The role of Wnt5 during Axon guidance

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Chapter 2

The Drosophila Wnt5 protein mediates selective axon fasciculation in the embryonic central nervous system

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The Drosophila Wnt5 protein mediates selective axon fasciculation in the embryonic central nervous system

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Abstract

The decision of whether and where to cross the midline, an evolutionarily conserved line of bilateral symmetry in the central nervous system, is the first task for many newly extending axons. We show that Wnt5, a member of the conserved Wnt secreted glycoprotein family, is required for the formation of the anterior of the two midline-crossing commissures present in each *Drosophila* hemisegment. Initial path finding of pioneering neurons across the midline in both commissures is normal in *wnt5* mutant embryos; however, the subsequent separation of the early midline-crossing axons into two distinct commissures does not occur. The majority of the follower axons that normally cross the midline in the anterior commissure fail to do so, remaining tightly associated near their cell bodies, or projecting inappropriately across the midline in between the commissures. The lateral and intermediate longitudinal pathways also fail to form correctly, similarly reflecting earlier failures in pathway defasciculation. Panneural expression of Wnt5 in a *wnt5* mutant background rescues both the commissural and longitudinal defects. We show that the Wnt5 protein is predominantly present on posterior commissural axons and at a low level on the anterior commissure and longitudinal projections. Finally, we demonstrate that transcriptional repression of *wnt5* in AC neurons by the recently described Wnt5 receptor, Derailed, contributes to this largely posterior commissural localization of Wnt5 protein.

Keywords: Drosophila; Wnt genes; CNS; Axon fasciculation; Commissural axon tracts; Longitudinal axon tracts

Introduction

The correct wiring of a nervous system requires that a large number of neurons stereotypically extend their axonal processes to make synaptic contacts with their muscle and neuronal targets. The leading portion of the axon, the growth cone, is faced with a bewildering number of routing decisions as it travels, frequently many hundreds of cell body diameters, to its target. Path finding relies on the growth cone receiving and interpreting guidance cues presented to it at intermediate points in its journey (Bate, 1976). While the growth cone likely integrates multiple signals at many points, the initial extension of axons has received the most scrutiny; several of the important attractive and repulsive guidance cues and their neuronal receptors have been identified (reviewed in Araujo and Tear, 2003; Dickson, 2002; Mueller, 1999).

In *Drosophila*, the majority of the neurons of the embryonic ventral cord are born near the ventral midline, a morphological and functional line of symmetry whose vertebrate equivalent is the floorplate (reviewed in Tessier-Lavigne and Goodman, 1996). Axon tracts in the mature embryo form a characteristic ‘‘ladder-like’’ structure reflecting the presence of two longitudinal

tracts that extend in the anterior –posterior axis and two commissural tracts, the Anterior (AC) and Posterior Commissures (PC) that bridge the longitudinal pathways in every segment.

The first choice for many newly extending axons is whether to cross the ventral midline. The axons of certain neurons, the ipsilaterals, do not cross the midline, but instead project with other longitudinal axons toward the anterior or posterior. Other axonal pathways, the contralaterals, cross the midline, extend along the longitudinal tracts and do not recross. The decision to cross or not and the prevention of repeated midline crossing are regulated by interactions between the extending axons and the Midline Glia cells (MG), specialized cells that emanate repulsive and attractive signals and underlie both the AC and PC at the ventral midline (reviewed in Jacobs, 2000; Klambt *et al.,* 1996). The Netrin and Slit proteins are among the best characterized of the midline-derived cues. Netrins, signalling through axonal Frazzled receptors, primarily act as attractants (Harris *et al.,* 1996; Mitchell *et al.,* 1996), although repulsive Netrin-dependent signalling has also been reported (Keleman and Dickson, 2001). Slit protein signalling through the axonal Robo family of receptor proteins repels axons away from the midline, thus preventing them from recrossing (Battye *et al.,* 1999; Kidd *et al.,* 1998, 1999; Rajagopalan *et al.,* 2000a, b). In addition to their midline roles, evidence has been provided that the expression domains of the three Robo proteins also delimit lateral domains within the longitudinal axon tracts (Rajagopalan *et al.,* 2000b; Simpson *et al.,* 2000a).

Initial outgrowth and path finding of pioneering axons is, at least in part, dependent on both their interactions with the glial cell scaffold (reviewed in Hidalgo, 2003) and their responsiveness to midline-derived cues. Subsequently, ‘‘follower’’ axons fasciculate with the pioneers to form multi-axon fascicles. Regulation of the relative balance between fasciculation and defasciculation through regulation of cell adhesion molecule activities at specific points allows individual axons to branch off to follow separate trajectories (reviewed in Goodman and Doe, 1993; van Vactor, 1998). Several cell adhesion proteins have been shown to act in the fasciculation of embryonic *Drosophila* axons. Embryos bearing mutations in the *Drosophila* NCAM ortholog, fasciculin II (fasII), display inappropriately defasciculated axons, whereas axons that overexpress fasII become hyper-fasciculated (Lin *et al.,* 1994). The Connectin (Conn) protein effects homophilic interactions between motoneurons (Nose *et al.,* 1997). The beaten path (beat-Ia) gene encodes an Ig domain-containing protein secreted from axonal growth cones (Fambrough and Goodman, 1996). In beat-Ia mutants, axons become hyperfasciculated in a manner suppressible by fasII and conn mutations, suggesting that Beat-Ia acts as a secreted anti-adhesive factor. There are 13 other Drosophila beat genes; interestingly, those four whose full ORF sequences have been determined encode transmembrane or GPI-linked proteins (Pipes *et al.,* 2001). Genetic interactions between beat-Ia and beat-Ic (encoding a transmembrane Beat protein) indicate their complementary functions, with beat-Ic and beat-Ia acting to increase and decrease adhesiveness, respectively. The Beat receptor(s) have not yet been identified.

Most of the mechanisms regulating guidance across the midline that have been uncovered thus far operate in both the AC and PC. Little is known about the mechanisms that underlie the choice of axons to go through either the AC or the PC. One gene implicated in this process is derailed (drl), a member of RYK subfamily of receptor tyrosine kinases (Bonkowsky *et al.,* 1999; Callahan *et al.,* 1995). Recent studies have demonstrated that interactions between the Drl and Wnt5 proteins play an important role in preventing AC axons from inappropriately crossing in the PC (Yoshikawa *et al.,* 2003). AC axons are less tightly fasciculated in the drl mutant (Callahan *et al.,* 1995; Speicher *et al.,* 1998), suggesting that Drl may act to regulate interneuronal adhesion.

wnt5 is a member of the Wnt gene family, a large group of evolutionarily conserved genes encoding secreted glycoproteins that play roles at many developmental stages in a variety of tissues (reviewed in Cadigan and Nusse, 1997). Among other roles in the nervous system (reviewed in Patapoutian and Reichardt, 2000), Wnt proteins act in cell fate determination (Baker *et al.,* 1999; Bhat, 1998; Chu-LaGraff and Doe, 1993; Deshpande *et al.,* 2001; Dorsky *et al.,* 1998; Garcia-Castro *et al.,* 2002; Muroyama *et al.,* 2002; Wilson *et al.,* 2001), synapse formation and maintenance (Hall *et al.,* 2000; Krylova *et al.,* 2002; Lucas and Salinas, 1997; Packard *et al.,* 2002) and as mitogens (Castelo-Branco *et al.,* 2003; Chenn and Walsh, 2002; Megason and McMahon, 2002).

Previously, we presented evidence that the *Drosophila* Wnt5 protein is found on the embryonic CNS axon tracts (Fradkin *et al.,* 1995). The *wnt5* gene encodes a highly unusual Wnt protein that bears a long amino terminal extension to the Wnt-homologous domain that contains no known conserved domains. In this report, we present a detailed analysis of the *wnt5* mRNA and protein expression domains and demonstrate a crucial role for Wnt5 in formation of the major axon tracts during embryonic CNS development through examination of embryos lacking *wnt5*. Wnt5 protein is required for the separation or defasciculation of early axonal projections that subsequently form the mature commissural and longitudinal connectives. We also provide evidence that the porcupine (porc) gene is a member of the Wnt5 signalling pathway and that the *wnt5* gene is itself one of the downstream targets.

Materials and methods

Generation and affinity purification of the Wnt5 antibody and immunoblotting

Antiserum against the same region of the Wnt5 prodomain carboxyterminus, as previously described (Fradkin *et al.,* 1995), was raised in rabbits and affinity purified except that a His-tagged immunogen, rather than trpE fusion protein, was used. Wnt5 in embryonic lysates was analyzed using standard Western blotting techniques and chemiluminiscent detection. Quantitation of Wnt5 protein normalized to a-tubulin loading controls was performed using a Lumi-Imager and LumiAnalyst software (Boehringer-Mannheim).

Immunohistochemistry and RNA *in situ* analysis

Antibody labelings (Patel, 1994), RNA *in situ*s (Tautz and Pfeifle, 1989), and staging of embryos (Wieschaus and Nusslein-Volhard, 1986) were performed as described. Affinity-purified Wnt5 antibody was used to stain unfixed embryonic ventral cords that were subsequently fixed in 4% formaldehyde before incubation with a fluorescent secondary antibody. Fixation with formaldehyde, paraformaldehyde, or Bouins' fixative before application of the primary antibody results in either no or variable staining results (data not shown). The following antibodies were used: rabbit antiRobo2 antisera (Rajagopalan *et al.,* 2000a), mAb BP102 (A. Bieber, N. Patel, C.S. Goodman, unpublished), mAb 1D4 (Vactor *et al.,* 1993), mAb anti-Sex-lethal M18 (Bopp *et al.,* 1991), mAb anti-Wrapper (Noordermeer *et al.,* 1998), rabbit anti-h-Galactosidase (Cappel), mAb 22C10 (anti-Futsch; Zipursky *et al.,* 1984), and the 9E10 anti-Myc mAb (Evan *et al.,* 1985). For visualization of the Sema2b-H -Myc expressing axon trajectories, embryos were fixed with paraformaldehyde and treated as described (Nagaso *et al.,* 2001); in other cases, formaldehyde-fixed embryos were stained. For double-fluorescent RNA *in situ*s and for fluorescent RNA *in situ*s in combination with fluorescent antibody staining, protocols developed by the Bier and McGinnes http://www.biology.ucsd.edu/~davek/index.html) and Frasch (Knirr *et*

al., 1999) groups were used. The posterior and anterior commissures were identified by their positions relative to the segmental border.

Generation of *wnt5* mutant alleles

A P-element (p[GT1]Wnt5BG00642, Bellen *et al.,* in press) inserted approximately 500 base pairs away from the *wnt5* start codon was used in a P-element mobilization screen (Tower *et al.,* 1993) to create small deletions removing regions of the *wnt5* gene. Fourteen lines that lack parts of the *wnt5* ORF were recovered by PCR screening. No Wnt5 protein can be detected in two of the 14 lines (lines $wnt5^{400}$ and $wnt5^{207}$) with the anti-Wnt5 antibody. The size of the deletions was determined by PCR of genomic DNA and sequence analysis (Fig. 2). A precise P-element excision line, $wnt5^{9}$, was generated and verified by sequence analysis for use as a control.

Generation of transgenic flies and genetics

To investigate the effects of ectopic Wnt5 expression, we generated transgenic flies bearing the full-length *wnt5* cDNA cloned downstream of the yeast GAL4 binding sites in the pPUAST vector (Brand and Perrimon, 1993). Twenty independent homozygous transgenic lines were generated and two of these, UAS-Wnt5 11C on the second chromosome and UAS-Wnt5 201 on the third chromosome, were used for ectopic expression studies using the following GAL4 transcriptional activator lines: Elav-GAL4 (Luo *et al.,* 1994), which drives expression in all neurons; ApGAL4 (Rincon-Limas *et al.,* 1999), which drives expression in subset of neurons in the medial longitudinal pathway; Ftz-neurogenic-GAL4 (Lin *et al.,* 1994) and Sca-GAL4 (Budnik *et al.,* 1996), which drive in subsets of neurons; Btl-GAL4 (Shiga *et al.,* 1996), which drives expression in the MGP and VUM neurons; Repo-GAL4, which drives expression in all lateral glia and Slit-GAL4 (Albagli *et al.,* 1996);and Sim-GAL4 (Golembo *et al.,* 1996),which express GAL4 predominantly in all midline glia. Several stocks were used to visualize specific neuronal pathways: Sema2b-H -Myc (K. Senti, B. Dickson, unpublished), Eg-GAL4 (Dittrich *et al.,* 1997),Ap-H -LacZ (Lundgren *et al.,* 1995), and UAS-H -LacZ (Benveniste *et al.,* 1998). Stocks bearing appropriate driver and reporter genes were generated in the $wnt5^{400}$ background. To express Wnt5 protein on all axons in a wnt5 mutant background, $wnt5^{400}$;UAS-Wnt5 flies were crossed with $wnt5^{400}$;ElavGAL4 flies, to express Wnt5 in all MG in a *wnt5* mutant background, $wnt5^{400}$;UAS-Wnt5werecrossedwith $wnt5^{400}$;Sim-GAL4 or $wnt5^{400}$;Slit-GAL4, and to express wnt5 in all lateral glia in a wnt5 mutant background, $wnt5^{400}$;UAS-Wnt5 flies were crossed to $wnt5^{400}$;RepoGAL4/TM3 flies. In addition, offspring were recrossed to allow the collection of embryo populations containing individuals bearing two copies of each of the transgenes. A UAS-Wnt5, Sim-GAL4 recombinant second chromosome was generated to study the effects of the X-linked porc mutation. Male porc embryos were distinguished from female porc/+ embryos by staining with the female-specific Sexlethal antibody. The drl^{RED2} (Bonkowsky *et al.,* 1999) and the porc PB16 (Eberl *et al.,* 1992) null alleles were used.

Results

wnt5 mRNA and protein are expressed predominantly on PC neurons

We examined the *wnt5* expression domains by RNA *in situ* analyses and antibody stainings using a Wnt5 antibody. *wnt5* is expressed predominantly in the CNS from stage 12 onward throughout embryonic development. *wnt5* mRNA was found in a large subset of presumptive

neurons (Fig. 1A). A double *wnt5* RNA *in situ* and anti-Myc antibody staining for endogenous *wnt5* mRNA and Elav-GAL4driven H -Myc protein demonstrates that *wnt5* is expressed in neurons (Figs. 1B). To localize *wnt5* mRNA-expressing cells with respect to the commissures, we performed double-fluorescent RNA *in situs* using *wnt5* and *drl* antisense probes, the latter labeling most AC neuronal cell bodies (Callahan *et al.,* 1995). *wnt5* mRNA was found predominantly in cell bodies near to and underlying the PC. *wnt5* RNA was also found in occasional cell bodies near the AC, but no overlap between $wnt5^{+}$ and drl^{+} cells was observed (Fig. 1C). A double *wnt5* RNA *in situ* and antibody labeling for the Repo protein showed that *wnt5* mRNA is not expressed by lateral glia (Fig. 1D). Likewise, a *wnt5* RNA *in situ* double staining with the anti-Wrapper antibody, which labels all MG, reveals that *wnt5* mRNA is not expressed by midline glia (Fig. 1E). During early stages of CNS development, Wnt5 protein is observed on cell bodies lateral to the ventral midline (Fig. 1F), and subsequently, on the axons projecting across the midline (Fig. 1G). During later stages of embryogenesis, Wnt5 protein accumulates primarily on the commissures with only weak staining apparent on the longitudinal pathways (stages 14, 16) (Figs. 1H,I). No staining is seen in the *wnt5* null mutant (Fig. 1J). Wnt5 expression on the PC was consistently higher than that on the AC (Figs. 1G–I) during all stages of embryonic CNS development.

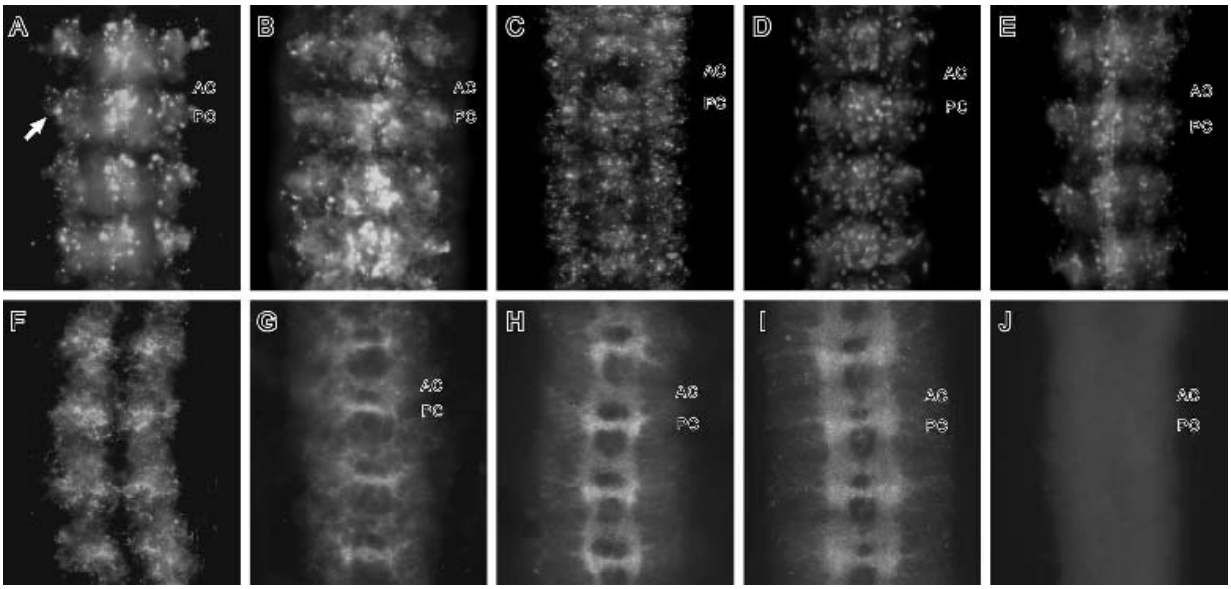

Figure 1. *wnt5* RNA and protein are most highly expressed in PC neurons. *wnt5* mRNA is expressed in cells predominantly associated with the PC midline and lateral clusters (arrow), but is not strictly excluded from the AC region (A). Overlap (yellow) of endogenous *wnt5* mRNA (green) and H -Myc protein (red) expressed in all postmitotic neurons using the Elav-GAL4 driver shows that *wnt5* mRNA is expressed in neuronal cell bodies (B). Double RNA *in situ* staining for endogenous *wnt5* mRNA (green) and drl mRNA (red, expressed in most AC neurons) shows that *wnt5* is predominantly expressed in PC-associated neuronal cell bodies that do not appear to express drl (C). The lack of overlap between endogenous *wnt5* mRNA (green) and Repo protein (red) expressed in all lateral glia indicates that *wnt5* mRNA is not expressed in lateral glia (D). The lack of overlap between endogenous *wnt5* mRNA (green) and Wrapper protein (red), detected with anti-Wrapper mAb, indicates that *wnt5* mRNA is not expressed in the MG (E). Wnt5 protein is found on neuronal cell bodies lateral to the midline at early stage 12 just before axon path finding across the midline (F). Wnt5 protein is found on AC, PC, and longitudinal axons at stage 13 (G), 14 (H), and 16 (I). At all stages, the protein is most abundant on PC axons. No Wnt5 protein is detected in the *wnt5* mutant at stage 16 (J). In all panels, anterior is up. Panels (A –E) depict stage 15 embryos. (see Appendix: Selected Color Figures)

Generation of *wnt5* null mutants

wnt5 mutant alleles were generated by imprecise excision of an adjacent P-element (Materials and methods). Two lines, $wnt5^{400}$ and $wnt5^{207}$, lacking Wnt5 protein as determined by anti-Wnt5

immunostaining and whole embryo Western blot analysis (Figs. 1J and 6E), were characterized initially by DNA sequence analysis and found to be lacking large regions of the Wnt5 ORF (Figs. 2B), suggesting that they are likely null alleles. *wnt5* mutants can be maintained as a homozygous stock; however, 19% of *wnt5* embryos, normalized to contemporaneous controls, fail to hatch (data not shown). Those embryos failing to hatch likely represent the subset with the most severe CNS defects described below (Fig. 3G). Once they hatch, *wnt5* mutant individuals have survival rates indistinguishable from controls at subsequent developmental stages (data not shown), suggesting that the lethality is restricted to the embryonic stage.

Data for $wnt5^{400}$ are shown, however, similar phenotypes were seen in $wnt5^{207}$. To confirm that both lines bear *wnt5* null mutations, we generated transheterozygous embryos with each of the *wnt5* mutant chromosomes over a deficiency [Df(1)E128; Engels and Preston, 1984] that completely removes the genomic interval containing *wnt5*. No increase in the severity of CNS phenotypes above those seen in the homozygous mutants was observed in any of the mutant-deficiency chromosome transheterozygotes using mAbs BP102 and 1D4 to label axonal projections (data not shown), indicating that they are null *wnt5* alleles. The *wnt5* 9 precise excision line appeared wild type for all markers used (data not shown).

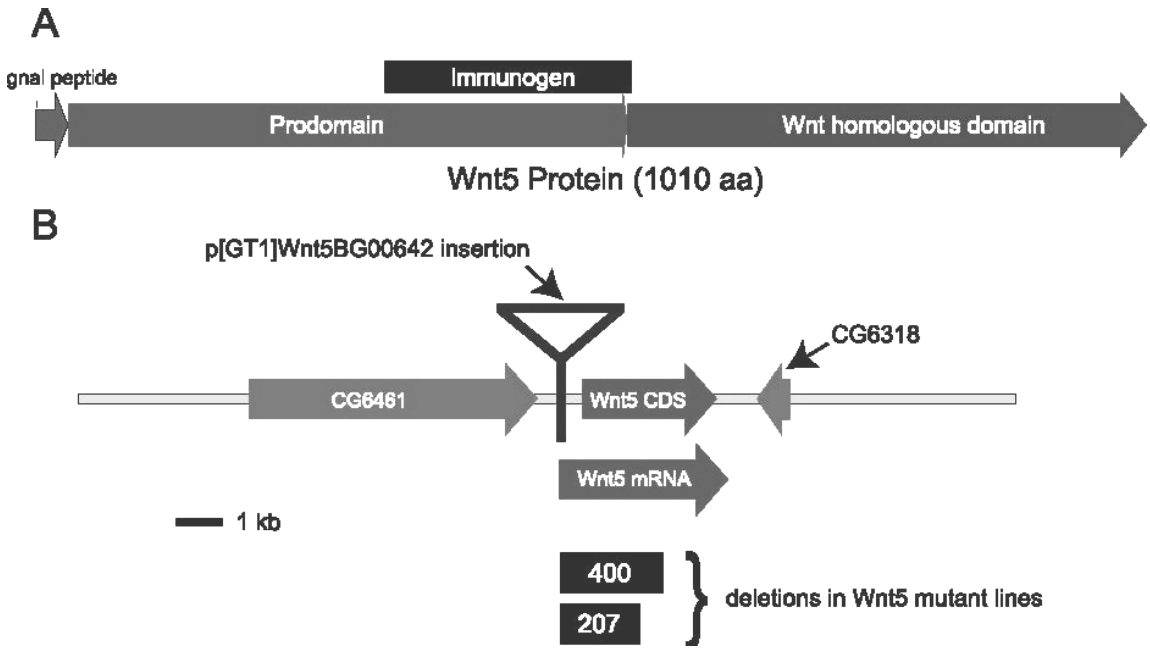

Figure 2. Wnt5 protein structure and *wnt5* loss-of-function mutants. The primary structure of the Wnt5 protein is shown in schematic form (A). The Wnt homologous region, the unique Wnt5 amino terminal extension, and the region used as an immunogen to generate anti-Wnt5 antisera are indicated. The *wnt5* genomic region on the X-chromosome is shown (B). A P-element located approximately 500 base pairs 5V to the predicted *wnt5* start codon was imprecisely excised to generate mutant lines that no longer express the Wnt5 protein. The location and sizes of the deletions in the $wnt5^{400}$ and $wnt5^{207}$ lines are shown.

Initial path finding across the midline is normal in *wnt5* mutants; however, the AC and PC commissures do not separate appropriately.

To understand the function of Wnt5 in CNS development, we used several cell-or lineage-specific mAbs to visualize the CNS axon trajectories in *wnt5* mutants. As visualized by mAb BP102, which stains all CNS neurons, the CNS scaffold in wild-type embryos matures into a characteristic ladder-like pattern with two commissures that cross the ventral midline in each segment and two longitudinal connectives that run along either side of the ventral midline (Figs.

3A–C; Klambt *et al.,* 1991). In *wnt5* mutants, pioneering BP102+ PC and AC commissural axons cross the midline at stage 12 as in the wild type (compare Figs. 3A and D). However, the AC and PC axons fail to subsequently separate at stage 13 in *wnt5* mutants (compare Figs. 3B and E). In the majority of *wnt5* embryos (67%, Table 1), the mature AC at stage 16 is much thinner than normal and several AC axons either do not cross or cross ectopically, projecting between AC and PC (compare Figs. 3C and F). In a more severely affected minority of embryos (approximately 10%), no AC is apparent (Fig. 3G).

To examine a specific AC-projecting lineage, we evaluated axon projections in embryos bearing the Sema2b-H -Myc transgene that labels three axons whose cell bodies lie just lateral to the AC. In wild-type embryos, Sema2b+ neurons cross the midline through the AC as late follower axons and subsequently turn anteriorly in the longitudinal connectives to fasciculate with their siblings (Fig. 3H and Senti and Dickson, unpublished). In *wnt5* mutant embryos, the majority (78%, n = 54 segments counted) of Sema2b+ axons do not enter the AC, but either extend minimally or inappropriately fasciculate with longitudinal projections and project ipsilaterally. A subset of Sema2b+ neurons (22%, n = 54 segments counted) cross the midline in a region between AC and PC (Fig. 3I).

Table 1. Restoration of longitudinal and commissural pathways in *wnt5* mutant embryos

Genotype	Number of Segments scored	Loss or thinning of AC (%)	Loss or thinning of PC (%)	Breaks in FasII^{+} fascicles (%)		
				Medial	Intermediate	Lateral
W^{1118}	251	0	0	0	0	3
$wnt5^{400}$ null	237	67	1	1	13	22
$wnt5^{400}$;UAS-Wnt5/+; Elav-GAL4/+	243	7	0	0	3	10
$wnt5^{400}$;UAS-Wnt5; Elav-GAL4	233	1	0	0	1	5
$wnt5^{400}$;UAS-Wnt5/+; Repo-GAL4/+	233	61	0	0	10	20
Sim-GAL4/+; UAS-Wnt5/+	243	97	0	ND	ND	ND
Sim-GAL4, UAS-Wnt5/+	351	17	0	ND	ND	ND
porc PB16; Sim-GAL4, UAS-Wnt5/+	380	2	0	ND	ND	ND

Stage 16/17 embryos were analyzed for commissural and longitudinal defects using mAbs BP102 and 1D4 (anti-FasII), respectively. ND indicates not determined.

The intermediate and lateral longitudinal fascicles are not formed correctly in *wnt5* mutants

To evaluate longitudinal projections in the *wnt5* mutant, we used three lineage-specific antibodies, mAb 1D4 (anti-FasII), mAb 22C10 (anti-Futsch), and anti-Robo2 (Materials and methods). During early path finding stages, mAb 1D4 labels, among others, the pCC neurons that pioneer the medial pathway (Fig. 4A), the innermost fascicle of the three FasII+ fascicles seen in the mature CNS (Lin *et al.,* 1994) (Fig. 4C). Early pioneering of the medial pathway is unaffected in *wnt5* mutant embryos (Fig. 4D) and the mature medial pathway is also not affected (Fig. 4F). In wild-type embryos, the ascending pCC and vMP2 axons form the first continuous longitudinal projection when they join the descending MP1 and dMP2 axons (Hidalgo and

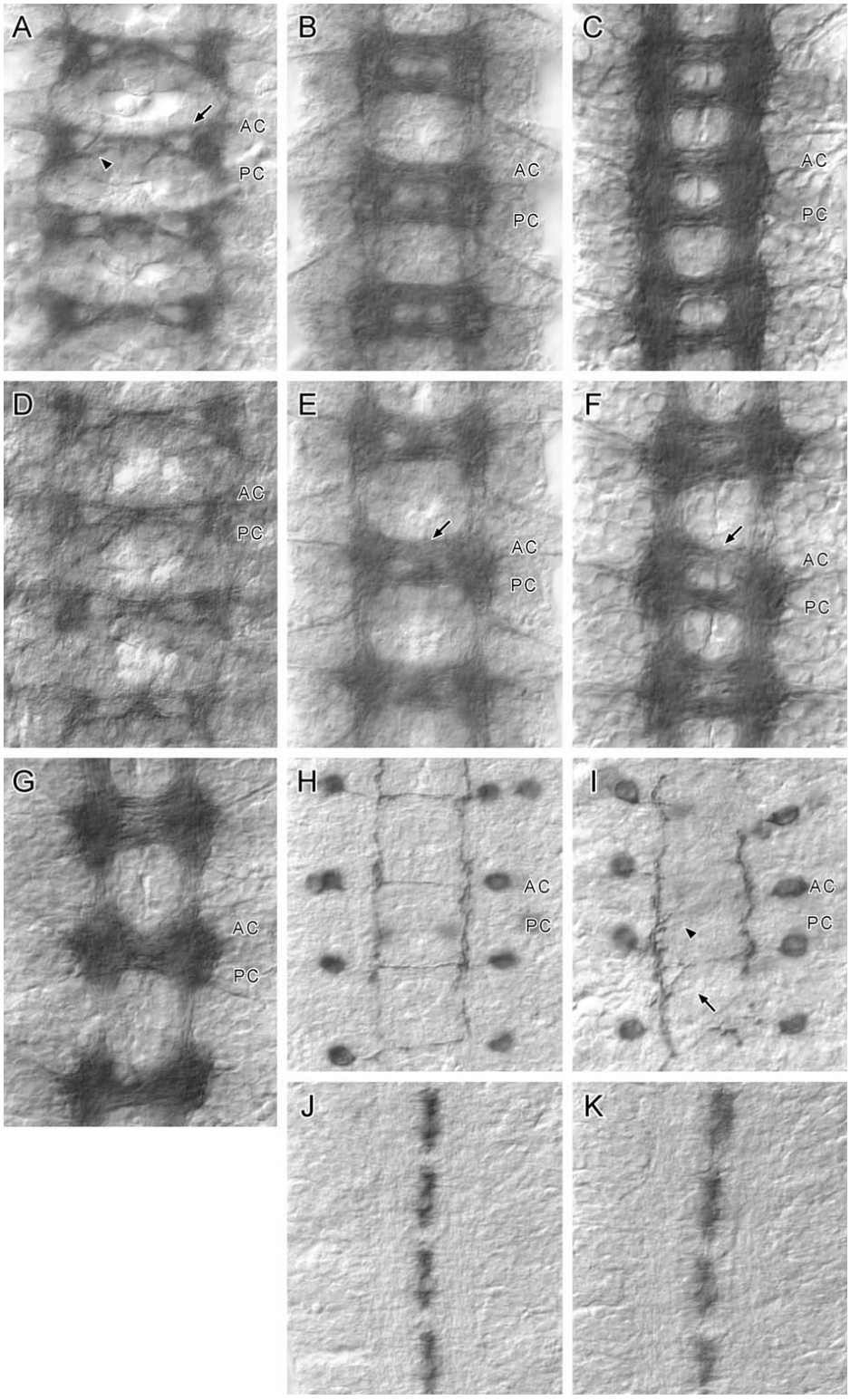

Figure 3 Abnormal commissural axonal projections in the *wnt5* mutant subsequent to a failure of the AC and PC to separate. (A –C) The development of the wild-type CNS axon tracts as visualized with mAb BP102 at stages 12 (A), 13 (B), and 16 (C) is shown. (D –F) In the *wnt5* mutant, early path finding of AC (arrow) and PC (arrowhead) BP102+ pioneers (stage 12) is apparently normal (compare A and D); however, the commissures subsequently fail to completely separate at stage 13, resulting in ‘‘fuzzy’’ commissures at stage 16 (compare E and F). In the embryos most affected (approximately 10%), the AC is absent (G). In panelsA –F, arrows indicate AC neurons; arrowheads point to PC neurons. The Sema2b+ neurons that crossthe wild-typemidlinein the AC areshown(H). In the *wnt5* mutant (I), many Sema2b+ axons fail to cross the midline, while a subset of axons ectopically cross the midline (arrow) and others inappropriately project ipsilaterally along the longitudinal tracts. Panels J and K show stage 16 embryos stained with the wrapper mAb that labels the MG. No differences between the wild type (J) or the $wnt5^{400}$ (K) MG numbers or location are observed. Anterior is up in all panels.

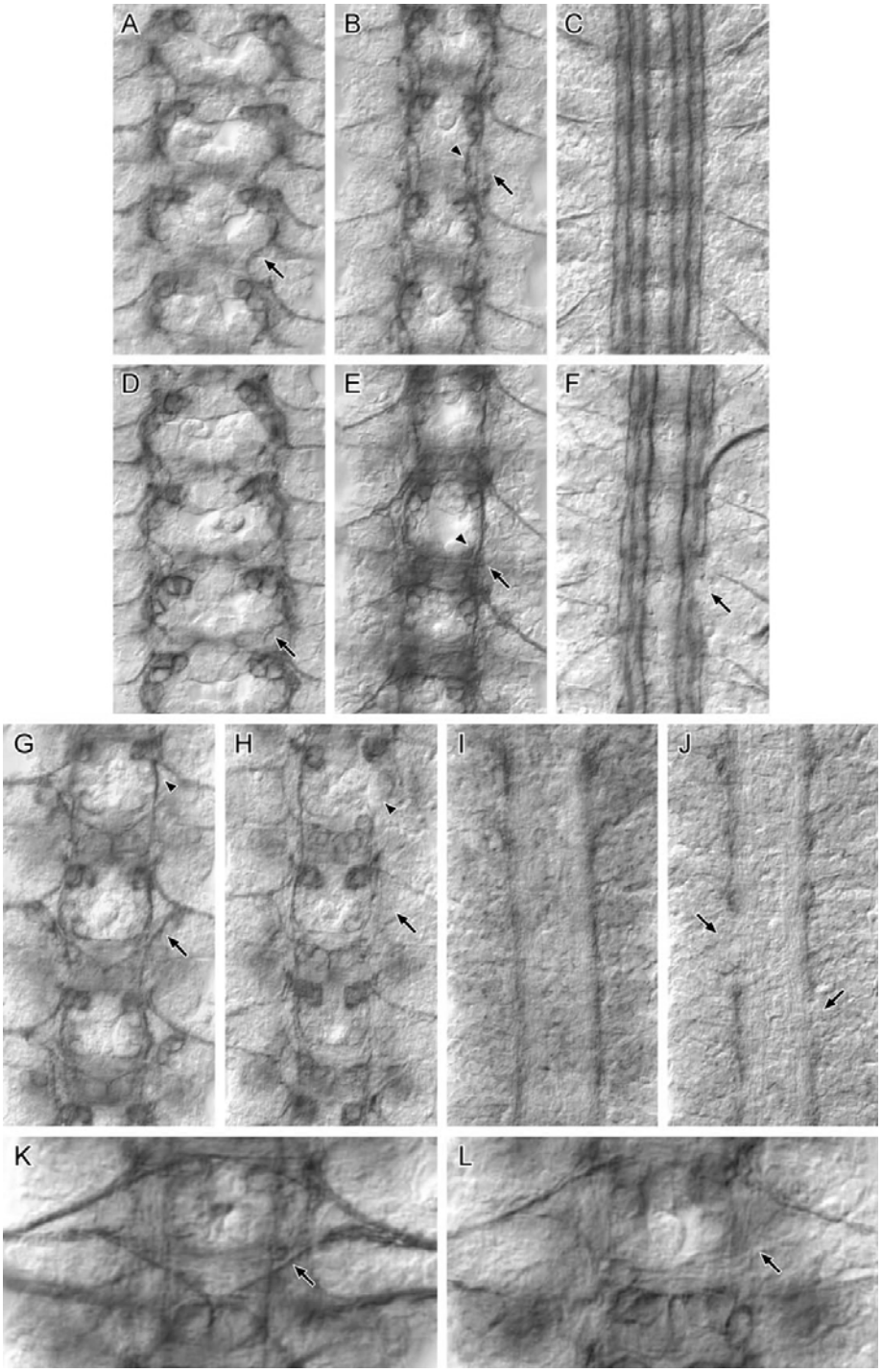

Figure 4. Early failures in longitudinal pathway projections lead to breaks in the mature lateral pathways. (A –C) Pioneering and mature longitudinal pathways as visualized by the anti-FasII mAb in wild type (A –C) and in *wnt5* mutant embryos (D –F) are shown. Longitudinal pathways are labeled with mAb 22C10 in wild type (G and K) and the *wnt5* mutant (H and L) and with anti-Robo2 in wild type (I) and the *wnt5* mutant (J). One of the earliest pioneers of the longitudinal pathway, the pCC (arrow), projects normally in the stage 13 *wnt5* mutant (compare A with D). However, at stage 14, the pCC/vMP2 inner fascicle (arrowhead) and the MP1/dMP2 outer fascicle (arrow), do not defasciculate in the *wnt5* mutant, but extend as a single pathway (compare B and E) or occasionally (in 5% of 100 segments examined) do not extend at all (see arrowhead in panel H). The MP1 and vMP2 cell bodies failing to extend axons are visible in another focal plane. At stage 16, the intermediate FasII+ pathway, which is pioneered by MP1, often shows breaks or fusion with the medial pathway (F, arrow). The VUM neurons, which normally bifurcate and fasciculate with RP2 and aCC to exit the CNS, often fail to do so in the *wnt5* mutant (arrow, compare G and H). The Robo2+ lateral pathway fails to form a continuous fascicle in the *wnt5* mutant at stage 16 (compare I and J, arrows). A higher magnification of the 22C10+ VUM axons is shown for wild type (K, arrow) and the *wnt5* mutant (L, arrow). Anterior is up in all panels.

Brand, 1997; Lin *et al.,* 1994). Later on, at stage 14, the MP1/dMP2 and the pCC/vMP2 pathways defasciculate and are associated only at the segment border, thereby forming an outer (MP1/dMP2) and an inner (pCC/vMP2) fascicle (Fig. 4B). This defasiciculation fails to occur in the *wnt5* mutant, resulting in a single thick fascicle (Fig. 4E). Furthermore, MP1 later pioneers the intermediate of the three FasII+ fascicles in wild-type embryos (Hidalgo and Brand, 1997) (Fig. 4C), but fails to do so in the *wnt5* mutant (Fig. 4F), resulting in breaks in the fascicle at stage 16.

The MP1 and vMP2 pathways can also be visualized with mAb 22C10 (anti-Futsch). Most of the pioneering MP1 and vMP2 axons extend and form the first longitudional pathway in *wnt5* mutant embryos, but rare (5 segments, n = 100 counted) breaks are observed in these pathways resulting from the failure of both the MP1 and vMP2 axons to fully extend (arrowhead, Fig. 4H). mAb 22C10 also labels the VUM neurons whose cell bodies are located at the PC midline and send their axons out anteriorly to subsequently bifurcate at the AC where they fasciculate with the RP2 and aCC axons to project laterally out of the CNS (Figs. 4G,K). In the *wnt5* mutant, VUM axons project incorrectly along the medial longitudinal pathway in 70% of segments (n = 198) (Figs. 4H,L), possibly due to inappropriate selective fasciculation. The cell bodies of the projections described (VUMS, MP1, vMP2, pCC, dMP2) were present at their wild-type locations in the *wnt5* mutant (data not shown).
We also examined the Robo2+ axons that project ipsilaterally through the lateral-most of the three FasII+ pathways in wild-type embryos (Fig. 4I). Initially, robo2 is expressed in many neurons (among others pCC, MP1, dMP2, vMP2; Simpson *et al.,* 2000a,b), but expression ceases in these neurons at stage 14, and from then on is present only in the lateral most FasII+ fascicle. Examination of *wnt5* mutants indicates that the Robo2+ axons initially extend in the mutant, but then stop in an apparently tightly fasciculated bundle by stage 14 (data not shown) and therefore fail to form the continuous lateral Robo2+ fascicle seen at stage 16 (compare Figs. 4J, I).

We interpret the phenotypes we observe in *wnt5* mutant embryos as resulting from defects in the abilities of the wnt5-responsive subset of axons to defasciculate sufficiently to extend or enter new pathways. However, they could also result from fate changes in cell lineages due to the absence of *wnt5*. To evaluate this possibility, we stained *wnt5* mutant embryos with anti-Repo to label all lateral glia and with several mAbs that label specific neuronal subsets: mAb 1D4, anti-Even-skipped, anti-Engrailed, and mAb 22C10. No obvious changes in the fates or numbers of these glia or neuronal cell bodies were detected in *wnt5* mutants (data not shown). The MG play important roles in commissural separation (Jacobs, 2000; Klambt *et al.,* 1996); therefore, commissural phenotypes could also result from the failure of the MG to migrate appropriately. Consequently, we visualized MG migration throughout embryogenesis using the anti-Wrapper mAb, which labels all of the MG. No obvious differences from wild-type embryos in the numbers and final positions of the MG were observed in *wnt5* mutants (Figs. 3J,K). Furthermore, the migration patterns of the MG throughout embryonic CNS development in the *wnt5* mutant appeared indistinguishable from those in the wild type (data not shown).

Rescue of the *wnt5* null phenotype by panneural *wnt5* expression

To understand where *wnt5* expression is required, rescue experiments were performed in which *wnt5* expression was restored in the *wnt5* null mutant either in all axons, the MG or the lateral glia through use of the UAS-GAL4 system (Brand and Perrimon, 1993). When Wnt5 was expressed in all CNS neurons (driven by Elav-GAL4), apparently complete rescue of the *wnt5* mutant longitudinal and commissural phenotypes was observed (Table 1; Figs. 5A–D).

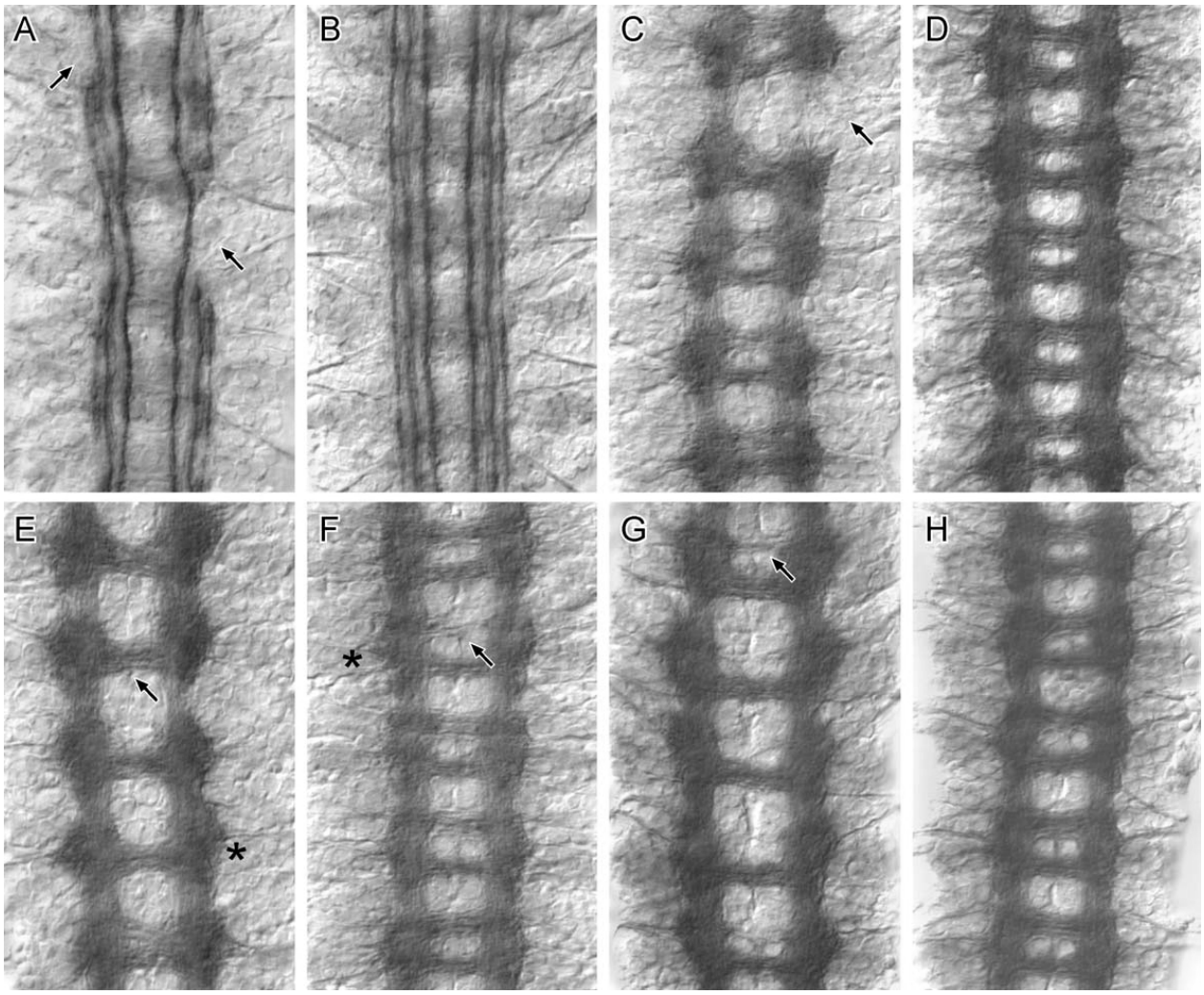

Figure 5. Panneural Wnt5 expression rescues the *wnt5* mutant phenotype and ectopic Wnt5 expression on MG results in the loss of the AC. The lateral FasII+ longitudinal pathways in $wnt5^{400}$ display frequent breaks (arrows) (A). Panneural expression of wnt5 driven by Elav-GAL4 rescues the mutant to wild-type appearance (B). The fuzzy or collapsed commissures and longitudinal thinning or breaks (arrow) in $wnt5^{400}$ is visualized with mAb BP102 (C). Panneural expression rescues both the commissural and longitudinal defects (D). Ectopic Wnt5 expression in the MG of $wnt5^{400}$;Sim-GAL4/+;UAS-Wnt5/+ embryos (one copy of each transgene) results in the loss of the AC and thickening of the longitudinal connectives in the region anterior to the PC (asterisk); thePC appears unaffected (arrow) (E). Ectopic MG Wnt5 expression in an otherwise wild-type background (Sim-GAL4/+;UAS-Wnt5/+) (one copy of each transgene) results in somewhat thinner ACs (arrow) and some thickening of the longitudinal connectives (asterisk) (F). High levels of ectopic MG Wnt5 expression in the wild type (Sim-GAL4;UAS-Wnt5) (two copies of each transgene) result in frequent thinning (arrow) and complete loss of the AC accompanied by significant longitudinal thickening (G). The midline Wnt5 overexpression phenotype (one copy each of UAS-Wnt5 and Sim-GAL4) is suppressed in hemi-and heterozygous zygotic porc PB16 mutant embryos (compare H with F). Anterior is up in all panels.

In contrast, when overexpressed in the lateral glia (using the Repo-GAL4 driver), no rescue of the *wnt5* null mutant phenotypes was observed using either single (Table 1) or double copies of driver and UAS-Wnt5 (data not shown). No rescue of the aberrant commissural or longitudinal pathways was observed in *wnt5* mutant embryos ectopically expressing Wnt5 protein at the midline (driven by SimGAL4). Instead, a striking phenotype was observed: although the PC appears normal, the AC fails to form (Fig.5E). This phenotype is highly penetrant: 96% of the embryos show this effect (Table 1). To investigate whether this phenotype could also be seen when Wnt5 protein is expressed at the midline in a wild type embryo, embryos with a single copy of Sim-GAL4 and UAS-Wnt5 in an otherwise wild type genetic background were

generated. A lower penetrance phenotype, in which 17% of the embryos lose the AC in one or two segments, was observed (Fig. 5F). This phenotype became increasingly more penetrant and severe when two copies each of Sim-GAL4 and UAS-Wnt5 (Fig. 5G) or two copies each of the stronger Slit-GAL4 driver and UAS-Wnt5 were present (data not shown), suggesting dose-dependent responses to ectopic midline Wnt5 expression.

The Sim-and Slit-GAL4 transgenes drive transcription from late stage 11 onward in most midline precursors and later on predominantly in the anterior (MGA), the medial (MGM), and the posterior (MGP) MG (Scholz *et al.,* 1997). To understand the relative importance of MGA/MGM vs. MGP *wnt5* overexpression in eliciting the midline over-expression phenotype, we expressed *wnt5* in the MGP using the Btl-GAL4 driver. This transgene drives expression from stage late 11 onward in a subset of neurons underlying the PC, including the VUMs, but is not expressed in the MGA and MGM. As Btl-GAL4-driven *wnt5* fails to suppress formation of the AC (data not shown), we conclude that *wnt5* ectopically expressed by the MGA and/or MGM likely mediates the Wnt5 midline expression phenotype.

porc is a member of the Wnt5 signalling pathway

To demonstrate the utility of the *wnt5* MG overexpression phenotype as a genetic tool to uncover members of the *wnt5* signalling pathway, we evaluated the ability of a mutant allele of porc to suppress this phenotype. The *porc* gene was previously shown to be required for the secretion of the Wnt protein Wg (Kadowaki *et al.,* 1996; Tanaka *et al.,* 2002; van den Heuvel *et al.,* 1993) and the Wnt5 protein (Tanaka *et al.,* 2002) and for *wg*-dependent signalling (Manoukian *et al.,* 1995; Noordermeer *et al.,* 1994; Siegfried *et al.,* 1994). The absence or reduction to single copy of the X-linked *porc* gene in embryos bearing one copy each of the Sim-GAL4 and UAS-Wnt5 transgenes completely suppressed the Wnt5 midline overexpression phenotype (Fig. 5H; Table 1), demonstrating that *porc* is not only required for Wnt5 protein secretion, but also for Wnt5 signalling.

The Wnt5 receptor, drl, represses *wnt5* transcription in the wild-type AC

Drl, a RYK family member protein expressed predominantly on the AC (Callahan *et al.,* 1995), has recently been shown to be a receptor for Wnt5 (Yoshikawa *et al.,* 2003). We therefore evaluated the possibility that the low levels of Wnt5 staining seen on AC neurons (Figs. 1G–I and 6A) reflected Drl-mediated binding and trapping of Wnt5 wild-type embryos. mAb BP102 was used to visualize all protein from PC neurons at the AC. Unexpectedly, examination of Wnt5 protein expression in a drl mutant background revealed that AC Wnt5 protein levels increased, resulting in similar levels to those seen on the PC (Fig. 6C). Western blot analyses of lysates made from wild type vs. drl mutant embryos indicate that overall levels of Wnt5 protein increase in the drl mutant (Fig. 6E). Quantitation of Wnt5 protein levels revealed that overall Wnt5 protein levels increase 2.2-fold in the drl mutant relative to wild type.
As the increase in AC-associated Wnt5 protein could reflect regulation of *wnt5* expression by *drl* at either transcriptional or post-transcriptional levels, we evaluated *wnt5* mRNA expression patterns by fluorescent double RNA *in situ*/antibody stainings in the *drl* null mutant vs. wild type embryos. mAb BP102 was used to visualize all CNS axon tracts and *wnt5* mRNA was detected using an -antisense probe. Comparison of the *wnt5* expression pattern, in wild type and the drl mutant indicates that *wnt5* mRNA expression expands into the AC (Figs. 6B, D). Thus, the presence of wild-type drl in AC neurons is required for the partial suppression *wnt5* transcription in those neurons, contributing to the marked difference observed between Wnt5 protein levels AC and PC.

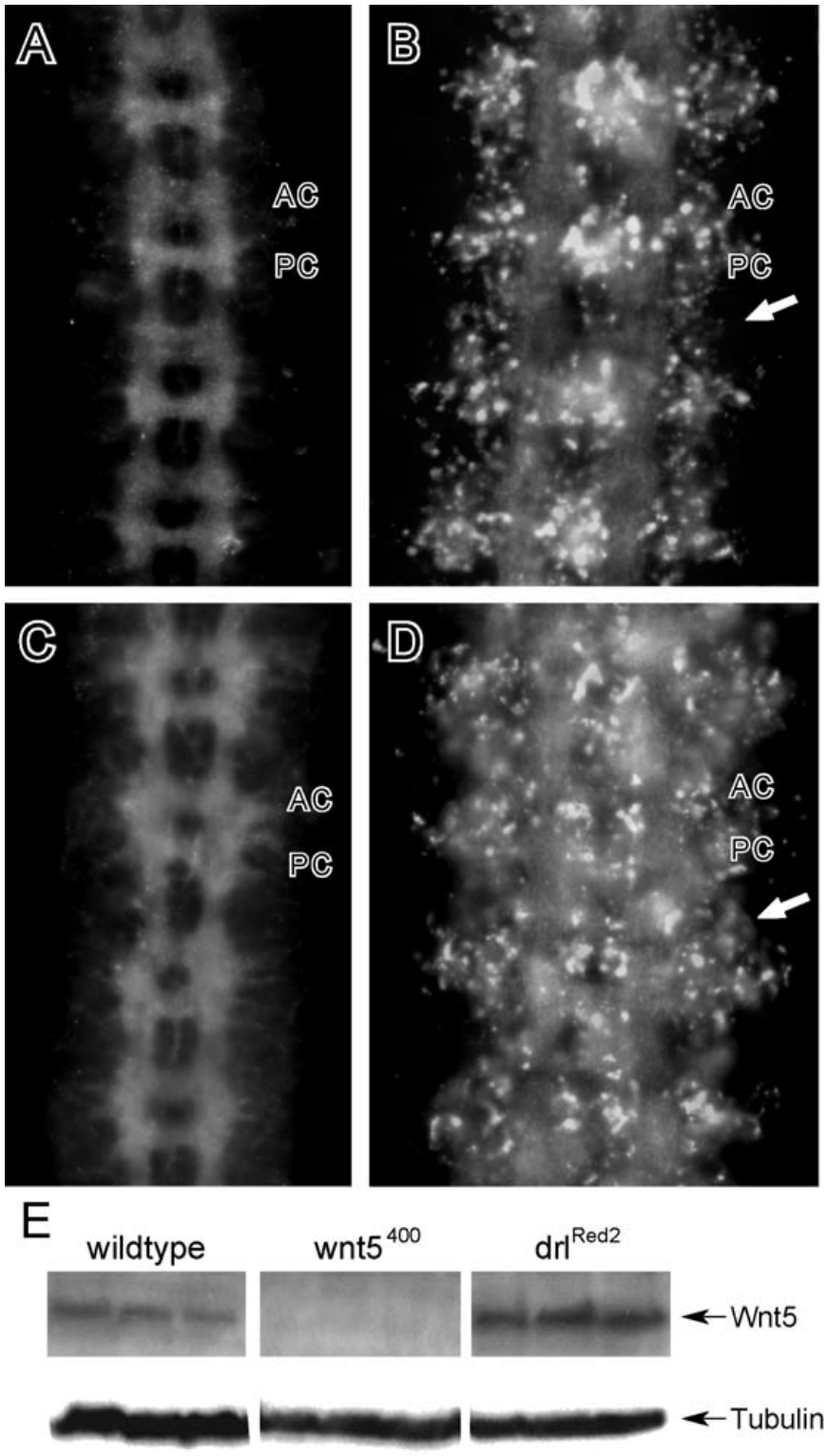

Figure 6. drl represses *wnt5* mRNA expression in AC neurons. In wild-type embryos, higher levels of Wnt5 protein are detected on the PC than the AC (A). In drlRED2 mutant embryos, equivalent levels of Wnt5 are detected on both commissures (C). A double RNA *in situ*/antibody staining (*wnt5* mRNA in green; panneurally staining mAb BP102 in red) on wild-type embryos is shown to indicate the normal expression domain of *wnt5* mRNA (B). In drlRED2, the *wnt5* mRNA-expressing domain expands to encompass the AC (D). Exposure times for the images in A and C, and in B and D were the same. Anterior is up for all panels. Immunoblot analysis (E) shows that overall embryonic Wnt5 protein levels reflect the increase apparent from the antibody and RNA *in situ* stainings. Quantitation of Wnt5 relative to the a-tubulin loading control revealed a 2.2-fold increase in Wnt5 protein in the drlRed2 mutant relative to wild type. (see Appendix: Selected Color Figures)

Discussion

In this report, we have presented evidence that the *Drosophila* Wnt5 protein plays an important role in establishing the architecture of the embryonic ventral nerve cord. We have demonstrated that *wnt5* mRNA is most highly expressed by a large number of neurons predominantly in lateral and midline clusters underlying the PC, but is also found in a few cell bodies more closely associated with the AC. *wnt5* is not apparently expressed by the midline or lateral glia cells. While Wnt5 protein is detected on both AC and PC axons, as well as the longitudinal axon tracts, Wnt5 protein is expressed at higher levels on the PC as compared with the AC from the earliest stages when axons begin to extend and throughout embryonic CNS development.

We have generated *wnt5* null alleles to determine the role of *wnt5* in CNS development. Early BP102+ commissural axons that pioneer first the PC, and subsequently the AC, are unaffected in the *wnt5* mutant. In the wild-type embryo, the BP102+ AC and PC pioneers and their early followers are closely associated near the midline, but subsequently separate at stage 13, to begin the formation of the two distinct commissures (Klambt *et al.,* 1991). This separation does not take place in the majority of *wnt5* mutant segments. Visualization of the Sema2b+ axons, followers that cross in the wild-type AC at early stage 15, reveals that they largely fail to do so in the *wnt5* mutant and fasciculate inappropriately with ipsilateral sibling longitudinal projections or cross the midline in the region between the AC and PC. We have visualized the PC axon trajectories only by panneural staining and in the Eg+ PC-crossing lineage (data not shown) and see no major alterations in the *wnt5* mutant.

In addition to the commissural defects in *wnt5* mutant embryos, we observed alterations to the longitudinal pathway projections. *wnt5* mutant embryos display differential phenotypes with respect to the FasII+ longitudinal pathways: the medial or innermost pathway is largely unaffected; however, breaks are found in the intermediate pathway and the lateral pathways. Supporting these observations, the Ap+ projections, which in wild type follow the ipsilateral medial pathway (Lundgren *et al.,* 1995), were unaffected by the absence of Wnt5 (data not shown). Visualization of the intermediate and lateral pathways with anti-Robo3 (data not shown) and anti-Robo2 (Rajagopalan *et al.,* 2000b; Simpson *et al.,* 2000a), respectively, further indicated that disruption became more severe in the pathways more lateral to the ventral midline. Analyses of the longitudinal pioneer neurons indicate that, when specific axons have to selectively defasciculate to pioneer new pathways, particularly the intermediate and lateral pathways, they do not do so in the absence of *wnt5*. Examination of the Robo2+ neurons indicates that after a limited period of extension, they stop and fail to form the continuous fascicle seen in the wild-type embryo. The *wnt5* longitudinal phenotypes are unlikely to simply reflect the failure of the axons that normally transit the AC to cross in that we see (1) early defasiculation defects in the longitudinal pioneering projections at times where the commissures themselves are being pioneered, (2) discontinuities in ipsilaterally projecting pathways that do not cross the midline, and (3) apparently normal Fas2+ longitudinal pathways in the comm null mutant (data not shown), where few if any axons cross in either commissure. We also observed that the VUM neurons, which normally aid in separating the commissures by sending their projections in between the PC and AC BP102+ pioneers, project abnormally in the *wnt5* mutant. Migration of the VUM cell bodies (data not shown) and the MG, which are also involved in establishing the physical separation between the AC and PC (Klambt *et al.,* 1991), do, however, occur normally in the *wnt5* mutant. Because the failure to establish the AC in the *wnt5* mutant occurs with a similar frequency (67%) to that observed for the abnormal VUM projections (70%), the VUM abnormalities likely contribute to the later failures of the follower axons to form the AC. The VUMs express *wnt5* mRNA at the time of commissure separation (data not

shown) and may therefore represent the chief source of Wnt5 protein mediating separation of the AC and PC.

How does *wnt5* mediate the formation of the AC and the lateral longitudinal pathways? Our data, including the observation that Wnt5 protein is most highly expressed on the PC, support the previously proposed role for PC-expressed *wnt5* as a repellent for Drl+ axons (Yoshikawa *et al.,* 2003), but reveal the likely nature of Wnt5-mediated repulsion required to effect the formation of the AC and more lateral longitudinal pathways. We suggest that the major role of *wnt5* is to mediate the selective defasciculation of the early commissural and longitudinal pathways necessary for them to separate into distinct commissures or pioneer new pathways, respectively. As defasciculation may be viewed as local repulsion or decreased attraction between axons, our interpretation of the early commissural defects in the *wnt5* mutant is therefore not at odds with Wnt5 acting as a PC-derived repellent. However, Wnt5 appears to act by facilitating the defasciculation of Drl+ axons from their siblings necessary for formation of the AC. The role of drl in the *wnt5* mutant longitudinal pathway defasciculation defects is presently unclear. Interestingly, studies of the drl mutant phenotype described the inappropriate defasiculation of axons lacking drl (Callahan *et al.,* 1995; Speicher *et al.,* 1998). We observe similar drl null phenotypes as previously reported; however, the drl CNS phenotypes are consistently less severe than those seen in the *wnt5* null (data not shown), suggesting that other genes may also interact with *wnt5* to effect selective fasciculation. We demonstrate in rescue experiments that the primary requirement for *wnt5* expression during embryonic CNS development is in neurons. Panneural *wnt5* expression rescued both the commissural and longitudinal defects. Strikingly, high levels of Wnt5 secreted throughout the CNS-driven by the Repo-Gal4 lateral glial cell driver in the *wnt5* mutant background fail to rescue, suggesting that neurons may process Wnt5 differently than the lateral glia. In support of this possibility, we (Fradkin *et al.,* 1995) and others (Yoshikawa *et al.,* 2003) have previously presented evidence that Wnt5 protein secreted from tissue culture cells and produced in embryos, respectively, is proteolytically processed.

Wnt5 overexpression at the AC midline (Sim-Gal4 driver), but not the PC midline (Btl-Gal4 driver) results in a failure to form the AC without noticeable effects on the PC. The noncrossing axons appear tightly associated in the longitudinals, suggesting that Wnt5 protein levels may be important in the regulation of axonal adhesion, with either too little or too much Wnt5 resulting in overly tight fasciculation. The Sim-Gal4-driven Wnt5 overexpression phenotype has also been interpreted to indicate that ectopic Wnt5 repulses all Drl+ AC axons (Yoshikawa *et al.,* 2003). However, given the current absence of data showing that Wnt5 can directly collapse Drl+ growth cones, it is equally possible that the overly tight fasciculation of those axons precludes their midline crossing.

We have shown that the differing levels of Wnt5 protein on the PC vs. AC are maintained, at least in part, from repression of *wnt5* transcription in AC neurons by Drl. Furthermore, we observe apparently normal CNS architecture when *wnt5* is pan-neurally expressed in a wild-type background (in Elav-GAL4 X UAS-Wnt5 embryos) resulting in high levels of Wnt5 protein on both commissures (data not shown). The question arises as to why such regulation of Wnt5 in the AC exists? One possibility is that, although high levels of Wnt5 protein on both commissures result in rescue of the *wnt5* mutant phenotype and a lack of an observable phenotype in the wild-type background, respectively, the active species of Wnt5 protein may be asymmetrically generated with respect to the commissure of origin, reflecting PC-vs. AC-specific Wnt5 protein processing. Alternatively, the Drl-mediated repression of AC *wnt5* expression may reflect a requirement for low levels of *wnt5* in AC axons while precluding the higher levels that effect defasciculation, likely via the Drl receptor.

We find that porc gene expression is required for the MG Wnt5 overexpression phenotype. As porc has previously been reported to aid in Wnt5 protein secretion (Tanaka *et al.,* 2002), our data indicate porc-mediated Wnt5 secretion is required for Wnt5 signalling in this overexpression assay. *porc* function appears to be limiting for Wnt5 secretion in this assay as reduction of *porc* gene dosage by half in the porc/+ females was sufficient to suppress the Wnt5 midline over-expression phenotype. Is porc required for wild-type Wnt5mediated signalling? The BP102+ axon tracts are disorganized in porc germline clone mutants lacking both maternal and zygotic porc (Tanaka *et al.,* 2002); however, interpretation of this phenotype is complicated by the requirement for porc in wg signalling, which in turn plays roles in segmentation (Manoukian *et al.,* 1995; Noordermeer *et al.,* 1994; Siegfried *et al.,* 1994) and neuroblast specification (Bhat, 1998; Chu-LaGraff and Doe, 1993).In porc zygotic mutants, the BP102+ axon scaffold is only slightly affected (data not shown); however, the maternally contributed porc mRNA may mask a requirement for porc in Wnt5 signalling in the zygotic porc mutant embryo. The demonstration of a requirement for porc in establishing the Wnt5 midline overexpression phenotype suggests that this assay will be a useful tool in uncovering novel members of the *wnt5* signalling pathway and its targets, which likely include cell surface adhesion proteins facilitating selective fasciculation and modulators of their activities.

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