Development 134, 273-284 (2007) doi:10.1242/dev.02653

β-Spectrin functions independently of Ankyrin to regulate the establishment and maintenance of axon connections in the *Drosophila* embryonic CNS

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α- and β-Spectrin are major components of a submembrane cytoskeletal network connecting actin filaments to integral plasma membrane proteins. Besides its structural role in red blood cells, the Spectrin network is thought to function in non-erythroid cells during protein targeting and membrane domain formation. Here, we demonstrate that β-Spectrin is required in neurons for proper midline axon guidance in the *Drosophila* embryonic CNS. In β-*spectrin* mutants many axons inappropriately cross the CNS midline, suggesting a role for β-Spectrin in midline repulsion. Surprisingly, neither the Ankyrin-binding nor the pleckstrin homology (PH) domains of β-Spectrin are required for accurate guidance decisions. α-Spectrin is dependent upon β-Spectrin for its normal subcellular localization and/or maintenance, whereas α-*spectrin* mutants exhibit a redistribution of β-Spectrin to the axon scaffold. β-*spectrin* mutants show specific dose-dependent genetic interactions with the midline repellent *slit* and its neuronal receptor *roundabout* (*robo*), but not with other guidance molecules. The results suggest that β-Spectrin contributes to midline repulsion through the regulation of Slit-Robo pathway components. We propose that the Spectrin network is playing a role independently of Ankyrin in the establishment and/or maintenance of specialized membrane domains containing guidance molecules that ensure the fidelity of axon repulsion at the midline.

KEY WORDS: Axon guidance, Midline, Repulsion, Roundabout, Slit, Spectrin, Ankyrin, *Drosophila*

INTRODUCTION

During central nervous system (CNS) development, growth cones navigate a series of choice points to find their appropriate targets. These guidance decisions are shaped by a balance of attractive and repulsive cues found in the extracellular environment that can act locally or at a distance (Tessier-Lavigne and Goodman, 1996). Slit ligands and Robo receptors play conserved roles in regulating midline axon crossing in both invertebrate and vertebrate nervous systems. In the *Drosophila* embryonic CNS, ipsilateral neurons (neurons that do not cross the midline) express the Robo receptor on their surface, which senses Slit to prevent midline crossing (Kidd et al., 1999; Kidd et al., 1998). By contrast, commissural neurons can overcome Slit repulsion because expression of Robo on their surface is prevented prior to midline crossing by the Commissureless (Comm) protein (Keleman et al., 2002). Thus, the specific receptors that a growth cone expresses as it encounters the midline greatly influences its guidance decision. The question of how guidance receptors and their downstream effectors are targeted to and distributed within functional domains of the growth cone plasma membrane remains key to the understanding of the mechanisms of axon path finding.

The Spectrin molecule, a long rod-shaped heterotetramer consisting of two α and two β subunits, is the defining element of a ubiquitous sub-membrane cytoskeletal network in nearly all metazoan cells (Bennett and Baines, 2001). Most β-Spectrin isoforms contain an actin-binding domain at their N termini, an Ankyrin-binding domain, and a pleckstrin homology (PH) domain near their C termini. The PH domain of β-Spectrin interacts with the plasma membrane in transfected COS cells (Wang et al., 1996). α-Spectrin contains an SH3 domain and two EF-hand motifs (Bennett and Baines, 2001).

In red blood cells, the Spectrin network was originally shown to have a role in supporting cell shape (Bennett and Chen, 2001); however, more recently it has been shown to participate in the formation of specialized membrane sub-domains (Bennett and Chen, 2001). Through its binding partner Ankyrin, the Spectrin network links many integral membrane proteins to the actin cytoskeleton. Genetic studies have revealed that many membrane proteins are mislocalized in the absence of Spectrin or Ankyrin (Dubreuil et al., 2000; Jenkins and Bennett, 2001; Komada and Soriano, 2002; Zhou et al., 1998). In addition, mutations in the vertebrate β-Spectrin and Ankyrin genes have been linked to various diseases, such as hereditary hemolytic anemias and spinocerebellar ataxia in humans, and auditory and motor neuropathies in mice (Ikeda et al., 2006; Mohler and Bennett, 2005; Parkinson et al., 2001). It has been proposed that Ankyrin and β-Spectrin are interdependent, and mutually stabilize the formation of polarized domains at axon initial segments and nodes of Ranvier (Komada and Soriano, 2002). Yet the relationship between Ankyrin and β-Spectrin, and the role that each plays in coordinately regulating microdomain assembly, is not well understood.

In *Drosophila*, β-*spectrin* mutants die before larval hatching and loss of β-Spectrin leads to a failure of Na,K ATPase accumulation at the basolateral domain of midgut epithelial cells (Dubreuil et al., 2000). More recently, studies using RNA interference (RNAi) techniques have demonstrated a role for β-Spectrin during nervous system development. Specifically, the β-Spectrin network was

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Accepted 19 September 2006

shown to be required pre-synaptically to maintain cell adhesion molecule (CAM) organization and neuromuscular junction stability (Pielage et al., 2005).

In a genetic screen for additional genes involved in midline axon guidance in the *Drosophila* CNS, we identified three β-*spectrin* alleles. β-*spectrin* mutant embryos display fused anterior and posterior commissures, and ectopic Fas2-positive neurons crossing the midline, suggesting a role for Spectrin in midline repulsion. Likewise, *α-spectrin* mutants exhibit very mild defects in midline repulsion, supporting the idea that it too has a minor role in regulating guidance. Additionally, in *α-spectrin* mutants, β-Spectrin is preferentially distributed to CNS axons, suggesting that α-Spectrin normally limits axonal accumulation of β-Spectrin. Genetic rescue experiments demonstrate that β-Spectrin functions in neurons and that the Ankyrin-binding domain is not required for guidance. This was surprising as Ankyrin is thought to be intimately associated with Spectrin in most cases. Furthermore, β-*spectrin* mutants demonstrate dose-dependent genetic interactions with the repulsive ligand *slit* and its receptor *robo* (but not with other guidance molecules), suggesting that they may be involved in the same pathway. Similar genetic interactions are observed within a small subpopulation of ipsilateral neurons, the apterous (Ap) neurons. Defects in the Ap neurons appear later in development, suggesting a role for β-*spectrin* in the maintenance of proper connectivity. Our data support a model in which the Spectrin network is important for the sorting and/or localization of molecules that contribute to and maintain midline repulsion.

Hülsmeier et al. (Hülsmeier et al., 2007) have reported that β-*spectrin* is the gene disrupted in the *karussel* mutant; *karussel* was previously isolated in a large-scale genetic screen for defects in axon guidance at the CNS midline (Hummel et al., 1999b). Our results, together with this complementary study, strongly support an important role for β-*spectrin* in the regulation of connectivity in the embryonic CNS.

MATERIALS AND METHODS

Genetics

The following stocks were used: (1) βspec^{em6}/FM7actinβgal; (2) βspec^{em21}/FM7actinβgal; (3) βspec^{G0108}/FM7actinβgal; (4) βspec^{G0198}/FM7actinβgal; (5) βspec^{G0074}/FM7actinβgal; (6) αSpec^{rg41}/Tm3UbxβGal; (7) αSpec^{lm88}/Tm3UbxβGal; (8) αSpec^{lm32}/Tm3UbxβGal; (9) slit^{2}, robo^{GA285}/CyOWgβgal; (10) slit^{2}, robo^{GA285}/CyOWgβgal; elavGAL4; (11) slit^{2},aptGAL4,UAS-TauMycGFP/CyOTubulinGal80; (12) aptGAL4, UAS-TauMycGFP/ CyOTubulinGal80; (13) robo^{GA285}, aptGAL4/CyOWgβgal; (14) robo^{GA285}/CyOWgβgal; UAS-TauGFP; (15) yw^{iso3}; (16) elavGAL4 (III); and (17) single-mindedGAL4 (simGAL4).

Genetic screen

To identify genes involved in midline guidance, we screened for defects in the eagle neurons, a subset of commissural neurons that require *frazzled* for midline attraction (D.S.G. and G.J.B., unpublished) using a reporter stock: *fra, UAS-TauMycGFP/CyTubulinGal80; eagleGAL4*. Reporter males were crossed to a collection of lethal p-element lines on the X chromosome. Embryos were fixed and stained using a 'large-scale collection' technique modified from Hummel et al. (Hummel et al., 1997). General axon guidance defects were identified using anti-BP102 and subset guidance errors were identified using anti-GFP.

Tissue-specific rescue

β*spec^{em6}/FM7actin*β*gal; UAS*β*Spectrin-Myc* flies were generated and crossed to *elavGAL4* or *simGAL4* males.

*α*Spec enhancement

β*Spec^{hypo}/FM7actin*β*gal; CxD/TM3Ubx*β*gal* virgins were crossed to *αSpec^{lm88}* or *^{rg41}/TM3Ubx*β*gal* males. Non-FM7, non-CxD virgins (β*Spec/+; αSpec/TM3Ubx*β*gal*) were crossed back to *αSpec/TM3Ubx*β*gal* males. Hemizygous β-*spectrin* and homozygous *α-spectrin* mutant embryos were identified by the absence of staining for Sex Lethal (Sxl), β-Spectrin or β-gal.

Structure/function study

Male flies carrying ubiquitously expressed wild-type (KW3A) or mutant transgenes (βSpecΔank or βSpecΔPH) were crossed to βSpec^{em6}/FM7actinβgal virgins. Embryos negative for Sxl and β-gal but positive for Myc were scored for rescue using an anti-1D4/Fas2 antibody.

Immunohistochemistry

The following primary antibodies were used: (1) mouse anti-1D4/Fas2 [Developmental Studies Hybridoma Bank (DSHB), 1:100]; (2) mouse anti-BP102 (DSHB, 1:100); (3) rabbit anti-Myc (Sigma-Aldrich, 1:500); (4) rabbit anti-β-Spectrin [337 (Byers et al., 1989; Dubreuil et al., 2000)]; (5) mouse anti-α-Spectrin (DSHB, 3A9, 1:25); (6) mouse anti-Sex Lethal (DSHB, M18, 1:1000); (7) mouse anti-β-gal (DSHB, 40-1a, 1:250); (8) Rb-anti-GFP (Molecular Probes, 1:500); (9) mouse anti-Robo (DSHB, 1:50). The following secondary antibodies were used: AlexaFluor488 goat anti-Rb (Molecular Probes, 1:500) and Cy3-conjugated goat anti-mouse (Jackson Laboratories, 1:1000). Stacks of images were obtained using a Leica DMIRE2 confocal and a 63× oil immersion objective. Stacks were generated using NIH ImageJ software.

Molecular biology

We confirmed the online p-element insert sequence annotation (http://flybase.bio.indiana.edu/) by recovering and sequencing the genomic DNA flanking the p-elements using standard protocols and primers (Huang et al., 2000). Although the p-element insertion sites associated with alleles *G0108* and *G0074* are only approximately 60 nucleotides apart from each other (Fig. 1A), *G0108* embryos display a much more severe defect than do *G0074* embryos.

RESULTS

Identification of β-*spectrin* mutants

Previous genetic screens looking for defects in CNS architecture have been instrumental in our understanding of axon guidance (Hummel et al., 1999a; Hummel et al., 1999b; Seeger, 1994). In an attempt to identify additional axon guidance molecules, we screened for global axon guidance defects and also more specifically for axon guidance errors within a subset of commissural neurons, the eagle neurons, labeled with GFP (see Materials and methods). We screened a collection of lethal p-elements on the X chromosome and identified a p-element line (0011819, allele *G0108*), in which hemizygous males exhibit a compressed axon scaffold containing fused anterior and posterior commissures. Public database (http://flybase.bio.indiana.edu) annotation indicates that the p-element disrupts the β-*spectrin* locus (Fig. 1A). There were two additional β-*spectrin* alleles (Fig. 1A) contained in the p-element collection (line 11799, allele *G0074*; and line 11922, allele *G0198*), both of which display phenotypes similar to the originally identified allele, although less severe (Fig. 1I). A strong loss-of-function (lof) β-*spectrin* allele, *em6*, and an allele that creates a truncated β-Spectrin protein, *em21* (Dubreuil et al., 2000), also have similar phenotypes (Fig. 1I). In addition, mutant p-element embryos show reduced levels of β-Spectrin protein (see Fig. S1 in the supplementary material). Taken together, these data indicate that the p-elements that cause axonal defects disrupt β-*spectrin* gene function.

β-*spectrin* mutants exhibit defects in midline repulsion

To better characterize the embryonic midline axon guidance defects associated with mutations in β-*spectrin*, we stained wild-type and mutant embryos with two neuronal markers, BP102 and Fas2/1D4. In wild-type embryos (or β-*spectrin* heterozygous embryos) stained

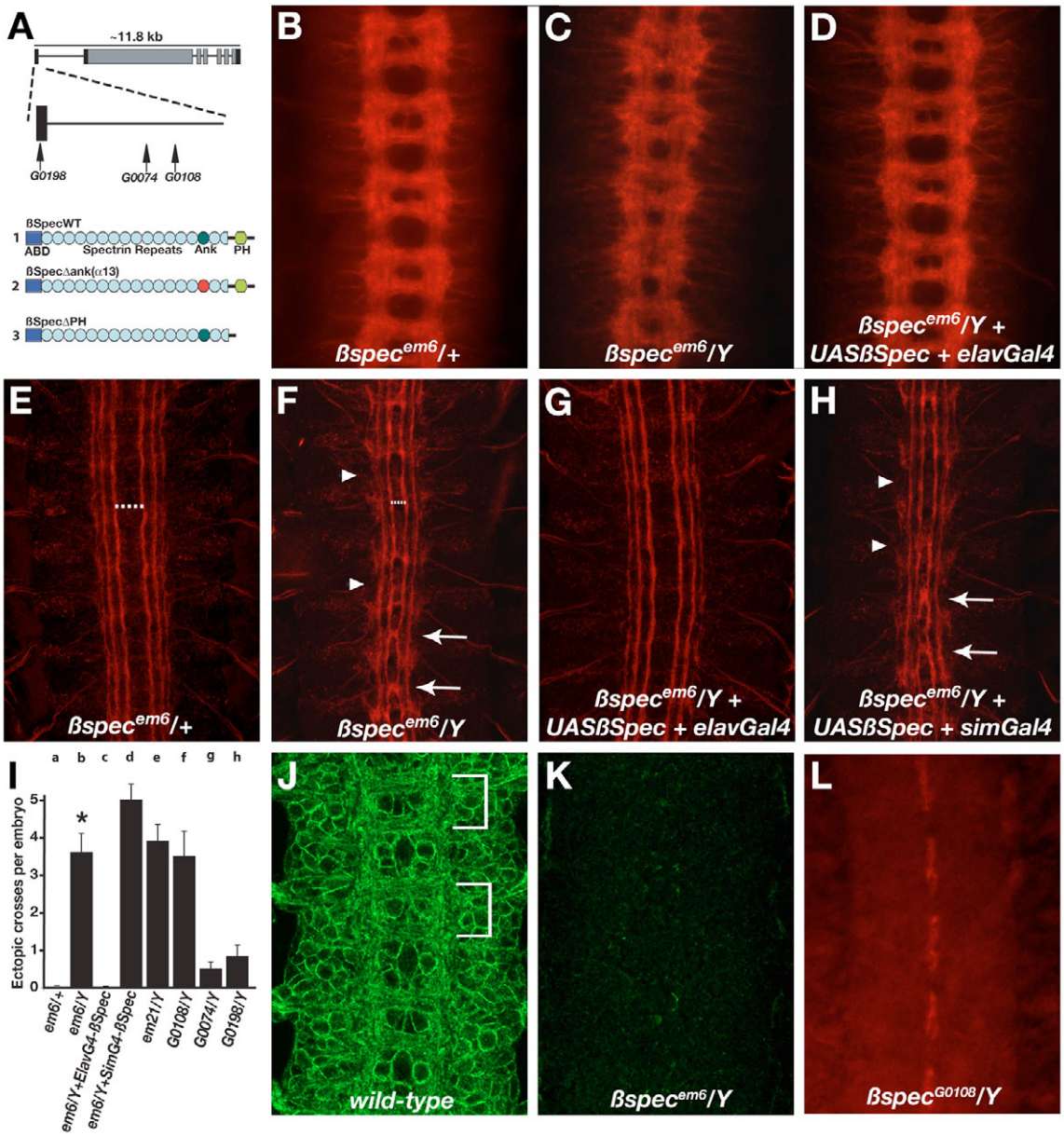

Fig. 1. β-Spectrin is required in neurons for midline axon guidance. (**A**, top) Expanded 5′ region of the β-*spectrin* genomic locus showing the location of each p-element insert. (Bottom) 1, Wild-type β-Spectrin (βSpecWT) protein including functional domains (ABD, actin-binding domain; ank, Ankyrin-binding domain; PH, pleckstrin homology domain); 2, βSpecΔank replaces segment 16 (the 15th repeat) of β-Spectrin with segment 13 of α-Spectrin; 3, βSpecΔPH places a stop codon at the beginning of the PH domain in β-Spectrin. (**B-D**) Stage 16 embryos stained with monoclonal antibody (Mab) BP102 to reveal the CNS axon scaffold. Anterior is up. (B) β-*spectrin* (*em6*) heterozygous embryos exhibit a wild-type ladder-like CNS architecture. (C) β-*spectrin* (*em6*) hemizygous embryos display fused anterior and posterior commissures reflecting a reduction in midline repulsion. (D) Rescue of β-*spectrin* (*em6*) phenotype by expressing wild-type β-Spectrin in all post-mitotic neurons with *elavGAL4*. (**E-H**) Late stage 16 embryos stained with anti-Fas2 Mab to reveal longitudinal axon pathways. Anterior is up. (E) β-*spectrin* heterozygous embryos have no Fas2-positive axon bundles crossing the midline. (F) β-*spectrin* (*em6*) hemizygous embryo. Medial ectopic Fas2-positive bundles cross the midline (arrows) and are closer together [distance between medial fasicles: wild-type, 13.7±1.37 μm; β*spec(G0108)*, 8.9±1.27 μm; compare E and F, dashed line]. Lateral Fas2-positive bundles contain longitudinal breaks (arrowheads). (G) β-*spectrin* mutant embryo expressing full-length wild-type β-Spectrin in all neurons using *elavGAL4*. Note complete rescue of medial and lateral longitudinal axon guidance defects. (H) β-*spectrin em6* embryo expressing full-length wild-type β-Spectrin in midline glia using *simGAL4*. β-*spectrin* mutant phenotypes in the longitudinal pathways (arrows and arrowheads) are still observed. (**I**) Quantification of ectopic crosses per embryo in genotypes: a, *em6/+* (n=15); b, *em6/Y* (n=18); c, *em6/Y,UAS-*β*Spec/+,elavGAL4/+* (n=15); d, *em6/Y,UAS-*β*Spec/+,simGAL4/+* (n=11); e, *em21/Y* (truncation) (n=18); f, strong p-element allele *G0108/Y* (n=14); g, presumptive hypomorphic p-element allele *G0074/Y* (n=13); h, presumptive hypomorphic p-element allele *G0198/Y* (n=14). Asterisk denotes significant difference in genotypes b and a, and b and c (P=8.027×10^{-7}; two-sample Student's t-test). There is no significant difference between genotypes b and d. Error bars indicate s.e.m. (**J**,**K**) Stage 15 embryos stained with polyclonal anti-β-Spectrin antibody. Anterior is up. (J) Wild-type β-Spectrin protein localizes to the plasma membrane around every cell of the CNS neuropil and to the axon scaffold (brackets). (K) β-Spectrin protein levels are severely reduced in β-*spectrin (em6)* mutant embryos. (**L**) β-*spectrin* (G0108) mutant embryo stained with an anti-wrapper antibody showing the appropriate number and location of the midline glia.

with BP102, the CNS has a uniform ladder-like axon scaffold, containing longitudinal axon bundles running along either side of the midline that are connected to each other by distinct anterior and posterior commissures in each segment (Fig. 1B). By contrast, β-*spectrin* mutant embryos have thicker anterior and posterior commissures that are often fused (Fig. 1C), phenotypes that are indicative of loss of midline repulsion. Because mutations that disrupt midline glial cell development and migration often give rise to phenotypes similar to those seen in β-*spectrin* embryos, we used antibodies against Wrapper (Noordermeer et al., 1998), and confirmed that the midline glia are present in the appropriate number and positions (Fig. 1L). The midline glia in β-*spectrin* mutants also express Slit, suggesting they produce appropriate repulsive guidance cues (data not shown).

Often, when midline repulsion is compromised, the most medial Fas2-positive longitudinal fascicle inappropriately crosses the midline and continues to extend on the opposite side (Kidd et al., 1998). Therefore, we investigated whether β-*spectrin* mutants had inappropriate Fas2-positive bundles crossing the midline. Similar to wild type, β-*spectrin* heterozygous embryos have no inappropriate crossovers (Fig. 1E,I). By contrast, β-*spectrin* hemizygous mutant embryos display ectopic Fas2-positive neurons crossing the midline (Fig. 1F,I). *em6*, *em21* and the p-element allele *G0108* show the strongest crossing defects, whereas the presumptive hypomorphic p-element alleles (*G0198* and *G0074*) have less severe defects (Fig. 1I). Additionally, when compared with wild type, β-*spectrin* mutant embryos show a reduced distance between opposing Fas2-positive medial longitudinal bundles (Fig. 1E,F), and the most lateral Fas2-positive bundles of axons appear to stall and fail to extend longitudinally (Fig. 1F, arrowheads). In *C. elegans*, mutations in β-*spectrin* (*unc-70/bgs-1*) also appear to affect neuronal function and axon outgrowth (Hammarlund et al., 2000; Moorthy et al., 2000), which suggests that *Drosophila* β-Spectrin may be playing a conserved role during the outgrowth of certain classes of axon.

β-Spectrin is required in neurons for midline axon guidance

To investigate where β-Spectrin functions during midline axon guidance, we examined β-Spectrin protein expression in wild-type and β-*spectrin* mutant embryos using an antibody that specifically recognizes full-length β-Spectrin (Byers et al., 1989; Dubreuil et al., 2000). Wild-type β-Spectrin localizes weakly to the axon scaffold and is targeted to the plasma membrane surrounding every cell within the midline neuropil (Fig. 1J). *em6* embryos have greatly reduced levels of β-Spectrin protein (Fig. 1K), yet we cannot rule out the possibility that trace levels may be maternally loaded. Strong and weak loss-of-function p-element alleles exhibit reduced levels and mislocalization (including lack of complete uniform cell body plasma membrane staining) of β-Spectrin protein (see Fig. S1 in the supplementary material).

The ubiquitous expression pattern of β-Spectrin did not give any insight into where β-Spectrin is required for its midline guidance function. Therefore, to investigate this, we tested two candidate cell types – neurons and midline glia – by expressing wild-type β-Spectrin protein in both cell types using the Gal4/UAS system in β-*spectrin* mutants. We found that the mutant phenotypes can be rescued by expressing wild-type UAS-β-Spectrin in all postmitotic neurons using *elavGAL4* (Fig. 1D,G). By contrast, expressing β-Spectrin in midline glia under the control of *simGAL4* does not rescue the β-*spectrin* phenotype (Fig. 1H). Importantly, overexpression of β-Spectrin throughout the CNS in a wild-type background using *elavGAL4* does not result in any obvious defects (data not shown). These data demonstrate that β-Spectrin is required in neurons but not in other cell types at the midline, and support the idea that β-Spectrin contributes to axon repulsion.

α-Spectrin plays a minor role during midline axon repulsion

Canonically, α- and β-Spectrin function together as a heterotetramer that ubiquitously associates with the plasma membrane (Bennett and Baines, 2001; De Matteis and Morrow, 1998). Therefore, we investigated whether α-Spectrin, like β-Spectrin, has a role during midline axon guidance. In contrast to what is observed in β-*spectrin* mutant embryos, *α-spectrin* mutants display only very mild defects in axon guidance. Specifically, these errors are seen just in certain hetero- and homoallelic combinations (Fig. 2A,C,F). When both copies of *α-spectrin* are removed in a hypomorphic hemizygous β-*spectrin* background, we see an increase in the number of Fas2-positive axon bundles ectopically crossing the midline (Fig. 2D-F). This genetic interaction supports the idea that *α-spectrin* modestly contributes to axon guidance. However, β-Spectrin appears to be playing a much larger role during this developmental process. Hulsmeier et al. independently identified additional *α-spectrin* alleles that do not display guidance defects (Hülsmeier et al., 2007). These data further support the idea that *α-spectrin* plays only a minor role during midline guidance.

α- and β-Spectrin depend on each other for proper localization

To investigate the relationship between α- and β-Spectrin, we examined their expression patterns in wild-type and single mutant backgrounds. Wild-type α- and β-Spectrin colocalize at the plasma membrane around every cell in the CNS and are weakly expressed on axon tracks (Fig. 3A-C). As noted above, β-*spectrin* (*em6*) mutant embryos exhibit severely reduced levels of β-Spectrin protein (Fig. 3D). Similarly, when image parameters are kept constant, α-Spectrin protein levels are also greatly reduced in β-*spectrin* mutants. However, the Photo Multiplier Tube (PMT) gain can be increased to detect low levels of α-Spectrin at the plasma membrane surrounding the cell bodies of the CNS, although the protein looks slightly more diffuse than in wild type (Fig. 3E, starred arrowheads). The fact that levels of α-Spectrin are dramatically reduced in β-*spectrin* mutant embryos suggests that localization and/or maintenance of α-Spectrin depends on β-Spectrin. However, the fact that we still see some α-Spectrin would suggest that there may be a minimal supply of maternally deposited β-Spectrin still present (although barely detectable by our antibody) in the β-*spectrin* mutant embryos that is able to localize α-Spectrin. We also cannot rule out the alternative possibility that there is a small pool of α-Spectrin that can localize independently of β-Spectrin. When we stain embryos carrying the hypomorphic allele (*G0108*) with anti-β-Spectrin and anti-α-Spectrin, we see reduced levels and mislocalization of both proteins. Intriguingly, α-Spectrin still colocalizes with β-Spectrin (see Fig. S1 in the supplementary material) even though the overall expression pattern is highly disrupted. This is consistent with the hypothesis that β-Spectrin contributes to the localization and/or maintenance of α-Spectrin.

As expected, *α-spectrin* mutants have reduced levels of α-Spectrin protein (Fig. 3H). The residual amount of α-Spectrin protein present in the embryo appears to still localize properly to the cell body plasma membrane, but not to axons (Fig. 3H, arrows), and is likely to be maternally contributed. In marked contrast to the clear dependence of α-Spectrin expression and/or stability on wild-type β-Spectrin, *α-*

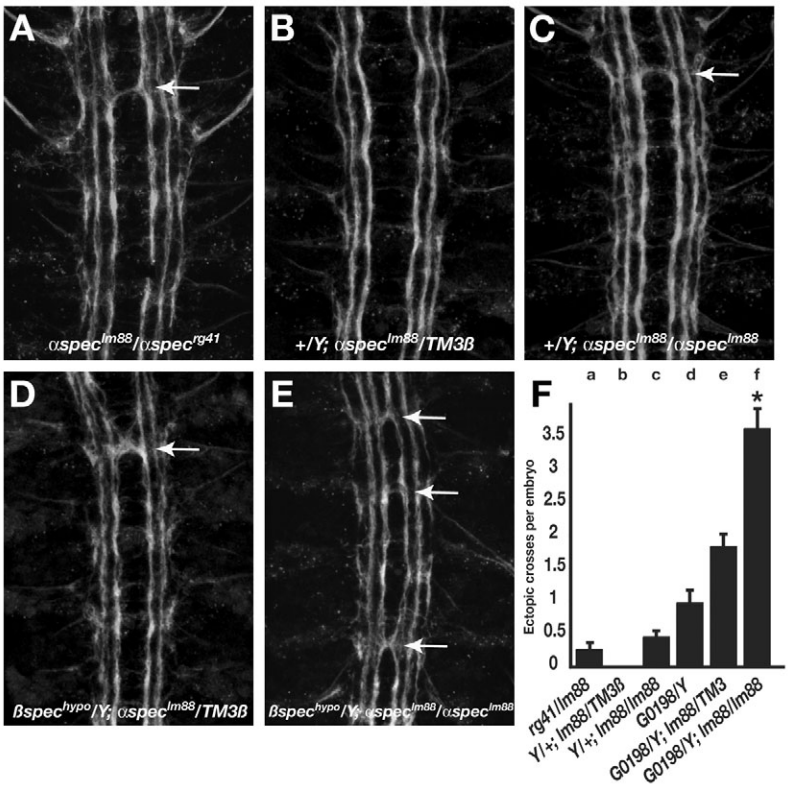

Fig. 2. *α-spectrin* weakly contributes to midline axon guidance. (**A-E**) Late stage 16 embryos stained with anti-Fas2 Mab. Anterior is up. (A) Heteroallelic *α-spectrin* (*lm88/rg41*) mutant embryos on average contain less than one Fas2-positive ectopic crossover (arrow). (B) *α-spectrin (lm88)* heterozygous embryos do not exhibit midline guidance errors. (C) Similar to the heteroallelic combination, *α-spectrin (lm88)* homozygous embryos display mild midline guidance defects. (D) Removing one copy of *α-spectrin (lm88)* mildly enhances the β-*spectrin* (*G0198*) hypomorphic phenotype. (E) Stronger genetic interactions are observed when two copies of *α-spectrin* are removed in β-*spectrin* hypomorphic mutants. Note the increase in Fas2-positive longitudinal axon bundles crossing the midline. (**F**) Quantification of single and double mutants. a, *α-spectrin(rg41)/ α-spectrin(lm88)* (*n*=19); b, *lm88/TM3*β (*n*=7); c, *lm88/lm88* (*n*=11); d, β-*spectrin(G0198)/Y* (*n*=14); e, *G0198/Y; lm88/TM3*β (*n*=6); f, *G0198/Y; lm88/lm88* (*n*=8). Asterisk denotes a significant difference between genotypes f and c, and f and d ($P=2.86\times10^{-5}$ and 0.000154 respectively; two-sample Student's *t*-test). Error bars indicate s.e.m.

spectrin mutants exhibit a noticeable redistribution of β-Spectrin to the axon scaffold (compare Fig. 3A with 3G). Thus, it appears that α-Spectrin is normally required to keep axonal levels of β-Spectrin low.

The Ankyrin-binding and PH domains are dispensable for β-Spectrin guidance function

Studies of βIV-Spectrin and Ankyrin-G mutant mice suggest that these two proteins may mutually stabilize each other, as well as CAMs and VGSCs, at axon initial segments and nodes of Ranvier (Komada and Soriano, 2002). Therefore, we were interested in testing whether there was a similar relationship between Ankyrin and β-Spectrin in *Drosophila*. Currently, *dank1* (*Drosophila ankyrin-1*; *ankyrin* – FlyBase) mutants are not available but their interaction can be studied by using a mutant β-Spectrin transgene in which Ankyrin-binding activity has been removed (Das et al., 2006). Specifically, this βSpecΔank transgene cannot rescue the localization defects of Dank1::EGFP in copper cells of β-*spectrin* mutants, suggesting that Ank1-binding is indeed disrupted. Surprisingly, when expressed ubiquitously throughout the embryo, βSpecΔank rescues the axon guidance defects as efficiently as does wild-type β-Spectrin (Fig. 4C,D,F). Because we know that β-Spectrin is required in neurons (Fig. 1), we attribute this rescue to its neuronal function. These data suggest that the role of β-Spectrin during midline axon guidance in *Drosophila* is independent of Dank1.

Interestingly, Ankyrin-independent membrane binding sites for β-Spectrin were identified in binding studies with NaOH-stripped membranes from rat brain (Davis and Bennett, 1994), and a reporter consisting of the PH domain of vertebrate βI-Spectrin fused to GFP binds IP3 and mediates targeting to the plasma membrane in COS cells (Wang et al., 1996). Therefore, to address whether the Ankyrin-binding or PH domains are required for precise localization of β-Spectrin in *Drosophila* neurons, we used the Myc epitope tag to monitor transgene expression. As expected, ubiquitously expressed full-length β-Spectrin localizes to axons and the plasma membrane of all cells in the neuropil (Fig. 4H,K). Similarly, βSpecΔank localizes with the same pattern as the full-length protein (Fig. 4I,L). By contrast, βSpecΔPH does not localize to the plasma membrane of cell bodies and the overall level of expression is reduced when compared with the other transgenes (Fig. 4J,M). However, we still observe some axonal staining (Fig. 4J). Despite disrupted cell body plasma membrane staining, βSpecΔPH completely rescues the midline guidance defects observed in β-*spectrin* mutant embryos (Fig. 4E), suggesting that the PH domain is not required for β-Spectrin to carry out its role in guidance. Together, these data suggest that the PH domain, but not the Ankyrin-binding domain, is required for proper membrane localization in the cell body. However, cell body plasma membrane localization does not appear to be required for function, as βSpecΔPH can still rescue the guidance phenotype despite partially defective distribution. This is consistent with the idea that a sub-population of axonally localized β-Spectrin is important during growth cone migration, and this localization is independent of the PH and Dank1-binding domains. However, we do acknowledge that these gain-of-function experiments may not reflect the true localization of endogenous proteins with similar deletions. For example, in our experiments, overexpressed full-length β-Spectrin appears more concentrated on axons than does endogenous protein (compare Fig. 1J with Fig. 4H).

Mutations in β-*spectrin* genetically interact with the Slit-Robo pathway

As has been previously shown, Fas2-positive axons ectopically cross the midline in β-*spectrin* mutant embryos. Given the similarity between β-*spectrin* and *robo* loss-of-function phenotypes, we speculated that β-*spectrin* might be influencing Slit-Robo-mediated repulsion. Therefore, we investigated whether mutations in β-

spectrin could enhance the guidance defects associated with heterozygous mutations in *slit* and *robo*. Observing a dose-dependent genetic interaction between a set of genes suggests that they may be involved in the same pathway. Indeed, mutations in β-*spectrin* demonstrate a genetic interaction with *slit* and *robo*. Specifically, β-*spectrin* hemizygous *em6* mutants, or *slit*, *robo* trans-heterozygous mutants, contain few ectopic Fas2-positive bundles crossing the midline (Fig. 5A,B,G). However, in combination, β-*spectrin* hemizygous mutations dramatically enhance the *slit*, *robo* trans-heterozygous phenotype (Fig. 5C,G), suggesting that these genes are involved in the same process, namely midline repulsion. Importantly, hypomorphic β-*spectrin* alleles (*G0198* and *G0074*)

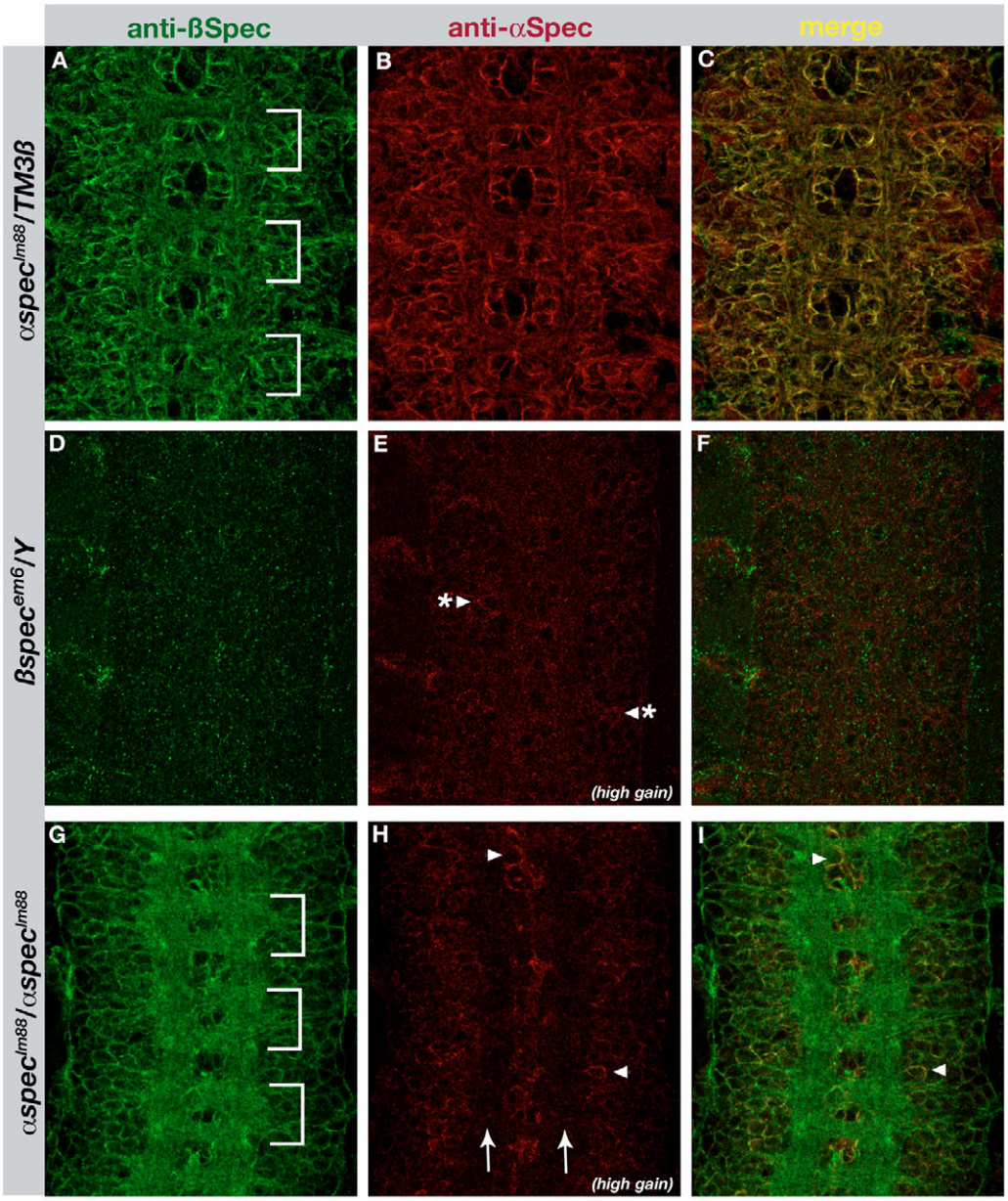

Fig. 3. Co-dependence of α- and β-Spectrin for proper protein localization. (**A-I**) Stage 15 embryos stained with polyclonal anti-β-Spectrin antibody (A,D,G) or with monoclonal anti-α-Spectrin antibody (B,E,H). A merged composite image of α- and β-Spectrin staining for each genotype is shown in C, F and I. (A-C) An *α-spectrin* heterozygous embryo. (A) β-Spectrin protein localizes to the plasma membrane surrounding all CNS cells and to the axon scaffold (white brackets). (B) α-Spectrin shows similar localization. (C) A merged image (yellow indicates colocalization). (D-F) A β-*spectrin (em6)* mutant embryo. (D) β-*spectrin* mutant embryos have significantly reduced β-Spectrin protein levels. (E) α-Spectrin protein levels are also reduced and are almost undetectable when confocal settings identical to those used for the image in B are applied, suggesting that β-Spectrin is required to maintain normal levels of α-Spectrin. When the Photo Multiplier Tube (PMT) gain is increased, low levels of α-Spectrin can be seen at the plasma membrane (starred arrowheads). (F) A merged image showing no colocalization. (G-I) An *α-spectrin* mutant embryo. (G) In *α-spectrin* mutants, β-Spectrin is redistributed to axons (white brackets). (H) *α-spectrin* mutants have low levels of α-Spectrin. When PMT gain is increased, residual α-Spectrin can be seen at the plasma membrane (arrowheads) but not on axons (arrows). (I) A merged image of α- and β-Spectrin localization in an *α-spectrin* mutant. Arrowheads depict cells in which α- and β-Spectrin colocalize (yellow). For all panels, the genotypes are listed on the left and antibodies are listed on top.

also robustly enhance the *slit*, *robo* trans-heterozygous phenotype (Fig. 5E-G). These dose-dependent interactions suggest that β-*spectrin* may be intimately involved in the Slit-Robo pathway. Furthermore, β-*spectrin* mutants seem to specifically modify the Slit-Robo pathway, as genetic interactions are not detected with either the Netrin/Frazzled or the Semaphorin/Plexin pathways (see Fig. S2 in the supplementary material).

Similar to the rescue of β-*spectrin* mutants observed by expressing wild-type β-Spectrin protein in all neurons, we also see rescue of the *slit*, *robo* enhancement by expressing wild-type β-Spectrin with *elavGal4* (Fig. 5D,G). Given these genetic interactions, we hypothesized that β-Spectrin may regulate the distribution of Robo in *Drosophila* midline CNS axons. However, we did not observe obvious changes in the levels or localization of Robo protein when β-*spectrin* mutant embryos were compared with wild type (Fig. 6). Nevertheless, β-Spectrin may affect another component of the Robo pathway downstream of the receptor.

In addition to defects in Fas2-positive neurons, we also see β-*spectrin* mutant phenotypes and genetic interactions with the Slit-Robo pathway within a small subpopulation of ipsilateral neurons, the apterous (Ap) neurons. In wild-type embryos, the Ap neurons approach the midline but never cross (Fig. 7A). In both *slit*/+ heterozygous and β-*spectrin* (*em6*) hemizygous mutant embryos, we observe mild midline errors (‘thick crosses’) when Ap neurons are labeled with Tau-Myc-GFP (Fig. 7B,C,H). Both null and hypomorphic mutations in β-*spectrin* enhance the *slit*/+

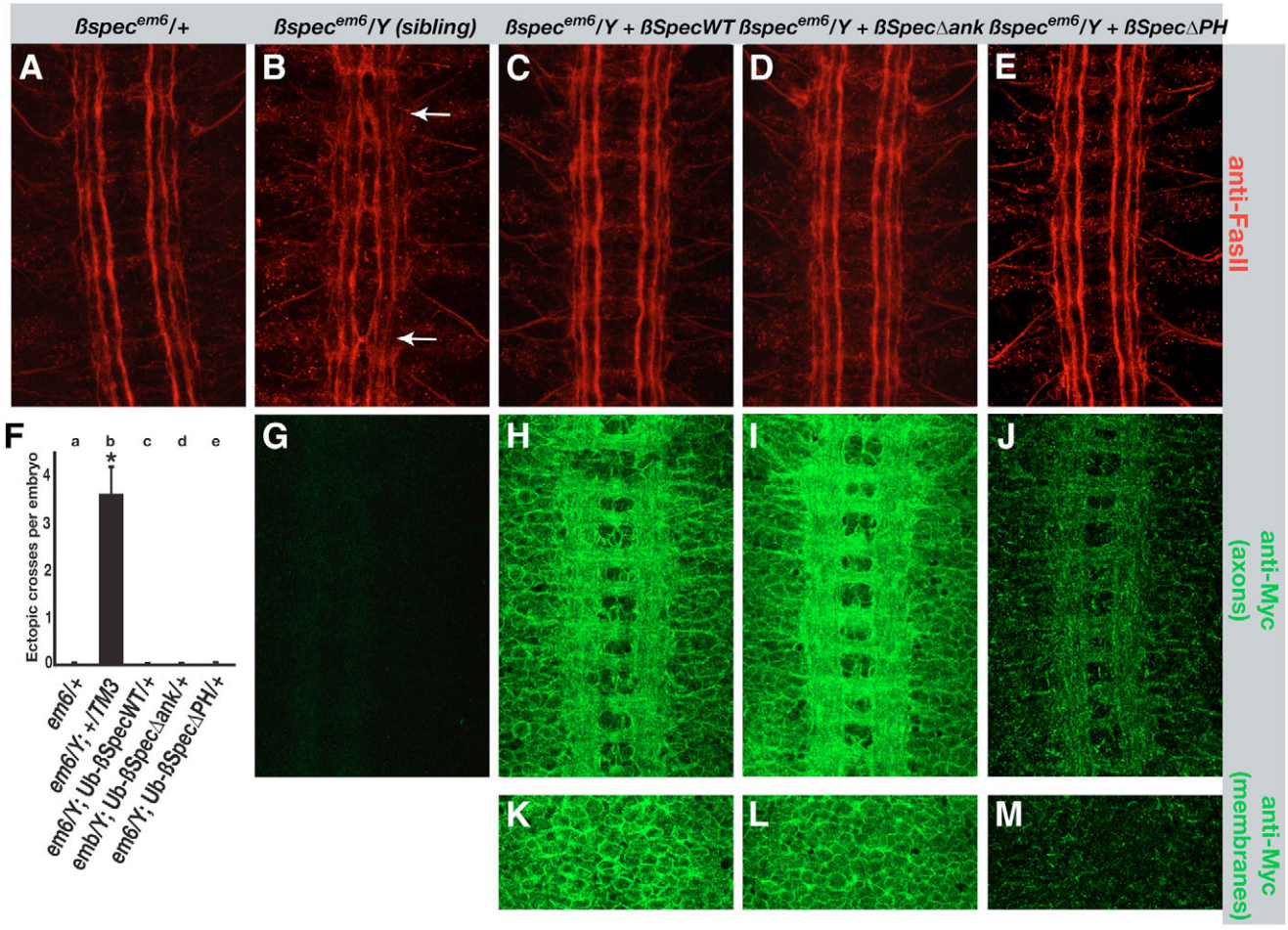

Fig. 4. The Ankyrin-binding and PH domains are not required for axon guidance. (**A-E**) Late stage 16 embryos stained with the anti-Fas2 Mab. Anterior is up. (A) β-*spectrin* heterozygous embryos have no Fas2-positive longitudinal bundles crossing the midline. (B) β-*spectrin (em6)* hemizygous embryos display Fas2-positive axons crossing the midline (arrows). (C) β-*spectrin* null hemizygous embryos ubiquitously expressing full-length β-Spectrin are rescued for both the medial ectopic crossing defect and lateral longitudinal breaks. (D) Ubiquitously expressing a form of β-Spectrin lacking the Ankyrin-binding domain (βSpecΔank) also rescues the midline axon guidance defects seen in β-*spectrin* mutant embryos. (E) Ubiquitously expressing a form of β-Spectrin lacking the PH domain (βSpecΔPH) also rescues β-*spectrin* mutant midline guidance errors. (**F**) Quantification of ectopic Fas2-positive midline crossovers in β-*spectrin* heterozygous, hemizygous mutant, and transgenic rescued backgrounds. a, *em6*/+ (*n*=15); b, *em6*/*Y* (*n*=13); c, *em6*/*Y*+*Ub*-β*SpecWT* (*n*=9); d, *em6*/*Y*+*Ub*-β*SpecΔank* (*n*=15); e, *em6*/*Y* + *Ub*-β-*SpecΔPH* (*n*=20). Asterisk denotes a significant difference between genotype b and genotypes a, c, d and e (P=1.22×10^{-6}; two-sample Student’s *t*-test). Error bars indicate s.e.m. (**G-M**) Same embryos as in B-E, stained with the polyclonal anti-Myc antibody. Anti-Myc stainings were performed at the same time and images were taken at the same confocal settings. (G) Sibling β-*spectrin* mutant embryos not expressing a transgene do not stain with the Myc antibody. Ubiquitously expressed wild-type β-Spectrin localizes to the axons (H) and to the plasma membrane at sites of cell contact (K). Ubiquitously expressed βSpecΔank localizes similarly to full-length transgenic protein to the axon scaffold (I) and to the plasma membrane (L). Ubiquitously expressed βSpecΔPH localizes to axons (J), although at reduced levels, but is no longer localized to the plasma membrane nor at sites of cell contact (M).

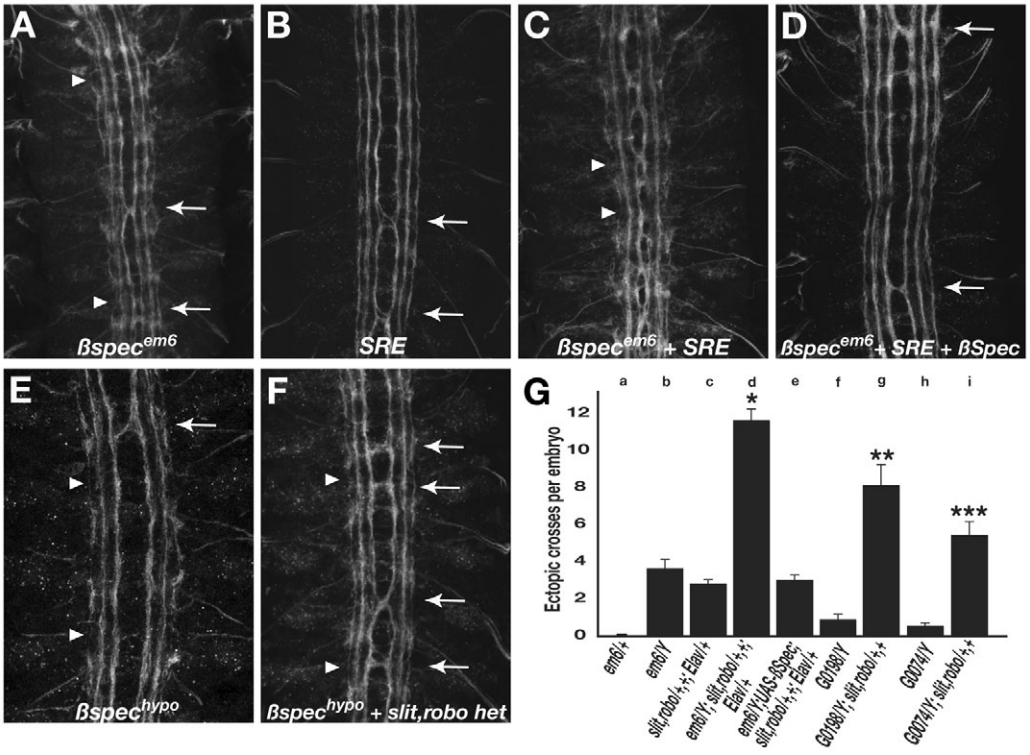

Fig. 5. Mutations in β-*spectrin* genetically interact with the Slit-Robo pathway. All embryos were dissected at late stage 16 and were stained with anti-Fas2 Mab. Anterior is up. In all panels, arrows indicate sites of medial axons ectopically crossing the midline and arrowheads point to lateral longitudinal bundle breaks. (**A**) β-*spectrin* null embryos exhibit ectopic Fas2-postitive axons crossing the midline and lateral longitudinal breaks. (**B**) *slit, robo/+; elavGal4/+* (SRE) heterozygous embryos contain approximately two to three Fas2-positive bundles crossing the midline. (**C**) Removing β-*spectrin* dramatically and specifically enhances the SRE defect. Note the effect is synergistic and not simply additive. (**D**) The genetic interaction seen in C can be suppressed by expressing wild-type UAS-β-Spectrin in all postmitotic neurons with *elavGal4*. Note that approximately two to three ectopic crosses (arrows) are still observed as a result of the SRE background; however, the distance between medial longitudinal bundles has widened and the lateral breaks have disappeared. (**E**) β-*spectrin* hypomorphic embryos have mild axon guidance defects. In this embryo, one medial longitudinal bundle crosses the midline (arrow) and lateral breaks are still observed (arrowheads). (**F**) Similar to the null allele, hypomorphic β-*spectrin* alleles also show specific dose-dependent genetic interactions in a *slit,robo/+* heterozygous background. (**G**) Quantification of single mutant phenotypes and genetic interactions. a, *em6/+* (n=15); b, *em6/Y* (n=18); c, *slit, robo/+; elavGal4/+* (n=19); d, *em6/Y; slit,robo/+; elavGal4/+* (n=9); e, *em6/Y; slit,robo/UAS-βSpec; elavGal4/+* (n=23); f, *G0198/Y* (n=14); g, *G0198/Y; slit,robo/+* (n=10); h, *G0074/Y* (n=13); i, *G0074/Y; slit,robo/+* (n=11). Asterisk denotes a significant difference between genotypes d and b, and d and e (P=2.71×10^{-8} and 1.13×10^{-11}, respectively; two-sample Student's t-test). Double asterisk denotes a significant difference between genotypes g and f (P=1.73×10^{-6}; two-sample Student's t-test). Triple asterisk denotes a significant difference between genotypes i and h (P=2.60×10^{-6}; two-sample Student's t-test). There is no significant difference between genotypes c and e. Error bars indicate s.e.m.

heterozygous phenotype (Fig. 7D,H). In contrast to pan-neural expression, which completely rescues the mutant β-*spectrin* guidance defects, the Ap neuron defect cannot be rescued by expressing full-length β-Spectrin in the Ap neurons (Fig. 7E,H). These data suggest a non-autonomous function for Spectrin for some classes of neuron and support the idea that the overall defective patterning of the entire CNS contributes to defects seen in the Ap neurons. *robo* mutants also display ectopic Ap neurons crossing the midline (Fig. 7F); however, this defect can be rescued by expressing full-length Robo in these neurons, demonstrating that Robo functions cell autonomously (Fig. 7G).

β-Spectrin contributes to the maintenance of CNS architecture

In the course of examining the strong midline crossing phenotypes that result from genetic interactions between β-*spectrin* and *slit* and *robo*, we were struck by the absence of defects in the early guidance of the pCC and dMP2 pioneer axons. Initially in β-*spectrin* mutant embryos, pCC and dMP2 make appropriate ipsilateral projections, and only later are crossing defects observed (data not shown). This is true even in the context of genetic interactions between β-*spectrin* and *slit* and *robo*, where severe midline crossing defects are observed later in development. This raised the possibilities either that guidance of the pioneer axons does not require β-*spectrin* function, whereas later navigating axons do, or alternatively that β-*spectrin* function is required for the maintenance of properly established connections. As it is no longer possible to resolve individual neurons at the time that midline crossing defects are observed with Fas2 antibodies, we analysed the behavior of the Ap neurons more closely to distinguish between guidance and maintenance functions.

Similar to what we have observed for the early behavior of the pCC and dMP2 axons, axons of the Ap neurons initially make the correct steering decisions in β-*spectrin* mutant embryos. Specifically, projection toward the midline is normal in early stage 14 embryos and axons make correct anterior ipsilateral turns at late stage 14 to early stage 15 (Fig. 8G,H), similar to wild type (Fig. 8A-C). However midline-crossing defects are observed by late stage 16

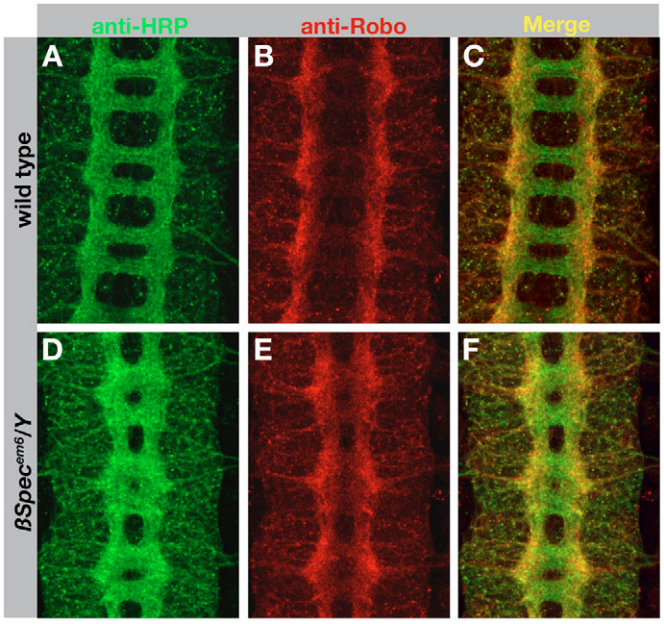

Fig. 6. The level and localization of Robo are not altered in β-*spectrin* null embryos. (**A**,**D**) Embryos stained with anti-HRP. (**B**,**E**) Embryos stained with anti-Robo. (**C**,**F**) Merged image for each genotype. β-*spectrin* mutant embryos (D-F) do not show reduction or mislocalization of Robo, although the distance between opposing sides of the midline is reduced when compared with wild-type embryos (A-C). Embryos were processed in parallel and images were taken at the same confocal settings.

(Fig. 8I). By contrast, *robo* mutants exhibit Ap neuron crossing defects during their initial extension toward the midline and throughout development (Fig. 8D-F). In addition, although β-*spectrin* mutants that are also heterozygous for *slit* show considerably stronger guidance and crossing defects, these defects only arise at later stages of CNS development (Fig. 8K,L). These observations suggest (1) that Slit-Robo repulsion is required not only for initial axon guidance, but also to maintain connections on the correct slide of the midline, and (2) that β-*spectrin* contributes to this later maintenance function.

DISCUSSION

In this study, we demonstrate that *Drosophila* β-Spectrin is required exclusively in neurons for normal repulsive midline axon guidance. Limiting β-*spectrin* shows a dose-dependent synergistic interaction with trans-heterozygous mutations in *slit* and *robo*. This genetic interaction suggests that β-Spectrin may be specifically contributing to Slit-Robo repulsion during growth cone migration. Our work also demonstrates that neither the Ankyrin-binding nor the Pleckstrin Homology (PH) domains of β-Spectrin are required for its role in repulsive guidance. Intriguingly, axonal localization is not disrupted when βSpecΔPH or βSpecΔAnk mutant proteins are overexpressed. These overexpression experiments suggest that β-Spectrin may localize to axons independently of the Ankyrin-binding and PH domains, and that axonal β-Spectrin is sufficient for growth cone migration. However, it is important to note that because these observations are based on overexpression studies, they may not completely reflect the localization of endogenous proteins with similar deletions. Finally, analysis of progressive developmental stages in β-*Spectrin* mutants suggests that, for at least some neurons, β-Spectrin is not required for the initial pathfinding of axons, but rather appears to be important for maintaining appropriate connectivity. Together, our findings support a role of the Spectrin network in contributing to the fidelity of axon repulsion at the *Drosophila* midline.

The role of β-Spectrin in midline repulsion during growth cone migration

The midline guidance defects we observe in embryos stained with BP102 and Fas2 antibodies suggest that β-Spectrin normally contributes to axonal migration and more specifically to axon repulsion. Considering that β-Spectrin has been shown to modulate the behavior of interacting membrane proteins within sub-membrane microdomains, and given that we observe specific dose-dependent genetic interactions with the Slit-Robo pathway, our data support the idea that the Spectrin cytoskeleton modulates the behavior of molecules that contribute to Robo repulsion. Many important signaling molecules must be coordinated downstream of guidance receptors so that navigating growth cones make appropriate decisions. For example, Dock, Pak and Rac contribute to midline repulsion by forming a complex with the Robo receptor upon Slit stimulation (Fan et al., 2003). Similarly, *Drosophila* Ena, a member of a protein family implicated in actin cytoskeleton regulation, functions cooperatively with Robo at a level downstream of the receptor (Bashaw et al., 2000). Additionally, the microtubule-associated protein CLASP (Chb – FlyBase) and the Abelson tyrosine kinase are required to induce restricted cytoskeletal events at the leading edge of growth cones (Lee et al., 2004). Along these lines, β-Spectrin may affect the ability of comparable proteins to signal effectively in the Slit-Robo pathway. Clearly, the Spectrin network cannot account for all Robo function, as many ipsilateral CNS neurons are still guided properly in β-*spectrin* mutants. Mutations in β-*spectrin* also enhance a *robo1* null loss-of-function mutation (data not shown), suggesting that the Spectrin network probably plays an additional role in repulsion outside of Robo1 signaling. It is important to note that our data do not rule out the alternative possibility that β-*spectrin* mutations may have general effects on growth cone migration. If so, then the specific interactions seen with *slit* and *robo* might reflect the fact that the Slit-Robo pathway is more susceptible to subtle perturbations than are the other guidance pathways tested.

β-Spectrin and the maintenance of axon connections

Our analysis of the effect of β-*Spectrin* mutations on the guidance of Ap neurons suggests that β-*Spectrin* is required not for the initial pathfinding of these axons, but rather for maintaining correctly established connections. Moreover, our genetic analysis suggests that in this context the Slit and Robo system also contributes to this maintenance function. Our observations support the idea that some level of Slit/Robo repulsion is required continuously to keep ipsilateral axons on their own side of the midline. A more dramatic example of pathway maintenance has been described in *C. elegans*, where mutations in the Zig genes lead to a 'flipping' of axon pathways over the midline (Aurelio et al., 2003; Aurelio et al., 2002). Additionally, in *Drosophila*, it has been shown that β-Spectrin is essential for synapse stabilization (Pielage et al., 2005). It will be interesting in the future to determine whether the maintenance function of *slit*, *robo* and β-*Spectrin* represents a repulsive mechanism distinct from the mechanism operating during pathway formation.

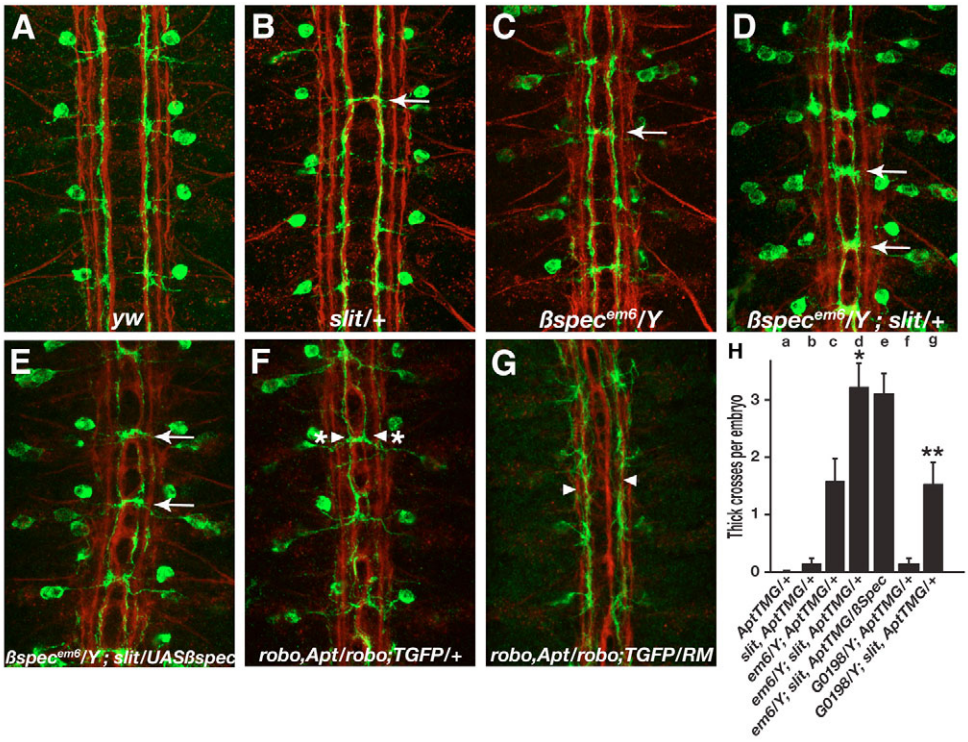

Fig. 7. β-Spectrin affects the guidance of the Apterous (Ap) neurons. (**A-E**) All embryos have *AptGal4, UAS-TauMycGFP* in the background. (**F**,**G**) Embryos have *AptGal4; UAS-TauGFP* in the background. (A) Wild-type embryos have no 'thick' bundles of Ap neurons crossing the midline. (B) *slit*/+ heterozygous embryos have less than one thick bundle of Ap neurons crossing the midline (arrow). (C) β-*spectrin* mutant embryos have approximately one to two thick Ap bundles crossing the midline (arrow). (D) Ap neuron guidance defects in a *slit*/+ heterozygous background are enhanced by removing β-*spectrin* (arrows). (E) The genetic interaction seen in D cannot be rescued by expressing UAS-β-Spectrin in the Ap neurons (arrows). (F) In *robo* mutants, Ap neurons in every segment cross and recross the midline (starred arrowheads). (G) Unlike what is observed in the β-*spectrin* background, Ap neuron defects seen in *robo1* mutants can be rescued by expressing UAS-RoboMyc specifically in the Ap neurons themselves (arrowheads). (**H**) Quantification of genotypes: a, *AptTMG*/+ (*n*=12); b, *slit, AptTMG*/+ (*n*=14); c, *em6/Y; AptTMG* (*n*=10); d, *em6/Y; slit, AptTMG*/+ (*n*=16); e, *em6/Y; slit, AptTMG/UAS-*β*Spec* (*n*=20); f, *G0198/Y; AptTMG*/+ (*n*=14); g, *G0198/Y; slit, AptTMG*/+ (*n*=13). Asterisk denotes a significant difference between genotypes d and c (*P*=0.0144; two-sample Student's *t*-test). There is no significant difference between genotypes d and e. Double asterisk denotes a significant difference between genotypes g and f (*P*=0.00139; two-sample Student's *t*-test). AptTMG, ApterousGal4, UAS-TauMycGFP.

Relationships between α- and β-Spectrin in the *Drosophila* CNS

Our work demonstrates that in the absence of β-Spectrin the stability of α-Spectrin is decreased. Thus, it appears that hetero-tetramer formation is required to maintain proper levels of α-Spectrin in the nervous system. By contrast, cell body plasma membrane localization of β-Spectrin is unaffected in *α-spectrin* mutants. Consistent with this result, it has been shown that β-Spectrin accumulates independently of α-Spectrin in *Drosophila* larvae (Dubreuil and Yu, 1994). This suggests that β-Spectrin recruitment to and stability at the cell body plasma membrane are independent of α-Spectrin, and supports the idea of an α-Spectrin-independent role for β-Spectrin in neurons (see below).

If α- and β-Spectrin function together as a hetero-tetramer, why then do we observe only mild axon guidance defects in *α-spectrin* single mutants? One explanation is that perhaps α-Spectrin has a higher maternal component that is able to compensate for a lack of zygotic expression. Indeed, *α-spectrin* mutants survive to a later developmental stage than do β-*spectrin* mutants. However, *α-spectrin* mutants also exhibit increased levels of axonally localized β-Spectrin (see below). Therefore, an alternative hypothesis could be that the preferential distribution of β-Spectrin to axons somehow compensates for a reduction of α-Spectrin, allowing neurons to make precise steering decisions. Lastly, and perhaps most interesting, β-Spectrin may function independently of α-Spectrin in neurons. This idea seems plausible given that β-Spectrin remains properly localized to the cell body plasma membrane in *α-spectrin* mutants.

As noted above, *α-spectrin* mutant embryos show an increase in the levels of axonal β-Spectrin, suggesting that α-Spectrin regulates the accumulation of β-Spectrin in axons. What does this shift in β-Spectrin distribution mean for the neuron? What are the signals that target β-Spectrin to axons and why does the localization change in *α-spectrin* mutants? One possibility is that the SH3 domain of α-Spectrin targets β-Spectrin to the cell body plasma membrane via a direct interaction with another protein. Another possibility is that, in the absence of α-Spectrin, β-Spectrin binds other axonally localized proteins to a greater degree, thus shifting the overall distribution.

Domain requirements for β-Spectrin axon guidance function

It was originally thought that Ankyrin-G is assembled upstream of βIV-Spectrin at axon initial segments (Jenkins and Bennett, 2001); however, later reports suggested that both Ankyrin-G and βIV-Spectrin are required for the localization and stability of one another,

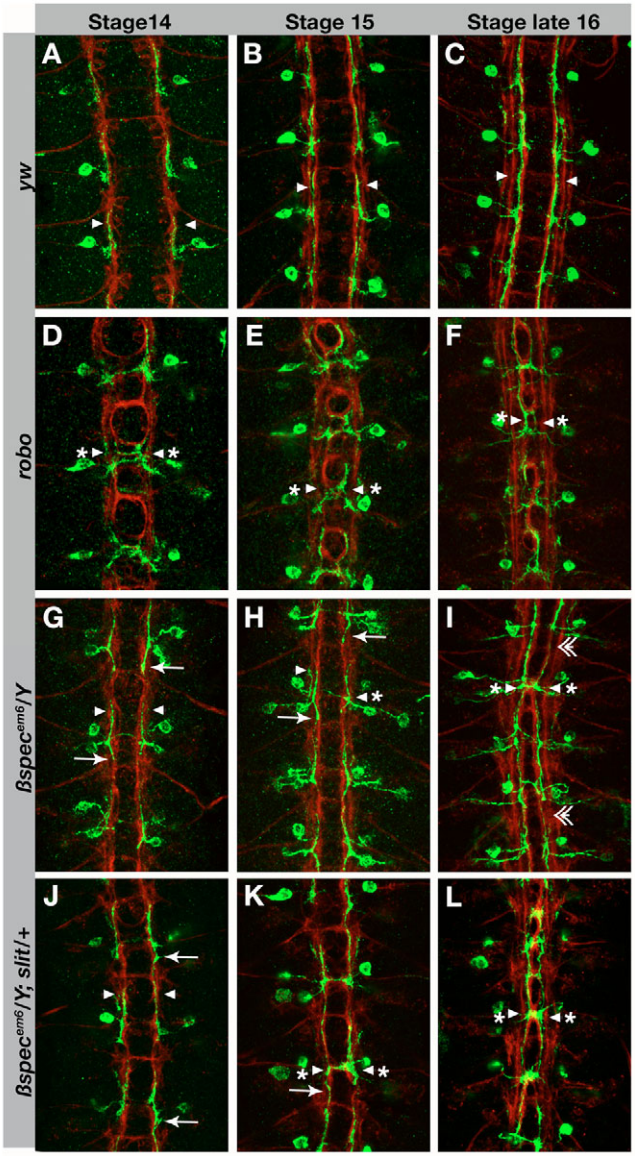

Fig. 8. β-Spectrin contributes to the maintenance of axonal connections. All embryos were stained with anti-Fas2 and anti-GFP, and contain aptGAL4 and UAS-TauMyc GFP in the background except for D-F; these embryos contain UAS-TauGFP. Anterior is up in all panels. Embryos of each genotype (left) were fixed, stained and dissected at the indicated stages (top). (**A-C**) *yw* embryos. (A) At stage 14, the Ap neurons in each segment approach the midline and make a stereotypical turn anteriorly (arrowhead). (B) At stage 15, neurons extend growth cones further anteriorly almost reaching the next segment. (C) At late stage 16, axons make one continuous bundle spanning the length of the embryo. Note that in wild-type embryos, the Ap neurons remain ipsilateral. (**D-F**) *robo* mutant embryos. In *robo* mutant embryos at stage 14 (D), Ap neurons do not respect the midline boundary and cross over to the other side (starred arrowhead). Later in development, axons continue to recross the midline as they extend anteriorly (E,F). (**G-I**) β-*spectrin*^{em6}/*Y* hemizygous embryos. (G) Similar to wild-type embryos at stage 14, the Ap neurons in β-*spectrin* mutants approach the midline, make an anterior turn, and do not cross the midline (arrowhead). Interestingly, in some segments, the Ap neurons also begin to extend processes slightly posteriorly (arrows). (H) At stage 15, most of the Ap neurons still respect the midline boundary, although we occasionally saw a stray axon crossing over (starred arrowhead). (I) Later in development, the Ap neurons lose sensitivity to the midline, fail to maintain their appropriate ipsilateral connections and cross the midline (starred arrowheads). Additionally, in some segments, we noticed that some axons do not extend all the way to the next segment (feathered arrowhead). (**J-L**) β-*spectrin*^{em6} hemizygous plus *slit* heterozygous embryos. (J) Even when one copy of *slit* is removed in a β-*spectrin* null background, stage 14 Ap neurons still do not cross the midline but begin to extend anteriorly. (K) By stage 15, some axons already lose sensitivity to the midline (starred arrowheads). (L) By late stage 16, Ap neurons in many segments now cross the midline (starred arrowheads).

as well as for the stability of VGSCs at axon initial segments and nodes of Ranvier (Komada and Soriano, 2002). Yet, in neonatal cardiomyocytes, Ankyrin-B is required for the proper distribution and levels of βII-Spectrin, and an Ankyrin-B protein lacking the Spectrin-binding domain still localizes properly. These data suggest that Ankyrin-B localization is independent of βII-Spectrin. We sought to test the functional importance of the protein domains of β-Spectrin during axon guidance, and the order of assembly of β-Spectrin and Ankyrin in *Drosophila* neurons.

We observe that a mutant form of β-Spectrin missing the *Drosophila* Ankyrin-binding domain (βSpecΔank) remains properly localized to cell bodies and axons. Importantly, a recent study has shown that the same βSpecΔank protein used in this study correctly accumulates at the plasma membrane of copper cells yet fails to accurately target an Ank-GFP fusion protein in epithelial cells (Das et al., 2006), suggesting that Ankyrin localization depends on β-Spectrin but not vice versa. In addition, βSpecΔank rescues the guidance errors seen in β-*spectrin* mutants. Taken together, our data suggest that Ankyrin-binding is not essential for β-Spectrin localization and guidance function in neurons, and that *Drosophila* β-Spectrin may be assembled upstream of Ankyrin.

It is important to note that *Drosophila* has another Ankyrin gene, *dank2* (*ank2* – FlyBase), which is expressed specifically in neurons (Hortsch et al., 2002). Therefore, perhaps accurate β-Spectrin targeting occurs via a direct interaction with Dank2. Keeping in mind that βSpecΔank is missing the conserved Ankyrin1-binding site, this logic would imply that β-Spectrin associates with Dank2 using a different binding site than that used for Dank1. There does appear to be an intimate relationship between Dank2 and β-Spectrin, as Dank2 is mislocalized and levels are reduced in β-*spectrin* mutants (D.S.G. and G.J.B., unpublished). Future experiments will help to establish whether Dank2 can bind β-Spectrin at a location different than that used by Dank1. If the mutant βSpecΔank does indeed bind to Dank2, it will be interesting to determine the functional significance of this interaction.

We also demonstrated that, although the PH domain of *Drosophila* β-Spectrin is not necessary for axonal localization and/or guidance function, it is required for appropriate localization to the cell body plasma membrane. From our experiments, it appears that axonally localized β-Spectrin is important for making accurate guidance decisions. In the future, it will be important to determine the region of β-Spectrin that is essential for axonal targeting and whether this domain is important for growth cone migration.

Implications for cell adhesion molecules in midline axon guidance

At nodes of Ranvier and axon initial segments, there is an intimate relationship between the Spectrin network, Ankyrin and CAMs (Lambert et al., 1997). For example, in Purkinje and granule cells,

Ankyrin-G localization precedes that of neural CAMs, and is required for proper CAM cluster assembly at axon initial segments (Jenkins and Bennett, 2001; Zhou et al., 1998). Other studies of CAMs have revealed that they can commonly act as contact-mediated attractive and repulsive signals for growing axons (Doherty and Walsh, 1994; Tanaka and Sabry, 1995). In *Drosophila*, pre-synaptically targeted β-*spectrin* RNAi disrupts the stability and organization of CAMs such as Fas2 and Neuroglian (Pielage et al., 2005), and *neuroglian* mutants exhibit defects in motoneuron pathfinding (Hall and Bieber, 1997). Furthermore, *neurotactin* mutant embryos exhibit midline axon guidance errors that appear to be similar to those observed in β-*spectrin* mutants, including ectopic midline crossing and longitudinal breaks (Speicher et al., 1998). Future studies should investigate whether there are functional links between CAMs and the Spectrin network in the context of midline axon guidance.

We thank Alex Kolodkin and the Bloomington Stock Center for fly stocks. We are grateful to the Bashaw lab for constructive comments and technical assistance/support. This work was supported by NIH GM 49301 to R.R.D. and NIH Grant NS046333 to G.J.B.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/2/273/DC1

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