.

The reduction of Robo expression seen in *lola* mutants is relatively modest (~40%). We know from previous experiments, however, that a 50% diminution in Robo is sufficient by itself to cause some inappropriate midline crossing (Kidd et al., 1998b), and this effect is strongly enhanced by a simultaneous 50% reduction in Slit (Kidd et al., 1999; Battye et al., 1999). Loss of *lola* causes a greater reduction than this in Slit levels. Thus, it is plausible that the change in Slit and Robo levels could account for much of the midline phenotype observed in embryos that bear strong *lola* mutations. But why are weaker *lola* alleles like $lola^{1A4}$ able to cause extra midline crossing when their effect on target gene expression is presumably proportionately less? It is likely that regulation of Slit and Robo expression is only one part of the control of midline crossing by *lola*, and that a significant contribution to the phenotype is made by changes in the expression of other, interacting guidance genes that are also controlled by *lola*. For example, aspects of the *lola* midline phenotype resemble details of the axon pattern observed upon mutation of genes encoding receptor tyrosine phosphatases (Sun et al., 2000) (E. G., unpublished), suggesting that these are good candidates for potential *lola* effectors. Moreover, we know that the *Notch*-dependent mechanism that promotes the alternative (longitudinal) trajectory of CNS axons also requires *lola* (Giniger et al., 1993; Seeger et al., 1993; Giniger et al., 1994). The multiplicity of genes contributing to the midline/longitudinal axon growth decision underscores the need for a gene, like *lola*, to coordinate the expression of all these cooperating guidance factors. We suggest that it is the combination of many quantitative effects, each individually modest, which together produce the profound effects of *lola* on axon patterning.

Many questions remain from these studies. First, though Lola itself is a transcriptional regulator, we do not know whether *robo* and *slit* are direct Lola targets or whether Lola initiates a longer chain of events leading only indirectly to *robo* and *slit*. For example, Lola could regulate other genes that themselves control the stability of *robo* or *slit* RNA or protein, or the splicing or translation efficiency of these genes. Analysis of this issue will require unambiguous identification of the exact *lola* isoforms required for expression of *robo* and *slit*, and characterization of their DNA-binding specificities in combination with their appropriate dimerization partner(s). Moreover, we have only characterized the accumulation of Robo and Slit protein in *lola* mutants, and not transcript levels. In our hands, the inherent variability of whole-mount RNA in situ hybridization has prevented sufficiently precise quantification of *robo* and *slit* RNA levels for this purpose. Nonetheless, the observation that ectopic expression of *lola 4.7* leads to ectopic expression of *slit* RNA strongly argues that *lola* is upstream of *slit* transcription, though it remains possible that Robo and Slit expression are also subject to *lola*-dependent regulation at some post-transcriptional level.

lola does not just regulate midline crossing, but also controls extension of some peripheral motor axons and orientation of lateral chordotonal neurons in the embryo, as well as pathfinding of some axons of the adult wing. In each case, it apparently establishes a precise balance of guidance factors, much as we have shown here (Giniger et al., 1994; Madden et al., 1999) (E. G., unpublished). How can one transcription factor exert such subtle control over such a diverse array of developmental events? This remains to be determined, but we have recently found that *lola* encodes a large number of protein isoforms (S. G., E. A. Greene and E. G., unpublished). At least in some cases, *lola* isoforms with different predicted DNA binding specificities are expressed in different tissue specific patterns, potentially allowing the regulation of distinct cohorts of downstream target genes. Moreover, we know that a single, direct Lola target gene can be activated by *lola* in one tissue and repressed in another (Cavarec et al., 1997). Both of these

properties are likely to contribute to the ability of Lola to modulate gene expression programs in distinct ways in different cells.

The problem of ensuring appropriate relative levels of multiple guidance genes is not unique to the midline crossing versus longitudinal growth decision in the fly CNS, but rather is an inherent feature of all axon guidance decisions (Winberg et al., 1998; Madden et al., 1999). We therefore imagine that *lola* is not unique in its property of co-regulating multiple, interacting guidance genes, but rather is the exemplar of a class of transcriptional regulators that will be found to be widespread in distribution and critical in importance in the regulation of axon patterning.

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