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The *Drosophila* RNA-binding protein ELAV is required for commissural axon midline crossing via control of *commissureless* mRNA expression in neurons

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Abstract

Drosophila ELAV is the founding member of an evolutionarily conserved family of RNA-binding proteins considered as key inducers of neuronal differentiation. Although several ELAV-specific targets have been identified, little is known about the role of *elav* during neural development. Here, we report a detailed characterization of the *elav* mutant commissural phenotype. The reduced number of commissures in *elav* mutant embryos is not due to loss or misspecification of neural cells but results from defects in commissural axon projections across the midline. We establish a causal relationship between the *elav* mutant commissural phenotype and a reduction in the expression of *commissureless*, a key component of the Robo/Slit growth cone repulsive signalling pathway. In the nerve cord of *elav* mutant embryos, *comm* mRNA expression is strongly reduced in neurons, but not in midline glial cells. Furthermore, specific expression of an *elav* transgene in posterior neurons of each segment of an *elav* mutant nerve cord restores *comm* mRNA expression in these cells, as well as the formation of posterior commissures. Finally, forced expression of *comm* in specific commissural neuron subsets rescues the midline crossing defects of these neurons in *elav* mutant embryos, further indicating that *elav* acts cell autonomously on *comm* expression.

Keywords: Hu; RNA-binding protein; Axon guidance; Midline crossing; Commissure; Commissureless; Neural development; Robo; Slit

Introduction

In the central nervous system (CNS) of animals with bilateral symmetry, a majority of axons (called commissural axons) cross the midline and connect their synaptic targets on the contralateral body side, whereas other axons do not cross the midline and project ipsilaterally (same body side). Directional axon extension toward and/or away from the midline depends on several receptor-ligand signalling events (reviewed in Dickson, 2002; Huber et al., 2003; Yoshikawa and Thomas, 2004). Two conserved main signalling molecules are known to control axonal growth across the CNS midline. Netrins and Slit are required to guide commissural axons toward the midline and to mediate axon repulsion from the midline, respectively. Netrins are secreted by cells of the CNS midline and can act as attractive guidance cues (Harris et al., 1996; Kennedy et al., 1994; Kidd et al., 1999; Serafini et al., 1994). In *netrin* mutants or in animals with mutations in their attractive receptor Fra/Dcc/Unc40, commissural axon tracts are thinner or missing, reflecting a failure to cross the midline (Fazeli et al., 1997; Harris et al., 1996; Hedgecock et al., 1990; KeinoMasu et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996; Serafini et al., 1996). In addition to Netrins, the CNS midline cells also express Slit proteins that can act as repulsive guidance cues (Battye et al., 1999; Brose et al., 1999; Zou et al., 2000). In *slit* mutants or

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in animals with mutations in their repulsive receptor Robo, commissural axons fail to leave the midline or can freely re-cross it (Battye et al., 1999; Brose et al., 1999; Kidd et al., 1998a, 1999; Li et al., 1999; Seeger et al., 1993; Long et al., 2004). In both mice and flies, modulation of the repulsive Slit activity is necessary to allow commissural growth cones to enter and exit the midline (Keleman et al., 2002; Marillat et al., 2004; Sabatier et al., 2004). In *Drosophila*, one such Slit modulator is Commissureless (Comm), a transmembrane protein. Loss-of-function mutations in *comm* result in a partial or complete loss of commissural axon bundles, reflecting increased growth cone repulsion from the CNS midline (Seeger et al., 1993; Tear et al., 1996). Comm is expressed both in midline glia and commissural neurons (Georgiou and Tear, 2002; Keleman et al., 2002; Tear et al., 1996). However, Comm seems to be required only in commissural neurons to allow their axons to cross the midline (Keleman et al., 2005). Cell-autonomous requirement for Comm in neurons suggests that commissural axon guidance depends on regulated expression of *comm* in neurons (Georgiou and Tear, 2002; Keleman et al., 2005; McGovern and Seeger, 2003). Comm, which specifies midline crossing, is believed to prevent the delivery of Robo to the growth cone by targeting newly synthesized Robo molecules for degradation (Keleman et al., 2002, 2005).

A saturating mutagenesis screen for mutations affecting the axon pattern in the *Drosophila* embryo (18,000 lethal lines) has identified only five genes (*fra*, *netrin*, *comm*, *schizo* and *weniger*) whose mutations lead to a reduction in the number of commissures without affecting cell fate at the midline (Hummel et al., 1999a). One of them, *weniger*, has been identified as an allele of *elav* (U. Lammel and C. Klämbt, unpublished results). Mutations in *elav* (*embryonic lethal abnormal vision*) lead to embryonic lethality with an abnormally formed CNS whose commissural and connective axon patterns are aberrant (Hummel et al., 1999a; Jimenez and Campos-Ortega, 1987). In the adult, loss-of-function mutant clones in the retina and optic lobes induce autonomous tissue degeneration (Campos et al., 1985). *elav* encodes an RNA-binding protein that is specifically expressed in all post-mitotic neurons from the earliest stages of their differentiation and is located predominantly in the nucleus (Robinow and White, 1991; Yannoni and White, 1999). In vertebrates, neural ELAV-like proteins (called HuB, C and D) that are antigens targeted in paraneoplastic encephalomyelitis (Szabo et al., 1991) are key inducers of neuronal differentiation in cultured cells (Akamatsu et al., 1999; Anderson et al., 2000; Antic et al., 1999). These proteins promote the stabilization and/or translation of specific target transcripts bearing the AU-rich element (reviewed in Keene, 2001). In *Drosophila*, ELAV has been shown to be essential for the formation of neural-specific splice forms of three pre-mRNAs, *neuroglian* (*nrg*), *erect wing* (*ewg*) and *armadillo* (*arm*) (Koushika et al., 2000). ELAV regulates alternative splicing of the last exon of *nrg* and *ewg* pre-mRNAs. In both cases ELAV binds to regions within the regulated intron that contain AU motifs (Lisbin et al., 2001; Soller and White, 2003), it can also bind to its own 3′untranslated region (UTR) (Borgeson and Samson, 2005).

Although some direct RNA targets for ELAV have been identified, it is not known for which aspects of neuron development these interactions are required. To better understand the role of ELAV during development of the nervous system, we performed a detailed characterization of the *elav* null mutant CNS phenotype focusing on the formation of commissures. This report shows that the *elav* mutant commissural phenotype results from a failure of most commissural axons to cross the midline, but that there is no neural cell loss associated with the lack of *elav*. Our study provides both genetic and molecular evidence that *elav* is required for proper expression of *comm* mRNA and that reduction of *comm* mRNA expression in commissural neurons in *elav* mutant embryos is responsible for the midline crossing defects observed.

Materials and methods

Drosophila lines and genetics

To analyze the phenotype of *elav* mutant CNS, $elav^5$ (provided by K. White) and $weniger^{E476}$ (provided by C. Klämbt) were used. $elav^5$ is a null allele with a deleted protein coding region (Robinow and White, 1991). $weniger^{E476}$ is an EMS-induced null allele of *elav* (U. Lammel and C. Klämbt, unpublished results). $weniger^{E476}$ is classified as a null allele based on the fact that no protein is made in hemizygous mutant embryos. In the figures, $elav^{weniger}$ refers to $weniger^{E476}$. For the analyses of genetic interaction, $comm^5$, $robo^{GA285}$ and $slit^2$ (null alleles) were used, and *TM6,P[Ubx-lacZ]* and *CyO,P[wg-lacZ]* balancers served to genotype embryos. For the rescue experiments, *UAS-elav* (provided by L. Théodore), *UAS-comm* (provided by A. Chiba) and *UAS-fra* (provided by B. Dickson) were used. These are UAS-P-element insertions in the second chromosome. The other stocks utilized were *en-GAL4* (provided by J.M. Dura), *eg-GAL4* (provided by A. Giangrande) and *UAS-τlacZ* (provided by M. Boyle). Expression of an *elav* transgene in *engrailed*-expressing neurons in *elav* mutant embryos was achieved by crossing females heterozygous for *elav* ($elav^5$) and *en-GAL4* to males homozygous for *UAS-elav*. Expression of a *comm* transgene in *eagle*-expressing neurons in *elav* mutants was achieved by crossing females heterozygous for *elav* ($elav^5$) and *eg-GAL4* to males homozygous for *UAS-comm,UAS-τlacZ*.

Histology

Immunocytochemistry was performed as described by Patel (1994). MAb BP102 and anti-ELAV MAb 9F8A9 (DSHB), anti-mouse- and anti-rabbit-Alexa secondary antibodies (Molecular Probes) were used at 1:1000 dilution. For anti-Comm staining, preadsorbed rabbit anti-Comm (gift of G. Tear) was used at 1:50 dilution. Preadsorbed rabbit anti-β-galactosidase (Molecular Probes) was used at 1:1000 dilution. *In situ* hybridizations on whole-mount embryos were performed according to standard protocols with digoxigenin-labelled antisense RNA probes (Tautz and Pfeifle, 1989). The digoxigenin-labelled antisense *comm* RNA probe was generated by *in vitro* transcription using T3 RNA polymerase (DIG RNA labelling kit, Boehringer) from a *comm* cDNA subclone (RE02011, ResGen) linearized with *Pvu*II.

Embryos carrying *lacZ*-expressing balancers were identified using anti-β-galactosidase. *elav* null mutant embryos ($elav^5$ and $elav^{weniger}$) lacking ELAV protein in the nervous system were genotyped by counterstaining using anti-ELAV antibodies. Developmental stages were determined according to Campos-Ortega and Hartenstein (1997). Segments of the CNS were examined in fillet-dissected embryos mounted in 1× PBS (50% glycerol). All images (except Fig. 4) were taken on a Leica DMR microscope equipped with a CCD camera and acquired with the Roper Scientific software (Roper Scientific). Confocal stacks (Fig. 4) were acquired on a Zeiss Axioplan 2 microscope and processed using the Metamorph imaging software. To quantify Comm expression in the CNS, average pixel intensity after background subtraction (equivalent in *elav*/+ and *elav* mutant CNS) was determined in each confocal section of a given stack using the ImageJ software thanks to the 3D-object-counter plugin recently

developed by Fabrice Cordelières (http://rsb.info.nih.gov/ij/plugins/track/objects.html).

Results

elav affects commissure formation

elav null mutations affect commissure formation in all neuromeres and lead to a reduction in the number of commissural fibers crossing the CNS midline (Figs. 1B, D, E and Fig. 2E). The commissural fibers are thinner than in wild type in 95% of the neuromeres ($n=351$, 32 embryos analyzed). The posterior commissure is missing entirely in 25% of the neuromeres (Fig. 1B and Fig. 2E, arrowheads) and both commissures are absent in 5% of the neuromeres (Fig. 1D, arrowhead). A greater distance between the longitudinal tracts is observed in more than 80% of the neuromeres of *elav* null mutant embryos (Figs. 1D, E and Fig. 2E, double arrows).

To analyze the *elav* mutant commissural phenotype in more detail, a Tau-β-galactosidase fusion protein was used to specifically label two subsets of neurons that send their axons across the midline into the anterior (EG) and posterior (EW) commissures (Dittrich et al., 1997) (Fig. 1C). In wild-type embryos, these axons cross the midline in 100% of the neuromeres ($n=182$, 23 embryos analyzed). In *elav* mutant embryos, EW commissural axons frequently fail to cross the midline and instead grow ipsilaterally (80% of the neuromeres, $n=205$, 26 embryos analyzed) (Figs. 1F and 6B, red arrowheads). Similarly, but to a lesser extent, EG commissural axons frequently do not cross the midline, preferentially extending on their own side (>50% of the neuromeres, $n=205$, 26 embryos analyzed) (Figs. 1F and 6B, black arrowheads). Using different cell markers, an effect on midline crossing was observed for other commissural axon subpopulations. Indeed, contralateral projections were found impaired in 30% of the neuromeres ($n=143$, 14 embryos analyzed) for the SP neurons (labelled with anti-Futsch) and no contralateral projections were seen for the RP3 neurons (labelled with anti-FasIII) in *elav* mutant embryos (100% of the neuromeres, $n=222$, 21 embryos analyzed) (Supplementary Fig. 1).

The absence of elav does not induce neural cell death

Reduction in the number and thickness of commissures in *elav* mutant embryos is not due to neural cell loss. Indeed, EW and EG neurons are present at least until stage 16 in embryos lacking *elav* (Fig. 1F). Using various cell markers (Eve, Odd, Futsch, Cas, FasII and FasIII), it was observed that all neuron subsets tested are present in *elav* mutant embryos during the period of commissure formation until late stages of

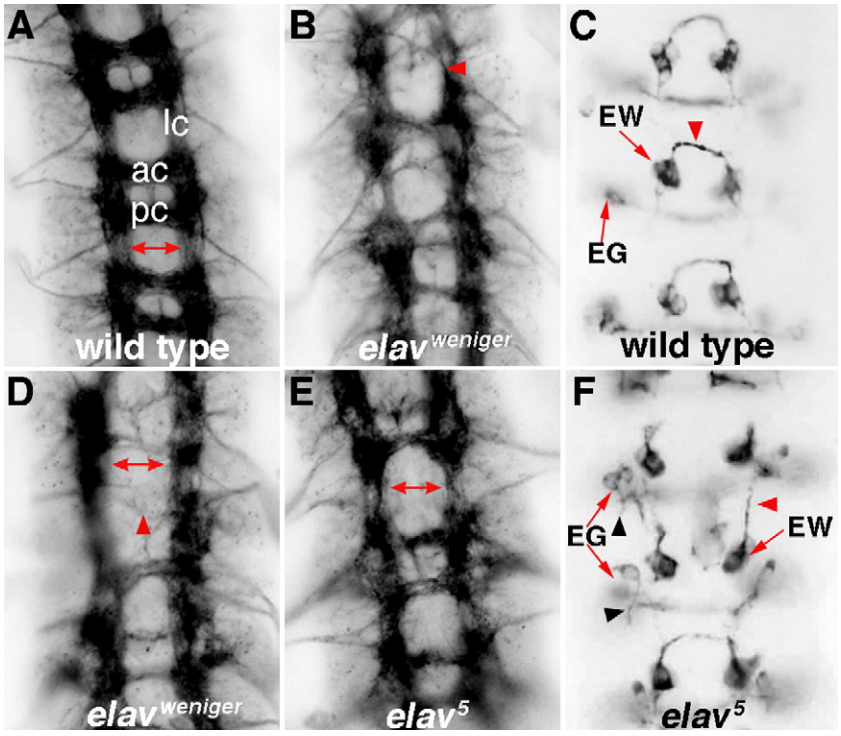

Fig. 1. *elav* is required for commissure formation. Dorsal views of dissected CNS preparations of stage 16 embryos. (A, B, D, E) Stained for the presence of all CNS axons using MAb BP102. (C, F) Stained for the presence of β-galactosidase. In these embryos, *eg-GAL4* drives the expression of UAS-tau:β-galactosidase in two subsets of commissural neurons (EW and EG). Anterior is up. (A) Wild-type embryos are characterized by a regular arrangement of longitudinal connectives (lc) and two commissures per segment, anterior (ac) and posterior (pc). (B, D, E) In hemizygous $elav^{weniger}$ and $elav^{5}$ mutant embryos, the number of commissures is strongly reduced. Some commissures are lacking and the commissural axons fibers that have extended across the midline are thinner than in wild type. *elav* null mutant embryos are also characterized by a higher distance between the longitudinal connectives (double arrows). (C) In wild-type embryos, the EW and EG axons cross the midline in the posterior and anterior commissure, respectively. (F) In $elav^{5}$ mutant embryos, EW and EG axons frequently do not cross the midline and make ipsilateral projections (arrowheads). Note that the cell bodies of the EW neurons are more distant from the midline than in wild type.

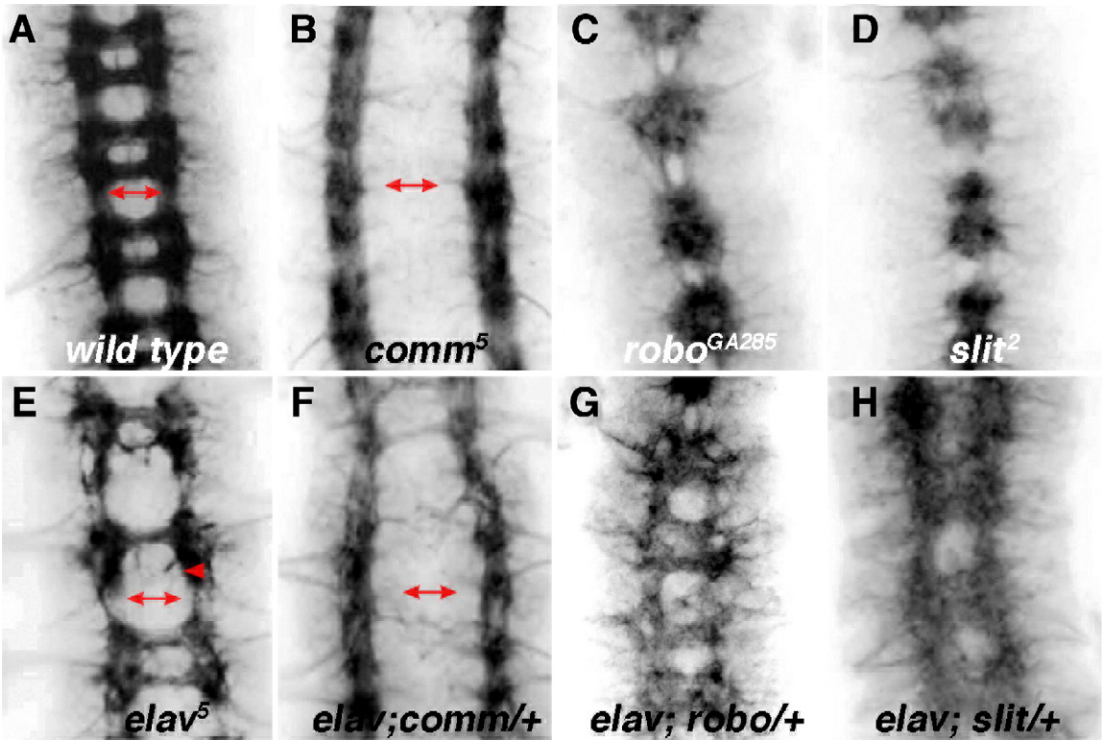

Fig. 2. *elav* interacts with *comm*, *robo* and *slit.* Dorsal views of dissected CNS preparations of stage 16 embryos. (A–H) Stained for the presence of all CNS axons using Mab BP102. Anterior is up. (A) In wild-type embryos, two commissures form in each neuromere. (B) $comm^{5}$ mutant embryos present a typical commissureless phenotype (100% of the neuromeres, $n=222$, 21 embryos analyzed). (C) $robo^{GA285}$ mutant embryos present a fused commissure phenotype in 100% of the neuromeres ($n=176$, 16 embryos analyzed). (D) $slit^{2}$ mutant embryos present a much severe fused commissure phenotype (100% of the neuromeres, $n=157$, 15 embryos analyzed). (E) $elav^{5}$ mutant embryos have reduced (or absent) commissures in 100% of the neuromeres ($n=351$, 32 embryos analyzed). (F) $elav^{5},comm^{5}$/+ mutant embryos display a commissureless-like phenotype. Commissural fibers frequently do not cross the midline in these embryos (90% of the neuromeres, $n=252$, 23 embryos analyzed). (G) The commissural phenotype of *elav* embryos is partially suppressed when the *robo* dosage is reduced. In $elav^{5},robo^{GA285}$/+ mutant embryos thicker commissural tracts are made in 50% of the neuromeres ($n=184$, 17 embryos analyzed). (H) In $elav^{5},slit^{2}$/+ mutant embryos many axons cross the midline leading to a fused commissures phenotype in more than 50% of the neuromeres ($n=167$, 16 embryos analyzed).

embryogenesis (Supplementary Fig. 1 and data not shown). In addition, glial cell markers (Slit and Wrapper) showed that in *elav* mutant embryos, midline glial cells are specified normally (e.g., they express Slit at normal levels), are present in normal numbers and have normal position and morphology (Supplementary Fig. 1 and data not shown).

elav interacts genetically with comm, robo and slit

The CNS phenotypes of *elav* null mutant embryos (i.e., reduction in the number of commissural fibers crossing the midline, increased distance between the opposite longitudinal tracts and more lateral position of the neuron cell bodies) is reminiscent to that of hypomorphic *comm* mutant embryos and amorphic *comm* mutant embryos especially at early stages (stage 12 to late 13) (Tear et al., 1996 and data not shown). To examine whether *elav* could be playing a role in the inhibition of Robo-mediated repulsion, we varied *comm* gene dosage in *elav* mutants. We found that *elav* mutant embryos that are heterozygous for null mutations in *comm* exhibit a commissureless-like phenotype (Fig. 2F, compare with 2B), whereas heterozygous *comm* embryos display no abnormalities (data not shown). In *elav;comm/+* embryos commissures are missing entirely in more than 80% of the neuromeres ($n=252$, 23 embryos analyzed) indicating that most commissural axons fail to cross the midline in these embryos (Fig. 2F). The longitudinal tracts frequently adopt a more lateral position (i.e., higher distance from the midline, double arrow) relative to *elav* mutant embryos (90% of the neuromeres, $n=252$, 23 embryos analyzed) (compare to Fig. 2E). Moreover, reduction of *robo* dosage partially suppresses the *elav* mutant commissural phenotype (Fig. 2G). Thicker commissural tracts (relative to *elav* mutant embryos) are made in more than 50% of the neuromeres ($n=184$, 17 embryos analyzed) in *elav,robo/+* mutant embryos. Similarly, reduction of one dose of *slit* frequently leads to a fused commissures phenotype (>50% of the neuromeres, $n=167$, 16 embryos analyzed) (Fig. 2H). These data show that *elav* interacts genetically with *comm*, *robo* and *slit.* By contrast, reduction of the *fra* dose neither enhances nor decreases the *elav* mutant commissural phenotype (Supplementary Fig. 2).

comm mRNA expression is strongly reduced in elav null mutant CNS

To further investigate the interaction between *elav* and *comm*, the pattern of *comm* mRNA expression was analyzed in *elav* mutant embryos by *in situ* hybridization. In wild-type embryos, *comm* mRNA expression in the CNS is strong and

dynamic (Figs. 3A–D and Keleman et al., 2002). *comm* mRNA is first expressed in few neurons and as development proceeds, in many more neurons. The number of *comm*-positive neurons reaches its height at stage 14 and then gradually declines. In the absence of *elav*, *comm* mRNA levels are reduced in the nerve cord and never reach the peak characteristic of stage 14 wild-type embryos. At stage 13, *comm* mRNA expression is always reduced in neurons close to the midline in *elav* mutant embryos (100% of the neuromeres, $n=225$, 21 embryos analyzed) (Fig. 3E, arrow). At stage 14, *comm* mRNA expression is reduced in 100% of the neuromeres of *elav* mutant nerve cords ($n=366$, 34 embryos analyzed) (Figs. 3F and 5E). Lateral neurons express little or no *comm* mRNA whereas neurons closer to the midline are less affected (Figs. 3F and 5E, arrows). Expression of *comm* mRNA is not reduced in the lateral epidermis (external to the brackets) of stages 13 and 14 *elav* mutant embryos, thus providing an internal control for staining specificity (Figs. 3E and F, compare to 3A and B). At stage 15, *comm* mRNA expression frequently appears slightly weaker in the nerve cord of *elav* mutant embryos than in wild type (80% of the neuromeres, $n=240$, 22 embryos analyzed) (Figs. 3G, H). Whereas *comm* mRNA expression is reduced in *elav* mutant CNS neurons, it is not affected in midline glial cells (Figs. 3E–H, arrowheads) where it persists until stage 16 as in wild-type embryos (data not shown). These results show that *elav* is required for proper expression of *comm* mRNA in the nerve cord and that the absence of *elav* affects neuronal expression of *comm*.

We also analyzed the expression pattern of Comm protein in *elav* mutant and wild-type CNS. The reconstructed confocal images presented in Fig. 4 show that, as for *comm* mRNA, Comm protein levels are reduced in the nerve cord of *elav* mutant embryos (Figs. 4B and D) compared to *elav* heterozygous embryos. At stage 14, a 4/5 reduction of Comm expression is observed in the *elav* mutant CNS (120 neuromeres, 13 embryos analyzed) (Fig. 4E). Although the Comm protein

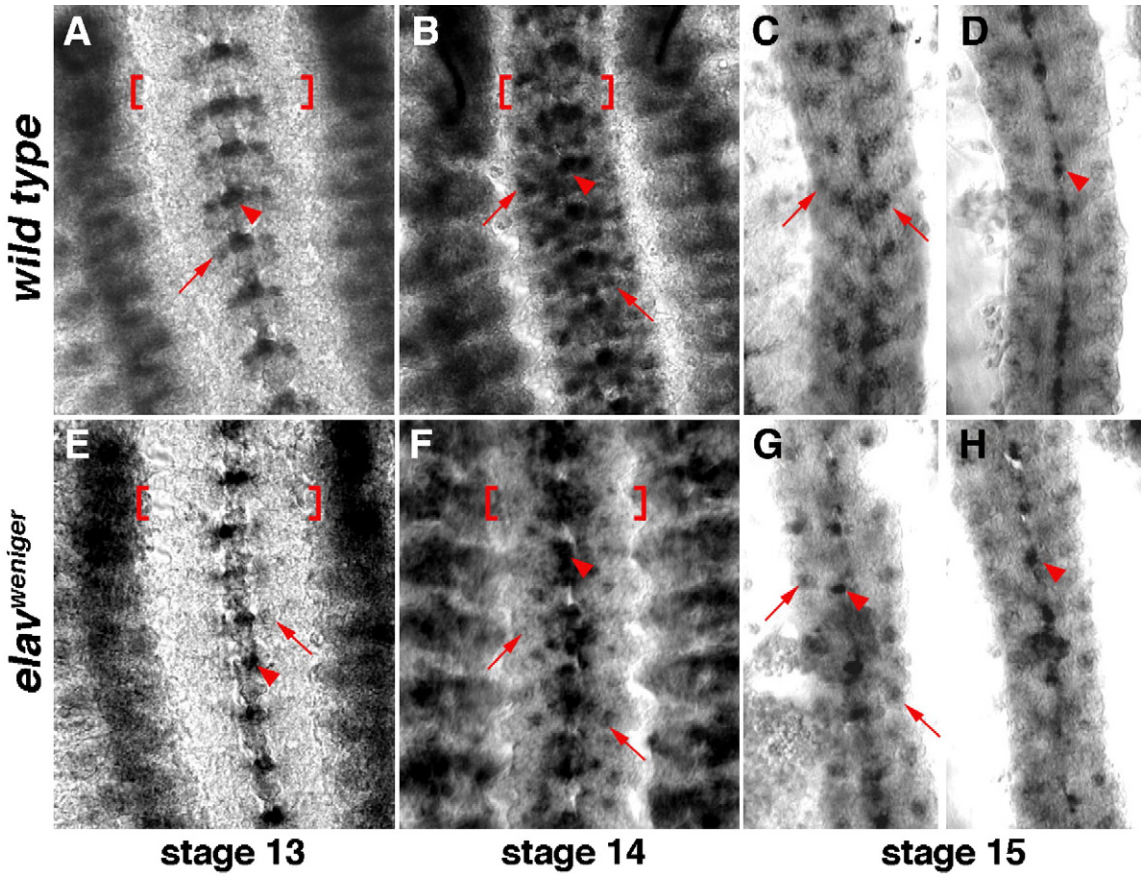

Fig. 3. *comm* mRNA expression is severely impaired in neurons of *elav* mutant CNS. Dorsal views of dissected CNS preparations of stage 13 to stage 15 embryos. (A–H) hybridized with a *comm* mRNA antisense probe. Anterior is up. Brackets in panels A, B, E, F delineate the CNS borders. The lateral epidermis is located on either side of the CNS. (A–D) Wild-type embryos. (A) During stage 13, *comm* mRNA starts to accumulate in the ventral nerve cord; it is detected in neurons close to the midline (arrow) and in midline glial cells (arrowhead). (B) At stage 14, *comm* expression is detected throughout the nerve cord and is expressed to high levels in lateral neurons (arrows). (C, D) At stage 15, *comm* mRNA expression has declined drastically in the nerve cord. Dorsal and ventral views of the same embryo are presented to distinguish expression in neurons (C, arrows) and midline cells (D, arrowhead). (E–H) *elav* null mutant embryos. (E) At stage 13, *comm* mRNA expression is reduced in neurons (arrow) whereas it is detected at normal levels in midline glial cells (arrowhead). *comm* mRNA levels are unaffected in the lateral epidermis. (F) At stage 14, *comm* mRNA levels are strongly reduced in the nerve cord and are not affected in the lateral epidermis. *comm* mRNA expression is not detected in lateral neurons (left arrow) but in few neurons closer to the midline (right arrow). Expression of *comm* mRNA is not affected in midline glial cells (arrowhead). (G, H) At stage 15, intensity of *comm* mRNA staining in the CNS is slightly lower in *elav* mutant embryos than in wild type. Dorsal and ventral views of the same embryo are presented. *comm* mRNA is detected in lateral neurons (G, arrows) and persist in midline cells (G, H, arrowheads).

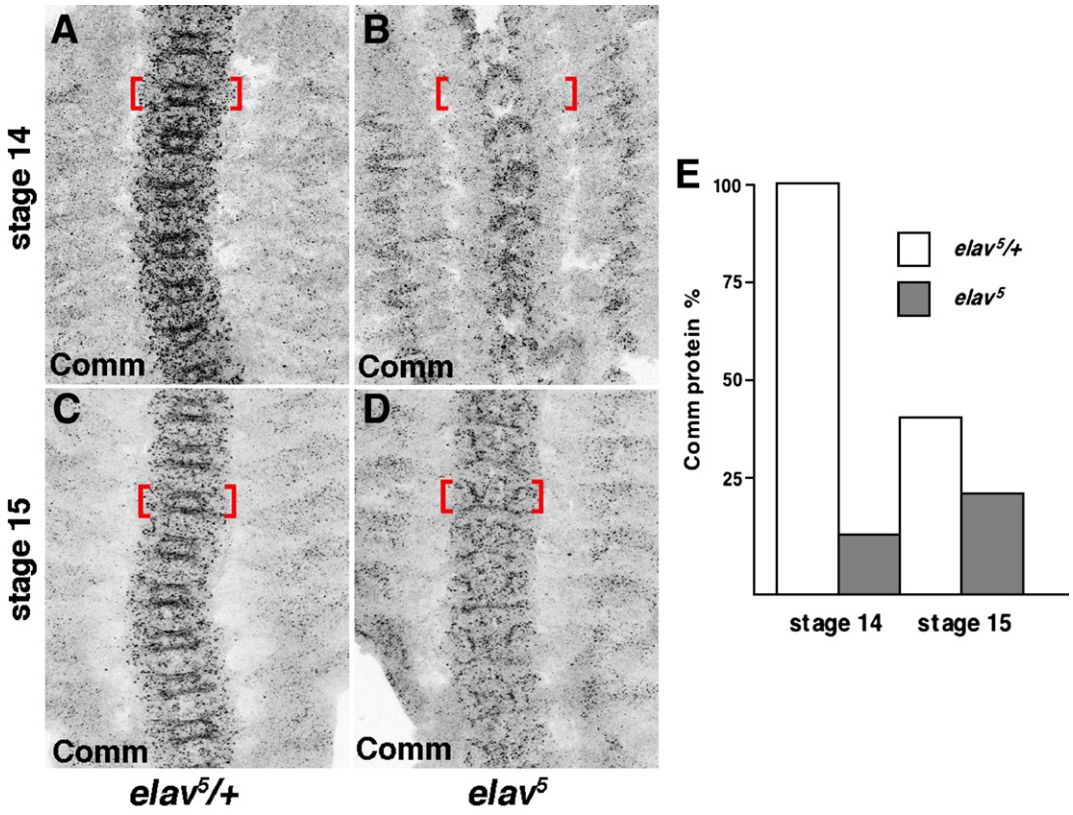

Fig. 4. Comm expression is strongly affected in the CNS of *elav* mutant embryos. (A–D) Dorsal views of dissected CNS preparations of stage 14 and early stage 15 embryos. Embryos are stained for the presence of Comm. The images correspond to untilted views of 3D reconstructions. Anterior is up. Brackets mark the CNS borders. (A, C) Stage 14 and stage 15 *elav* heterozygous embryos. (B, D) Stages 14 and 15 hemizygous *elav* mutant embryos. (A) At stage 14, Comm expression is at its height in the nerve cord of *elav* heterozygous embryos. It is detected in numerous vesicles (dots) in the whole nerve cord and in the anterior and posterior commissures. (B) At stage 14, Comm staining is severely affected in the entire nerve cord of *elav* mutant embryos. (C) At stage 15, Comm expression in the nerve cord of *elav* heterozygous embryos is weaker than at stage 14. (D) At stage 15, Comm staining is still reduced in the entire nerve cord of *elav* mutant embryos. (E) Quantification of Comm expression in stage 14 and stage 15 CNS. Histograms show the relative fluorescence intensities of *elav* heterozygous and *elav* mutant CNS, normalized against the intensity of *elav* heterozygous CNS at stage 14.

level increases slightly in *elav* mutant nerve cords at stage 15, it is about half the level seen in wild type (145 neuromeres, 16 embryos analyzed) (Fig. 4E). These results confirm that *comm* expression (both mRNA and protein) is strongly reduced in *elav* mutant CNS and never reaches the peak observed in stage 14 wild-type embryos.

Comm protein expression in the embryonic midline glia is dynamic. It is first detected at high levels in a subset of midline cells during stage 12 and then decreases (Tear et al., 1996). It becomes more difficult to detect in midline glial cells at stage 14 (Figs. 4A, C). At stage 13, Comm protein expression levels in midline glial cells are equivalent in *elav* mutant and wild-type embryos (Supplementary Figs. 1B, F). Altogether, these results show that whereas Comm expression is affected in neurons of *elav* mutant embryos, expression in midline glia is not.

Expression of an elav transgene in posterior neurons restores both comm mRNA expression in these cells and posterior commissure formation in elav null mutant embryos

The fact that two independent null alleles of *elav* (*elav*^{5} and *weniger*^{E476}) induced the same CNS phenotypes (reduction in the number of commissures, altered *comm* mRNA expression) strongly suggested that downregulation of *comm* mRNA was indeed due to the absence of *elav*. Expression of an *elav* transgene in the *elav* pattern using the GAL4/UAS system (Brand and Perrimon, 1993) rescued lethality of *elav* embryos as well as all other postembryonic functions of *elav* (Yannoni and White, 1999, and data not shown). To further confirm the relationship between reduced *comm* mRNA expression and reduction in the number of commissures in *elav* mutant embryos, we expressed an *elav* transgene in a subset of CNS neurons in *elav* mutant embryos using the GAL4/UAS system (Brand and Perrimon, 1993). Two commissures per neuromere are already formed in wild-type embryos at stage 14 (Fig. 5A) and neurons in the nerve cord of these embryos express high levels of *comm* mRNA (Figs. 3B and 5D). In contrast to wild type, only thin commissural fibers have crossed the midline in each neuromere of *elav* mutant embryos at stage 14 (100% of the neuromeres, $n=408$, 37 embryos analyzed) (Fig. 5B) and *comm* mRNA expression is reduced in 100% of the neuromeres ($n=360$, 36 embryos analyzed) (Figs. 3F and 5E). Most of *engrailed*-expressing neurons send their axons across the midline into the posterior commissure (W. Joly and F. Maschat, personal communication). When *elav* expression is induced in *engrailed*-expressing neurons, a posterior commissure is frequently formed

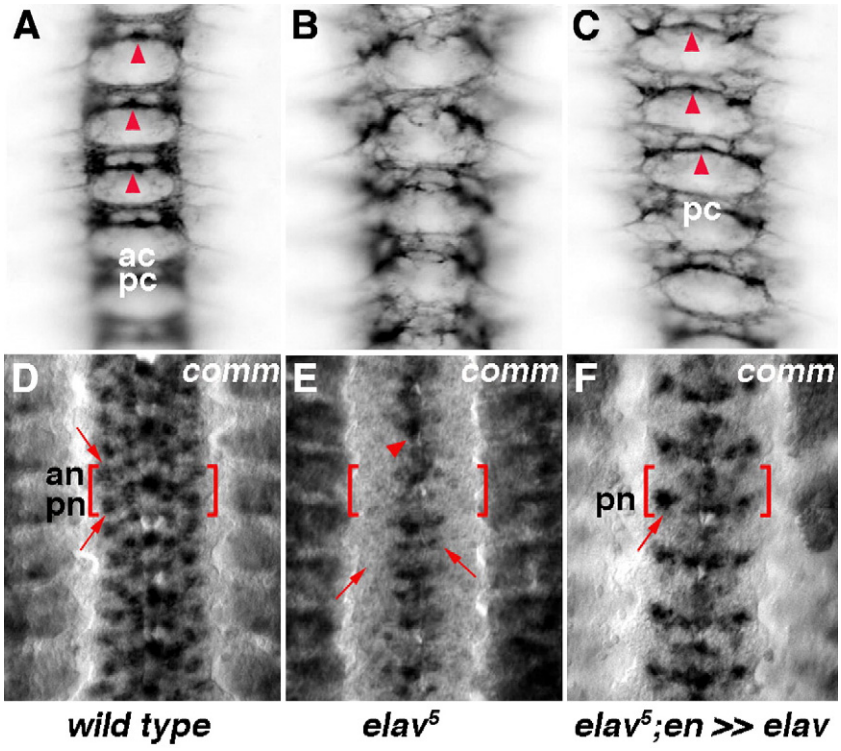

Fig. 5. Expression of *elav* in posterior neurons rescues posterior commissure formation and *comm* expression in *elav* mutant embryos. Dorsal views of dissected CNS preparations of stage 14 embryos. (A–C) CNS stained for the presence of all axons using MabBP102. (D–F) Hybridized with a *comm* mRNA antisense probe. Brackets delineate one neuromere. Anterior is up. (A) Wild-type embryos present two commissures per neuromere, anterior (ac) and posterior (pc). (B) In *elav* null mutant embryos commissures do not form at this stage and few commissural axons have crossed the midline. (C) Following expression of UAS-*elav* in posterior neurons using en-GAL4 driver, a posterior commissure is restored in each segment in *elav* mutant embryos (arrowheads). (D) In wild type, *comm* mRNA levels reach their highest intensity in the nerve cord, being detected both in anterior (an) and posterior (pn) neurons in each neuromeres (arrows). (E) In *elav* null mutant embryos, *comm* mRNA expression is strongly reduced in the nerve cord. (F) Following expression of an *elav* transgene in posterior neurons of each segments of the nerve cord, *comm* mRNA expression is restored in the corresponding neurons (arrow) in *elav* mutant embryos. *en ≫ elav* refers to *en-GAL4;UAS-elav.*

in *elav* mutant CNS (>90% of the neuromeres, $n=220$, 20 embryos analyzed) (Fig. 5C, arrowheads). In addition, *comm* mRNA expression is always restored to normal levels in posterior neurons in such embryos (100% of the neuromeres, $n=198$, 18 embryos analyzed) (Fig. 5F, arrow). These results confirm that inhibition of *comm* mRNA expression in CNS neurons of *elav* mutant embryos is indeed due to the lack of *elav* in these cells and suggest that fewer commissures are formed in *elav* mutant embryos because commissural neurons express lower levels of *comm* mRNA.

Forced expression of comm in commissural neuron subsets rescues midline crossing in elav null mutant embryos

Pan-neuronal expression of a *comm* transgene using the UAS/GAL4 system (Brand and Perrimon, 1993) has drastic effects on midline axon guidance, leading to a dominant fused-commissures phenotype (Kidd et al., 1998b, and data not shown). To determine whether commissural axons do not cross the midline in *elav* mutant embryos because *comm* mRNA expression is downregulated in the corresponding neurons, we attempted to rescue the *elav* mutant midline crossing phenotype by expression of a *comm* transgene in only a subset of commissural neurons (EW and EG neurons) (Fig. 6). In *elav* mutant embryos, EW and EG axons often do not cross the midline as mentioned earlier (Figs. 1F and 6B, arrowheads). In contrast, when *comm* is expressed in these *elav* mutant neurons, EW and EG axons once again cross the midline (80% and 95% of the neuromeres, respectively, $n=163$, 21 embryos) (Fig. 6C, arrowheads). We confirmed that expression of *comm* per se is not sufficient to induce wild-type EW and EG commissural axons to re-cross the midline (data not shown). The specificity of the rescue was also tested by inducing expression of Fra (the attractive receptor for Netrin) in EW and EG neurons. Expression of a *fra* transgene in these neurons does not modify the proportion of contralateral projection defects in *elav* mutant embryos. For example, EW axons fail to cross the midline in 75% of the neuromeres in such embryos ($n=72$, 9 embryos analyzed) (Fig. 6D, arrowhead). Thus, providing Comm in *elav* commissural neuron subsets specifically rescues the midline crossing defects of their axons. This result supports the notion that many commissural neurons do not send their axons across the midline in *elav* mutant embryos because they express low or insufficient levels of Comm protein. It also further indicates that *elav* exerts a cell-autonomous control on *comm* expression in commissural neurons.

Discussion

In this paper, we report the characterization of the commissural phenotype of *elav* null mutant embryos providing

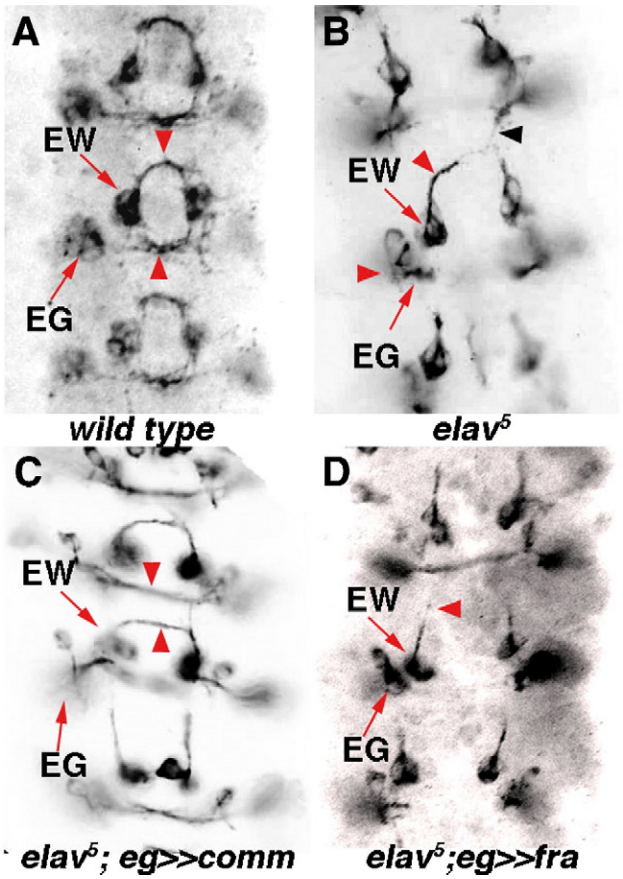

Fig. 6. Expression of *comm* in commissural neuron subsets rescues their midline crossing defects in *elav* mutant embryos. Dorsal views of dissected CNS preparations of stage 16 embryos. (A–D) Stained for the presence of Tau:β-galactosidase to visualize cell morphology (axons and cell bodies). Tau:β-galactosidase is expressed in EW and EG commissural neurons using an *eg-GAL4* driver. Anterior is up. (A) In wild-type embryos, EW and EG neurons extend their axon across the midline. (B) In *elav* null mutant embryos, most of the EW and EG axons do not cross the midline but grow ipsilaterally (arrowheads). (C) Specific expression of *comm* in the EW and EG neurons rescues their axon midline crossing in *elav* mutant embryos (arrowheads). (D) In contrast, expression of *fra* in the EW and EG neurons has no effect on their axon midline crossing defects in *elav* mutant embryos. *eg≫comm* and *eg≫fra* refer to *eg-GAL4;UAS-comm* and *eg-GAL4;UAS-fra*, respectively.

further insights into the role of the RNA-binding protein ELAV in neuronal development. The principal finding of this study is that the reduced number and abnormal structure of commissures in *elav* null mutant embryos is caused, at least in part, by reduction of *comm* expression in commissural neurons leading to failure of these cells to extend their axons across the CNS midline. Therefore, our results identify *elav* as an axon guidance gene and they also provide the first example of a gene whose function is necessary for *comm* expression in neurons.

elav is required for commissural axon growth across the CNS midline

In the *Drosophila* embryonic nerve cord, a large majority (90%) of the neurons are commissural (Bate and Martinez Arias, 1993). In *elav* null mutant embryos many commissural axons do not grow toward the midline preferentially extending on their own side (Fig. 7). This leads to a reduction in the number and thickness of the commissural tracts formed. When an *elav* transgene is specifically expressed in posterior neurons of each nerve cord segment, posterior commissures are formed once again in *elav* mutant nerve cords (Fig. 5), suggesting that *elav* is cell-autonomously required for commissural axon midline crossing. We found that the commissural phenotype in *elav* mutant nerve cords is not due to neural cell loss (Fig. 1 and Supplementary Fig. 1) in contrast to what was previously suggested by others (Jimenez and Campos-Ortega, 1987). In addition, our observations show that neurons and midline glial cells are correctly specified in embryos lacking *elav* (Supplementary Fig. 1 and data not shown) indicating that the commissural axon midline crossing defects in *elav* mutant embryos do not result from changes in neuronal identities nor from an indirect effect on midline glial cell fate. The reduced number and thickness of the commissural tracts in *elav* null mutant embryos (Fig. 1) could be the result of a delay in the differentiation of neurons. However, the normal spatial and temporal expression pattern of a number of neuronal markers confirms that neurons are born at the right stage and indicate that neurons differentiate properly in embryos lacking *elav*.

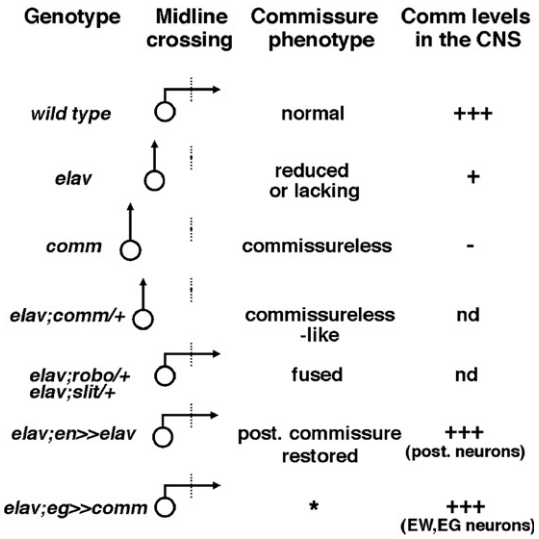

Fig. 7. Schematic of the commissural axon guidance decisions in different *elav* backgrounds. The midline is symbolized by a dashed-line. In wild-type embryos most CNS axons extend along a commissural pathway and cross the midline. The CNS is symmetrically organized, with the same neurons on either side of the midline. In *comm* null mutant embryos, all commissural axons fail to cross the midline. Neuron cell bodies are located in an extreme lateral position. In *elav* null mutants, many commissural axons do not cross the midline and the position of neuron cell bodies is slightly more lateral than normal. In *elav;comm/+* mutant embryos, most commissural axons fail to cross the midline as in *comm* embryos. Neuron cell bodies are displaced more laterally than in *elav* mutants. In *elav;robo/+* and *elav;slit/+* mutant embryos, a greater proportion of CNS axons can cross the midline than in *elav* embryos. When an *elav* transgene is expressed in posterior neurons of a given segment in *elav* mutant embryos, the axons of these neurons extend across the midline once again forming a posterior commissure. When *comm* is expressed in specific commissural neuron subsets in *elav* mutant embryos, axon midline crossing is rescued for these neurons. *The midline crossing rescue for a small subset of axons does not modify the commissural phenotype. nd, not determined.

Thus, the wrong axon path-finding decisions of a great proportion of commissural neurons in *elav* mutants are likely to be the result of an altered program of axon guidance in CNS neurons within the period of commissure establishment.

elav acts within the Robo/Slit signalling pathway

Reduction in the number of commissural fibers crossing the midline in *elav* mutant embryos can be interpreted either as a decrease in growth cone attraction towards the midline and/or an increase of growth cone repulsion from the midline. An earlier genetic interaction study between *weniger* and *fra* suggested that *weniger* (identified as an allele of *elav*, U. Lammel and C. Klämbt, unpublished results) does not act within the attractive Fra/Netrin signalling pathway (Hummel et al., 1999b). In this paper, we provide genetic and molecular evidence that *elav* specifically acts within the repulsive Robo/Slit signalling pathway (Fig. 7) and not within the attractive Fra/Netrin signalling pathway (Supplementary Fig. 2). We show that *elav* interacts genetically with the key components of the Robo/Slit signalling pathway (*comm*, *robo* and *slit*) in a dose-sensitive manner (Fig. 2). In particular, a strong dominant enhancement of the *elav* mutant commissural phenotype is observed when the *comm* gene dose is reduced, suggesting that *elav* and *comm* work together in the same pathway. Our findings that *comm* mRNA levels are strongly reduced in *elav* mutant nerve cords and that expression of an *elav* transgene in neuron subsets specifically restores *comm* mRNA expression in these cells show that *elav* is necessary for proper neuronal expression of *comm* (Figs. 3 and 5). The commissural phenotype in *elav* mutant embryos is reminiscent of that of hypomorphic *comm* mutant embryos, where *comm* function is decreased (Tear et al., 1996). Given that *comm* expression is decreased in the absence of *elav*, the commissureless-like phenotype induced when the *comm* gene dose is reduced (50%) in *elav* mutant embryos (Fig. 2) suggests comparisons with that of strong hypomorphic *comm* mutants. In addition to that, our results reinforce the notion that *elav* acts within the Robo/Slit signalling pathway via the control of *comm* mRNA expression by establishing a causal relationship between the *elav* mutant commissural phenotype and the inhibition of *comm* mRNA expression in neurons (Fig. 7). Finally, our finding that forced expression of *comm* in specific commissural neuron subsets significantly restores their axon midline crossing in *elav* mutant embryos (Fig. 6) suggests that the lack or decrease of *comm* mRNA may be the primary reason for the failure of many *elav* mutant commissural axons to cross the midline.

Proper neuronal comm mRNA expression requires elav

The complex and highly dynamic pattern of *comm* mRNA expression during commissure establishment as well as the cell-autonomous neuronal requirement for *comm* suggests that commissural axon guidance is dependent on regulated expression of *comm* in neurons (Keleman et al., 2002, 2005; McGovern and Seeger, 2003). In this paper, we show that the lack of *elav* specifically alters *comm* expression (both RNA and protein) in the embryonic nerve cord within the period of commissure establishment and not in other tissues such as the epidermis and the gut where *comm* is also expressed (Fig. 3 and data not shown). We found that, in the nerve cord of *elav* mutant embryos, *comm* mRNA levels are reduced in neurons where *elav* in expressed, but not in midline glial cells where *elav* is absent. No difference in the expression levels of various nuclear (Eve, Odd, Cas) and membrane markers (FasII, Futsch) in the nerve cord was noted between *elav* mutant and wild-type embryos (Supplementary Fig. 1 and data not shown), indicating that *comm* downregulation in *elav* mutant nerve cords is specific and that the absence of *elav* does not affect overall gene expression in neurons. In addition, our findings that expression of an *elav* transgene in neuron subsets restores *comm* mRNA expression in these cells (Fig. 5) and that expression of a *comm* cDNA in specific commissural neuron subsets rescues axon midline crossing in *elav* mutant embryos (Fig. 6) indicate that *elav* controls *comm* mRNA expression in a cell autonomous fashion. Taken together, the rescue experiments and the expression data support the model in which *comm* functions in neurons rather than in midline glia for midline crossing (Keleman et al., 2005).

In wild-type embryonic nerve cords, *comm* mRNA expression peaks at stage 14 when commissures are formed, and declines drastically thereafter between stages 15 and 16. It has been shown that *comm* is necessary for midline crossing and that the temporal expression pattern of *comm* mRNA in neurons coincides with midline crossing (Keleman et al., 2002). In the nerve cord of *elav* mutant embryos, *comm* mRNA levels are always reduced compared to wild type and never reach the intensity of the peak observed in wild type even in the later stages (Fig. 3). This confirms that the reduced number and thickness of commissures in *elav* mutant embryos do not simply result from a temporal delay in the kinetics of *comm* mRNA expression in the nerve cord but from the fact that a great proportion of commissural neurons do not express proper levels of *comm* mRNA.

In situ hybridizations with specific antisens RNA probes did not reveal any difference in the expression level of *comm* pre-mRNA in the nerve cord of *elav* mutant and wild-type embryos, suggesting that *elav* does not affect synthesis or stability of *comm* pre-mRNAs (data not shown). By contrast, the levels of *comm* mRNA expression in the nerve cord are low in the absence of *elav* compared to wild type (Fig. 3), suggesting that defects in the accumulation of *comm* mRNAs occur in neurons. Because neuronal transcription of *comm* does not seem to be affected in *elav* mutant embryos, at what levels *elav* could interfere on post-transcriptional regulation of *comm* expression? *elav* has been shown to promote the synthesis of the neural-specific splice isoform of several pre-mRNAs (Koushika et al., 2000). Although *comm* does not seem to be alternatively spliced, it is possible that *elav* could promote or inhibit constitutive splicing of *comm*. We wished to determine, by quantitative RT-PCR, if *comm* splicing was affected in *elav* mutant nerve cords; however, this turned out to be technically challenging. Indeed, because the lack of *elav* does not alter expression of *comm* mRNA in the epidermis (Figs. 3E–F) or in

the gut (not shown), dissection of the nerve cords from formaldehyde-fixed embryos was absolutely required and makes it impossible to quantify *comm* transcripts only in one tissue (data not shown). Alternatively, *elav* could have a primary role in regulating *comm* mRNA stability in the cytoplasm. In support of this, vertebrate homologues of ELAV have been implicated in regulating stability and translatability of their mRNA targets (reviewed in Keene, 2001) and evidence for 3′ UTR-dependent autoregulation of *elav* has been reported (Samson and Chalvet, 2003). In addition, ELAV localization in neurons is not strictly nuclear especially at early stages and the function of ELAV could be necessary in this cell compartment. Finally, ELAV could be necessary for correct nuclear export of *comm* mRNAs. Indeed, TAP/NXF1, a nuclear export factor responsible for exporting the majority of cellular mRNAs to the cytoplasm (reviewed in Erkmann and Kutay, 2004) has been shown to interact specifically with HuD, a vertebrate homolog of ELAV (Saito et al., 2004).

ELAV encodes the founding member of the Hu family of RNA-binding proteins. These proteins are found in RNP complexes composed of a number of RNA-binding proteins including some from other protein families (reviewed in Agnès and Perron, 2004). In *Drosophila*, ELAV has been shown to interact physically with another RNA-binding protein from the same family, called FNE that is also expressed in neurons (Giot et al., 2003; Samson and Chalvet, 2003). It would be interesting to determine in the future if *comm* pre-mRNAs and/or mRNAs are found in RNP complexes together with ELAV and if *comm* RNAs can be bound by ELAV *in vivo*. Such a study should help to determine whether control of *comm* mRNA expression by ELAV is direct or indirect via expression of a gene upstream of *comm*.

elav may have multiple roles in the CNS

The function of *elav* is essential for proper development of the nervous system. Indeed, mutations in *elav* have been shown to induce a highly disorganized structure of the embryonic nerve cord with abnormal commissural and connective axon patterns (Hummel et al., 1999a; Jimenez and Campos-Ortega, 1987), suggesting that *elav* could play several roles during development of this tissue. The search for ELAV's targets has been undertaken with the idea that these would reveal some of the roles that *elav* might play in neuron differentiation. Hence, ELAV has been shown to be essential to promote neural-specific splicing of three pre-mRNAs (*arm*, *nrg* and *ewg*) (Koushika et al., 2000). However, it is not known for which step and/or processes of neural development these interactions are required and potential roles of *elav* in cell–cell adhesion and/or axon fasciculation can only be inferred from phenotypic analyses of *arm* and *nrg* mutant embryos and expression studies (Bieber et al., 1989; Loureiro and Peifer, 1998; Hall and Bieber, 1997).

In this paper, we have focused on the *elav* mutant commissural phenotype in the embryo and searched for its earliest causes. We found that commissural axon guidance across the midline is severely impaired in *elav* mutant nerve cords and established a causal relationship between this phenotype and the inhibition of *comm* mRNA expression in neurons (Fig. 7). An interesting feature of our study is that although *elav* mutations lead to very complex phenotypes these can be dissected by meticulous phenotypic analyses.

elav may play other roles in commissure formation. For instance, careful analysis of the *elav* mutant commissural phenotype reveals that some commissural axons cross the midline in the wrong commissures in *elav* embryos (e.g., EG axons extending in the posterior commissure; Fig. 6B, black arrowhead). This phenotype, reminiscent of that of *derailed* loss-of-function mutants (Bonkowsky et al., 1999), suggests that *elav* could also be involved in the control of axon routing across the CNS midline. Finally, we found that some ipsilateral neurons extend their axons inappropriately across the midline in *elav* mutant embryos (data not shown), suggesting that *elav* may also be required for the proper expression of other molecules involved in axon guidance at the midline because the ipsilateral neurons are *comm*-negative neurons (Georgiou and Tear, 2002; Keleman et al., 2002).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.09.028.

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