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Distinct functions of α-Spectrin and β-Spectrin during axonal pathfinding

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Cell-shape changes during development require a precise coupling of the cytoskeleton with proteins situated in the plasma membrane. Important elements controlling the shape of cells are the Spectrin proteins that are expressed as a subcortical cytoskeletal meshwork linking specific membrane receptors with F-actin fibers. Here, we demonstrate that *Drosophila karussell* mutations affect *β-spectrin* and lead to distinct axonal patterning defects in the embryonic CNS. *karussell* mutants display a *slit*-sensitive axonal phenotype characterized by axonal looping in stage-13 embryos. Further analyses of individual, labeled neuroblast lineages revealed abnormally structured growth cones in these animals. Cell-type-specific rescue experiments demonstrate that β-Spectrin is required autonomously and non-autonomously in cortical neurons to allow normal axonal patterning. Within the cell, β-Spectrin is associated with α-Spectrin. We show that expression of the two genes is tightly regulated by post-translational mechanisms. Loss of β-Spectrin significantly reduces levels of neuronal α-Spectrin expression, whereas gain of β-Spectrin leads to an increase in α-Spectrin protein expression. Because the loss of *α-spectrin* does not result in an embryonic nervous system phenotype, β-Spectrin appears to act at least partially independent of α-Spectrin to control axonal patterning.

KEY WORDS: Spectrin, *Drosophila*, Growth cone, Nervous system

INTRODUCTION

The development of multicellular animals is tightly linked to the evolution of a dynamic cell capable of forming and stabilizing manifold types of differentiation. This is most beautifully seen in the developing nervous system. Here, neurons migrate to their final destinations while, at the same time, the growth cones of the developing axons migrate towards their targets. In addition, dendritic specializations are formed and are kept in a dynamic equilibrium.

The dynamic changes of cell shape, as well as the subsequent stabilization of a specific form, demand a molecular machinery that can sense and transmit extracellular signals to the cytoskeleton. An important structural element that links cell adhesion proteins in the cell membrane to the F-actin cytoskeleton is the sub-membranous Spectrin network. Spectrins were first identified as important determinants defining the biconcave shape of erythrocytes. Erythrocytes that lack the Spectrin-based cytoskeleton loose their shape and stability, resulting in severe anemia in humans (Gallagher, 2004; Tse and Lux, 1999). Now, Spectrins are recognized as a large class of proteins ubiquitously expressed during development. The Spectrin proteins organize an extended protein network just below the plasma membrane by linking different actin fibers and many other proteins by numerous interaction motifs, such as the SH3 domain in α-Spectrin (α-Spec) (Bialkowska et al., 2005; Nedrelow et al., 2003). Furthermore, a pleckstrin-homology (PH) domain in β-Spectrin (β-Spec) allows its direct binding to membrane lipids (Williams et al., 2004). One of the best-characterized adaptor proteins that binds to Spectrin is Ankyrin, which can mediate interaction with other cell-membrane-associated receptors or channel proteins (Bennett and Chen, 2001; De Matteis and Morrow, 2000).

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Generally, Spectrins are long rod-like structural proteins and have been found in all metazoan species. α- and β-Spectrin form antiparallel dimers that associate in a head-to-head fashion to form an $(\alpha\beta)_2$ hetero-tetramer (Bennett and Baines, 2001). In humans, Spectrins are found in all cells, and several different α- and β-Spectrin isoforms exist (Bennett and Baines, 2001; Berghs et al., 2000; Dhermy, 1991). In *Drosophila*, only one *α-spectrin* gene, one *β-spectrin* and one *β_H-spectrin* gene have been described (Dubreuil et al., 1989; Dubreuil et al., 1990; Lee et al., 1993; Thomas and Kiehart, 1994; Thomas et al., 1998). The *Drosophila* α- and β-Spectrins share about 60% sequence identity with their human homologs, whereas the β_H-Spectrin isoform shares only 34% with its human homolog. β_H-Spectrin expression is found only in epithelial cells, whereas α- and β-Spectrin are ubiquitously expressed during development.

Within *Drosophila* epithelia, the $(\alpha\beta)_2$-Spectrin tetramer is found at basolateral membranes, whereas the $(\alpha\beta_H)_2$-Spectrin tetramer is localized to the apical membrane domain only (de Cuevas et al., 1996; Dubreuil et al., 1997; Lee et al., 1997; Pesacreta et al., 1989). At cell-cell contact zones, the basolateral $(\alpha\beta)_2$-Spectrin is recruited to Neuroglian, a *Drosophila* homolog of the L1-cell adhesion molecule, via the adaptor protein Ankyrin. An additional protein recruited to the basolateral cell membrane is the Na^+/K^+-ATPase (Dubreuil et al., 1996; Dubreuil et al., 1997; Dubreuil et al., 2000; Nelson and Veshnock, 1987). A functional correlate of these interactions has recently been demonstrated in the neuromuscular junction. Here, reduction of both α- and β-Spectrin not only leads to a mis-localization of Neuroglian and Fasciclin II, another cell adhesion protein, but finally results in a destabilization and retraction of the synapse (Featherstone et al., 2001; Pielage et al., 2005).

In *Drosophila*, mutations in all spectrin genes have been identified. Flies lacking *β_H-spectrin* function show reduced viability only and surviving flies exhibit relatively mild phenotypes, arguing against an essential function in determining epithelial-cell polarity (Zarnescu and Thomas, 1999). In contrast to *β_H-spectrin*, *α-spectrin* and *β-spectrin* are both essential genes. Only 50% of the embryos lacking zygotic *α-spectrin* expression reach the larval stages, and the

ones that do die in the first-instar stage (Dubreuil et al., 2000; Lee et al., 1993). The survival rate of homozygous-mutant *β-spectrin* animals is even further reduced, and less than 10% of the mutant animals are able to leave the egg shells (Dubreuil et al., 2000). Clonal analyses have revealed essential functions of *α-spectrin* in the polarity of follicle cells (Lee et al., 1997; Thomas et al., 1998). Although α-Spectrin and β-Spectrin are both found in a common protein complex, *β-spectrin*-specific functions have been described (Dubreuil et al., 2000). Epithelial cells of the midgut lacking *α-spectrin* normally position the Na^+/K^+-ATPase in their cell membrane. By contrast, *β-spectrin* mutants show an abnormal Na^+/K^+-ATPase distribution, suggesting that β-Spectrin can function independent of α-Spectrin.

Both α- and β-Spectrin are required during neuronal development. In *C. elegans*, it has been shown that *β-spectrin* is required for normal axonal outgrowth and fasciculation (Hammarlund et al., 2000). In mammalian axons, the Spectrin proteins are required to stabilize transmembrane proteins at the nodes of Ranvier (Lacas-Gervais et al., 2004; Yang et al., 2004). In *Drosophila*, α- and β-Spectrin have been shown to be involved in synapse organization and stability (Featherstone et al., 2001; Pielage et al., 2005). Here, we report the characterization of two mutants that were previously identified in a large phenotypic screen for genes affecting axonal pattern formation in the *Drosophila* embryo (Hummel et al., 1999a; Hummel et al., 1999b). We demonstrate that *klötzchen* and *karussell* (*kus*) encode α-Spectrin and β-Spectrin proteins, respectively.

The *kus* (*β-spectrin*) phenotype is characterized by a *slit*-sensitive crossing of the CNS midline by Fasciclin II-expressing axon fibers. Most prominently, we detected enlarged growth cones in single-cell analyses. Cell-type-specific genetic-rescue experiments demonstrated a requirement for β-Spectrin in cortical neurons. This requirement, in part, may include non-autonomous effects, because single neurons cannot be rescued in homozygous-mutant *kus* embryos. We demonstrate that α-Spectrin protein levels are tightly coupled to the levels of β-Spectrin by post-translational mechanisms, suggesting that *α-spectrin* mutants may share *β-spectrin*-mutant phenotypes. However, within the nervous system, β-Spectrin appears to act independently of α-Spectrin.

We further demonstrate that *klötzchen* mutants, which were initially selected based on a phenotype distinct from β-spectrin mutants, affect the *α-spectrin* locus. We show that a reduction in α-Spectrin levels render the animal very sensitive to background mutations and temperature. *klötzchen*-mutant flies that do not carry a background mutation did not show a mutant CNS phenotype and, similarly, germline clones of hypomorphic alleles did not result in an abnormal nervous system phenotype. Thus, within the *Drosophila* nervous system, *β-spectrin* appears to act independently of *α-spectrin* to stabilize neuronal growth cones.

MATERIALS AND METHODS

Generation of antibodies

To generate β-Spectrin-specific antibodies, we cloned 1686 bp starting from the ATG of β-spectrin into pQE31. The resulting fusion protein was expressed and purified according to the manufacturer's instructions (Qiagen), and was used to immunize rabbits (Davids, Regensburg).

Generation of transgenes

To generate a UAS::*β-spectrin* construct we used the cDNA clone *AT24411* (BDGP), which contains the 5′ region of the *β-spectrin* mRNA. The 3′ third of the mRNA was cloned via a RT-PCR approach (details are available on request). The resulting clones were subcloned in pUAST and the sequence was verified by sequencing. Subsequent germline transformation was performed according to standard procedures. Several independent insertion lines were tested, which all showed similar effects. An UAS::*β-spectrin*^{dsRNA} construct was made using a 500 bp fragment from the 5′ region of the *β-spectrin* open reading frame (ORF). Several independent transgenic lines were established. Only lines that led to a lethal phenotype when crossed to *daughterless*-Gal4 were used in this study.

Genetic analyses

EMS mutagenesis of isogenic chromosomes has been described by Hummel et al. (Hummel et al., 1999a). All complementation analyses were performed at 25°C under standard conditions. The duplication *Dp(1;3)B*^{S3i}*D2* (provided by R. Dubreuil, University of Illinois, Chicago, IL), which rescues the lethality of five *kus* mutant chromosomes, was used. To remove the background lethal mutations from the *α-spectrin*$^{E2-26}$ mutation, we first exchanged most of the third chromosome using the rucuca multi-marker chromosome. Subsequently, all recessive mutations of the rucuca marker were removed following recombination. The resulting *E2-26* chromosome could be rescued to full viability using an *ubi:α-spectrin* mini-gene (Lee et al., 1993). The following alleles were used: *α-spec*^{lm88}, *α-spec*^{rg41} (Lee et al., 1993); *α-spec*$^{E2-26}$, *α-spec*$^{D4-65}$, *α-spec*$^{N2-141}$, *β-spec*^{G113}, *β-spec*^{E175}, *β-spec*^{E292}, *β-spec*^{H127}, *β-spec*^{L105}, *β-spec*^{M046}, *β-spec*^{S012} (Hummel et al.1999a); *α-spec*$^{N-2}$, *α-spec*$^{P-2}$, *α-spec*$^{S-1}$, *α-spec*$^{1.3}$, *α-spec*$^{1.2.1}$ (this work); *β-spec*^{em6}, *β-spec*^{em15}, *β-spec*^{em21} (Dubreuil et al., 2000); *slit*$^{B1-32}$ (Hummel et al., 1999a); and *gcm*^{P1} (Jones et al., 1995). To identify mutant animals, we employed GFP- or *lacZ*-labeled balancer chromosomes. The FRT elements *ubi*::GFP FRT19A and *rsp17*^{4} P[*white*$^+$]70C FRT80B were used, and mitotic recombination was induced by an *ey*::Flp transgene (Bloomington stock center). The following Gal4 strains were used: *ptc*::Gal4, *da*::Gal4, *ap*::Gal4, *elav*::Gal4 (Bloomington Stock Center); *sim*::Gal4 (Scholz et al., 1997); and *repo*::Gal4 (provided by B. Jones, University of Mississippi, USA).

Labeling and antibodies

Immunohistochemistry was performed as previously described (Hummel et al., 1999a). The following antibodies were used: mouse anti-Wrapper (Noordermeer, 1998); anti-α-Spectrin, anti-Repo, BP102, anti-Fasciclin II (Developmental Studies Hybridoma Bank); rabbit anti-β-Galactosidase (Cappel); anti-myc 9E10 (Santa Cruz); anti-GFP (Invitrogen); anti-Kette (Bogdan and Klambt, 2003); anti-HRP-Cy5 (Dianova); and rabbit anti-β-Spectrin (this work). DiI labeling of individual neuroblast cell clones was performed as previously described (Bossing and Technau, 1994; Bossing et al., 1996).

RESULTS

The *karrussell*-mutant phenotype

We have previously identified seven independently induced mutations in the *Drosophila* gene *kus* in a large-scale mutagenesis screen for defects in embryonic axon pattern formation (Hummel et al., 1999a; Hummel et al., 1999b). All *kus* mutants were characterized by their having a similar unique CNS phenotype that was not found in any other complementation group (Hummel et al., 1999a; Hummel et al., 1999b). In particular, stage-14 *kus*-mutant embryos were characterized by axon structures that loop out into the CNS cortex, a phenotype that was never observed in wild-type embryos (Fig. 1C). Interestingly, the circular axon pattern phenotype is resolved in older embryos and, at the end of embryogenesis, commissures appear to be fused, which is often indicative of CNS-midline defects (Klämbt et al., 1991). In addition, the longitudinal axon tracts are found in closer proximity to the CNS midline and are often thinner as compared to the wild type (Hummel et al., 1999a; Hummel et al., 1999b) (Fig. 1D). All *kus* mutants that have been identified lead to very similar mutant phenotypes.

karrussell mutations affect the *β-spectrin* gene

We were able to rescue the lethality of five *kus* alleles using the chromosomal duplication *Dp(1;3)B*^{S3i}*D2*, which affects the cytological interval 16A-D. The subsequent complementation

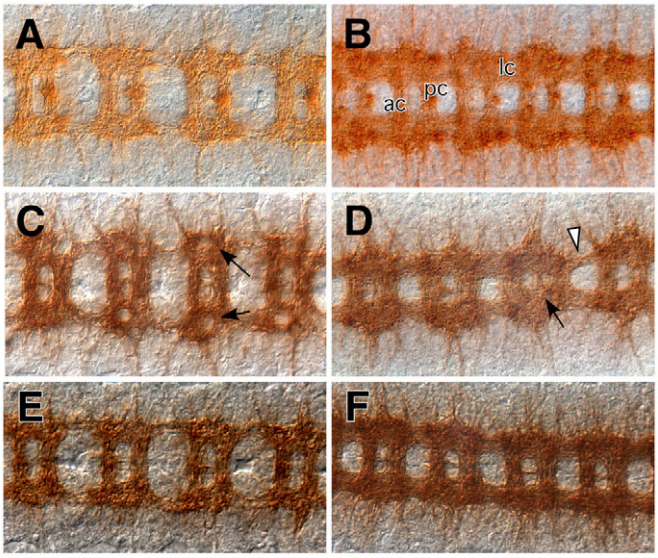

Fig. 1. The *karussell* phenotype. Dorsal view of dissected embryonic nervous systems stained for BP102 expression. Anterior is to the left. (**A**) In wild-type stage-14 embryos, commissures are established as regular axon fascicles. (**B**) In wild-type stage-16 embryos, the typical ladder-like axon pattern is established. Two clearly separated commissures (anterior commissure, ac; posterior commissure, pc) are found in every neuromere. Neuromeres are connected by the longitudinal connectives (lc). (**C**) In stage-14 hemizygous-mutant *β-spectrin*^{kusS012} embryos, characteristic loops are found at the point where commissural axons normally enter the connectives (arrows). (**D**) Stage-16 hemizygous *β-spectrin*^{kusS012} embryo. The longitudinal connectives are found closer to the midline and appear thinner (arrowhead); in addition, the segmental commissures are not clearly separated and appear slightly fused (arrow). (**E**,**F**) Hemizygous *β-spectrin*^{kusS012} embryos that express *β-spectrin* under the control of the *elav* promoter. The axonal phenotype is rescued.

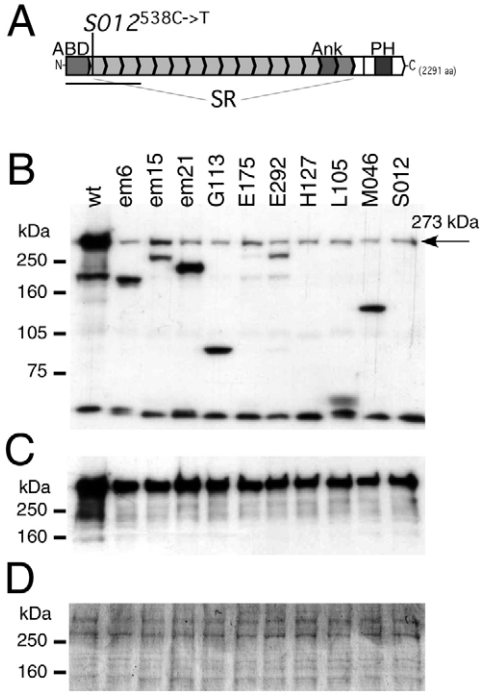

Fig. 2. Molecular characterization of *β-spectrin* mutants. (**A**) Schematic drawing of the 2291-amino acid large β-Spectrin protein. The position of the S012 mutation is indicated. The bar indicates the part of the β-Spectrin protein used for immunization. (**B**) Western blot of cell extracts derived from stage 15-17 embryos probed with a polyclonal antiserum generated against a β-Spectrin fusion protein. First lane: wild type (wt), the 280 kDa large β-Spectrin protein and a 200 kDa degradation product is visible. The 50 kDa band is a background band recognized by the polyclonal antibody. Mutant *β-spectrin* embryos were selected using GFP-carrying balancer chromosomes. The genotype is indicated. In *em6*, *em15*, *em21*, *G113*, *E292*, *L105* and *M046* hemizygous embryos, truncated β-Spectrin proteins are detected. *E175*, *H127* and *S012* hemizygous-mutant embryos lack detectable β-Spectrin expression. (**C**) The same membrane was probed for expression of α-Spectrin. Notice the strong reduction of α-Spectrin expression in all genotypes, regardless of whether residual β-Spectrin protein can still be detected or not. (**D**) Coomassie staining of same membrane to demonstrate equal loading. ABD, actin-binding domain; SR, spectrin repeats; Ank, ankyrin domain; PH, pleckstrin homology domain.

assays that we performed showed that *kus* alleles cannot complement the lethality of the previously described *β-spectrin* alleles *em15* and *em21* (Dubreuil et al., 2000). To further confirm that *kus* encodes β-Spectrin, we sequenced the first 4000 bp of the *β-spectrin* ORF in three mutant *kus* alleles and found a mutation resulting in a stop codon at position 538 of the deduced *β-spectrin* ORF in the allele *S012* (Fig. 2A). In addition, we assayed β-Spectrin protein expression in homozygous-mutant *kus* embryos that were selected using a *twist*-GFP FM7 balancer chromosome. When using a newly generated antiserum against the N-terminus of β-Spectrin, most *kus* mutants revealed altered β-Spectrin protein expression in western blot experiments (Fig. 2B). The previously described allele *em15* and our allele *E292* encode proteins that presumably lack the C-terminal PH domain, but may retain the Ankyrin-binding domain. The strongest previously known *β-spectrin* allele, *em6*, generates a 190 kDa large protein and thus may not be a complete null. The proteins encoded by *G113*, *L015* and *M046* are all significantly shorter compared with the wild-type protein. In the *L015* mutant, the remaining protein appeared to encompass only two or three Spectrin repeats. Interestingly, all truncated β-Spectrin proteins were relatively stable.

In protein extracts of hemizygous-mutant *E175*, *H127* and *S012* mutants, we could not detect any β-Spectrin protein. Sequence analysis predicted that *S012* mutants could generate a 20 kDa large protein fragment, which, however, was not detectable in western blot experiments. In summary, these data show that *kus* mutants affect the *β-spectrin* locus. Despite these differences in β-Spectrin protein sizes in the different *kus* and/or *β-spectrin* mutations, we did not observe significant qualitative differences in the axonal phenotypes between these alleles. This indicates that a functional, full-length β-Spectrin protein is required for normal nervous system development.

Neuronal expression of *β-spectrin* rescues the *karussell* phenotype

It was previously shown that β-Spectrin is expressed ubiquitously during *Drosophila* development (Dubreuil et al., 2000). Within the developing nervous system, β-Spectrin is expressed in all neurons (see Fig. S1A in the supplementary material). Superficially, expression in the axonal compartment appeared somewhat lower when compared with the neuronal cell bodies. However, relatively high levels of β-

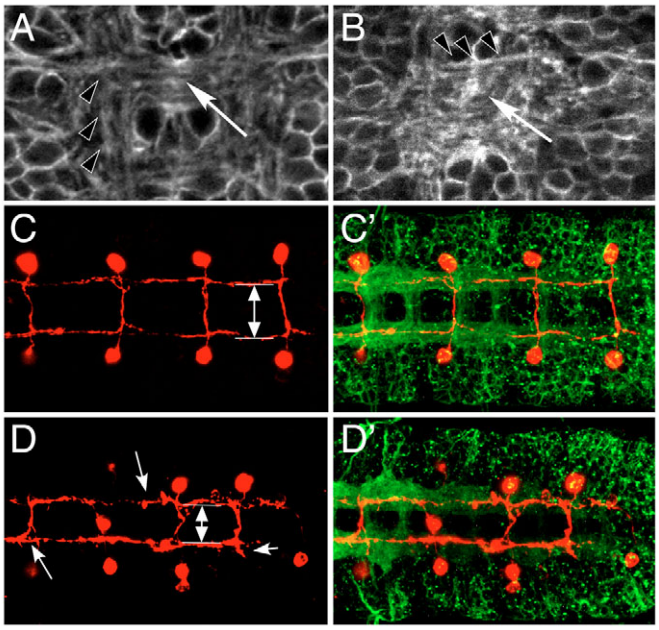

Fig. 3. Expression of β-Spectrin in the nervous system. Confocal views of dissected nervous systems of wild-type stage-16 embryos stained for expression of β-Spectrin (white), axonal membranes (anti-HRP, green) or the SemaIIbτMyc marker (red). (**A**) In wild-type embryos, β-Spectrin expression is found in neuronal cell bodies and specific axonal fascicles (arrowheads). The arrow denotes increased levels of β-Spectrin at the CNS midline. (**B**) In homozygous-mutant *gcm* embryos, lateral glial cells are absent. β-Spectrin can still be detected at specific fascicles (arrowheads) and at the CNS midline (arrow). (**C**,**C′**) The SemaIIbτMyc marker is expressed by only one neuron per hemineuromere. This neuron is positioned at the lateral margin of the neuropil. SemaIIbτMyc-positive axons project across the midline in the anterior commissure and then make a sharp turn to follow a specific path in the longitudinal connective. (**D**,**D′**) In hemizygous-mutant kus^{S012} embryos, the SemaIIbτMyc-positive neurons appear normally specified, but show irregular positions in the nerve cord, often being displaced towards the CNS midline. The SemaIIbτMyc-positive fascicles are found closer to the CNS midline (double-headed arrow indicating distance in C and D) and the fascicle morphology appears changed. Often, ectopic projections are found that may correspond to enlarged growth cones (arrows). In addition, the precision in axonal pathfinding is lost; however, we did not observe ectopic crosses of the CNS midline.

Spectrin expression can be found on specific axonal fascicles in both the connectives and the commissures (Fig. 3A, arrowheads). Within the commissures, the level of β-Spectrin expression is further modulated and appears highest at the CNS midline (Fig. 3A, arrow). Co-expression with Wrapper, a specific marker for the midline glial cells (Noordermeer et al., 1998), demonstrated an overlap of this β-Spectrin expression domain with the midline glial cells (see Fig. S1C,C′ in the supplementary material). Unfortunately, the resolution of the confocal microscope did not allow us to determine whether the enhanced levels of β-Spectrin expression at the midline were due to axonal or glial β-spectrin expression.

Co-expression with the glial marker Repo (Campbell et al., 1994; Halter et al., 1995; Xiong et al., 1994) demonstrated that β-Spectrin is also expressed in the longitudinal glia (see Fig. S1D,D′ in the supplementary material). As glial processes invade the longitudinal connectives, β-Spectrin expression within the longitudinal connectives might be due to glial expression. To discriminate between glial versus axonal expression, we analyzed *gcm* mutants, which have no lateral glial cells (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). *gcm*-mutant embryos still showed a fasciculated β-Spectrin staining pattern, indicating that axons and not glial cells are the major source for β-Spectrin staining within the longitudinal connectives (Fig. 3A,B).

To further test whether expression in neurons or glia is required for the β-$spectrin^{kus}$ axon phenotype, we performed cell-type-specific rescue experiments using Gal4/UAS-mediated expression of *β-spectrin*. Ubiquitous expression of a UAS-*β-spectrin* construct induced by a *daughterless*::Gal4 driver rescued hemizygous β-$spectrin^{kus}$-mutant animals to full viability (data not shown). Following expression of *β-spectrin* in all lateral glial cells using a *repo*::Gal4 driver, we did not observe any alterations in the phenotypic strength (data not shown). Similarly, when *β-spectrin* was expressed in all CNS-midline cells using the *sim*::Gal4 driver, the β-$spectrin^{kus}$-mutant phenotype was not rescued (data not shown). However, when we expressed *β-spectrin* in all postmitotic neurons using the *elav*::Gal4 driver, we noted a complete rescue of the axonal patterning defects (Fig. 1). However, neuronal expression could not rescue the lethality associated with β-$spectrin^{kus}$ mutants, demonstrating that *β-spectrin* has additional essential functions outside of the nervous system (Dubreuil et al., 2000). To further test the requirement of β-$spectrin^{kus}$ for Slit-Roundabout (Robo) signaling, we performed single-cell rescue experiments using both *sim*::Gal4 and *apterous*::Gal4 (*ap*::Gal4) driver strains. When *ap*::Gal4 was used to express β-Spectrin no phenotypic rescue of their trajectory is observed in only a few cortical neurons and the β-Spectrin-expressing fascicles are located in a unchanged position compared with the *kus* mutant (Fig. 4B-C′). Similarly, we failed to obtain cell-specific rescue in the MP1 fascicle following the expression of *β-spectrin* using the *sim*::Gal4 driver (Fig. 4D-F′).

These results suggest that β-$spectrin^{kus}$ is required in cortical neurons for normal pathfinding and, furthermore, indicate that community effects are important in steering growth cones to their correct targets.

β-Spectrin is required for axonal morphogenesis

To understand better how β-Spectrin affects axonal pattern formation, we employed additional single-cell markers that reveal contralateral projections. In wild-type embryos, the SemaIIb:τmyc marker (Rajagopalan et al., 2000) is expressed in only one neuron per hemineuromere; this neuron projects its axon across the midline and the axon then follows a specific path within the longitudinal connectives (Fig. 3C,C′). In β-$spectrin^{kus}$ mutants, specification of the SemaIIb neurons is not affected and the overall axonal trajectories are unchanged. However, the structure of the SemaIIb:τmyc-positive axon fascicle is severely altered in these mutants and the precision of axonal pathfinding is disrupted (Fig. 3D,D′). Although the normally straight axonal projection across the midline appeared irregular, ectopic crossings of the CNS midline were never observed (Fig. 3D,D′). In addition, the position of the SemaIIb:τmyc-expressing cell bodies was often shifted towards the CNS midline.

Very similar observations were made when analyzing the progeny of single labeled neuroblasts in stage-16 embryos (Fig. 5). Following the labeling of 210 individual DiI-labeled neuroblasts in 70 β-$spectrin^{kus}$-mutant embryos, we found mostly normal projection patterns and did not observe contralateral-projecting axons that ectopically crossed or illegitimately re-crossed the CNS midline. However, axons displayed abnormal varicosities and additional small, ectopic side-branches (Fig. 5). Most prominently, we noted alterations in the structure of the growth cones, which appeared enlarged with sometimes extensive, filopodia-

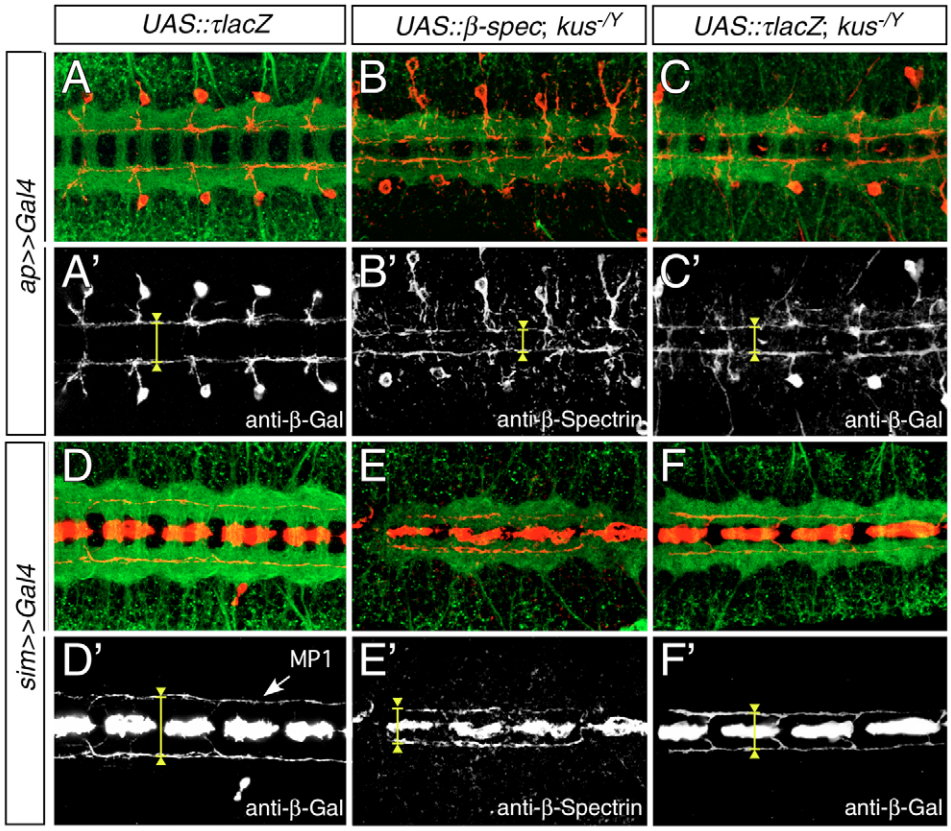

Fig. 4. Non-autonomous effects of *β-spectrin* on axonal patterning. Dissected preparations of stage-16 embryos stained for HRP (green) and β-Galactosidase or β-Spectrin, as indicated. (**A**,**A′**) The *ap*::*Gal4* driver directs *τlacZ* expression into a small subset of ipsilateral-projecting neurons. (A′) Notice the distance between the β-Galactosidase-expressing fascicles (yellow bar). (**B**,**B′**) Following expression of *β-spectrin* in the *ap*::*Gal4* pattern in *kus* mutants, the axonal trajectories are found at the same distance from the CNS midline as in hemizygous-mutant *kus*-mutant embryos (**C**,**C′**). (**D**,**D′**) The *sim*::*Gal4* driver directs *τlacZ* expression into the MP1 neurons and some midline cells. Notice the distance of MP1 fascicles to the midline (yellow bar). (**E**,**E′**) Following expression of *β-spectrin* in the *sim*::*Gal4* pattern in *kus* mutants, the MP1 axons are found at the same distance from the CNS midline as in hemizygous *kus*-mutant embryos (**F**,**F′**).

like processes (Fig. 5A′,C′; arrowheads). Thus, β-Spectrin appears to be required to establish or maintain the structure of growth cones needed for precise axonal patterning.

β-spectrin^{kus} interacts with *slit*

A hallmark of the *β-spectrin*^{kus} phenotype is that the longitudinal connectives are located closer to the CNS midline in mature embryos (Hummel et al., 1999a; Hummel et al., 1999b) (Fig. 1). Correlating with the slight collapse at the CNS midline, we noted mild axon-crossing defects in hemizygous-mutant *β-spectrin*^{kus} animals. Whereas, in wild-type embryos, Fasciclin II-positive axon tracts never cross the CNS midline, we observed rare ectopic crosses of the midline in *β-spectrin*^{kus}-mutant embryos (Fig. 6). As midline crossing of Fasciclin II-positive axons is often sensitive to the dose of the midline repellent Slit, we performed gene-dosage experiments. Heterozygous *slit*-mutant animals, as well as *β-spectrin*^{kus}/+; *sli*/+ double-heterozygous female embryos, never showed ectopic crossing of Fasciclin II-positive axon tracts (Fig. 6). When we removed one copy of *slit* in a hemizygous *β-spectrin*^{kus} embryo, the number of neuromeres with ectopic midline crosses increased significantly ($P<0.001$; *t*-test), suggesting that *kus* function is somehow integrated in the Slit-

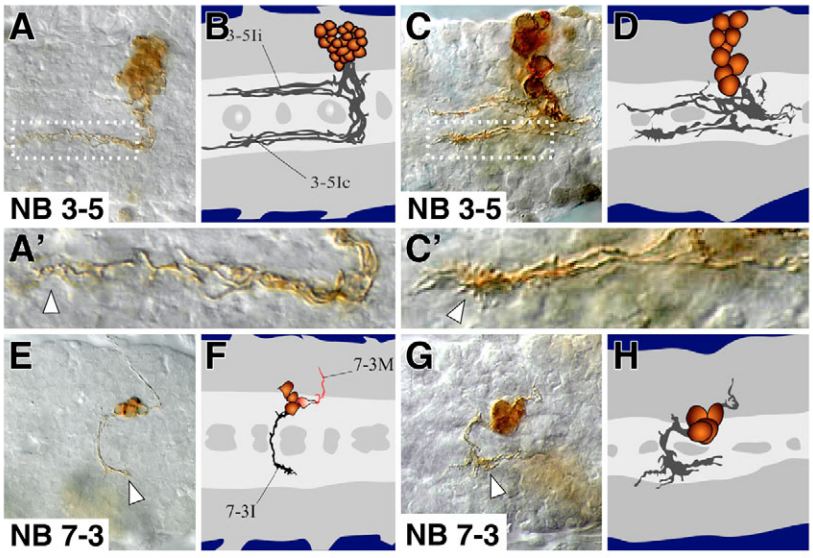

Fig. 5. Morphology of the *karussell*-mutant neurons. Individual neuroblasts were filled using the DiI methodology. (**A**,**E**) DiI-labeled lineages of neuroblasts NB 3-5 and NB 7-3 in a wild-type embryo. (**A′**) Higher magnification of the dotted area in A. The arrowhead denotes the growth cone. (**B**,**F**) Camera lucida drawings of wild-type lineages of NB 3-5 and NB 7-3. (**C**,**G**) DiI-labeled lineages of neuroblasts NB 3-5 and NB 7-3 in a hemizygous *kus*-mutant embryo. (**C′**) Higher magnification of the dotted area in C. The arrowhead denotes the enlarged growth cone in the *kus* mutant. (**D**,**H**) Camera lucida drawings of these preparations reveal principally normal projection patterns, but altered fiber morphology and enlarged growth cones. No ectopic crossing of the CNS midline was observed.

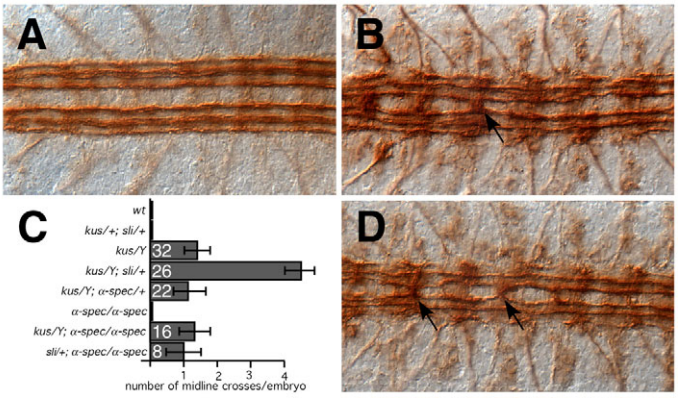

Fig. 6. *β-spectrin*^{kus} interacts with *slit*. (**A**) In wild-type stage-16 embryos, three distinct fascicles can be recognized following Fasciclin II staining. The most medial fascicle never crosses the CNS midline (see C for quantification). (**B**) In hemizygous-mutant *β-spectrin*^{kus} embryos, the inner fascicles appear to cross the midline (arrow). (**D**) Hemizygous-mutant *kus* embryo also lacking one copy of *slit*. The number of ectopic midline crosses increases significantly (arrows). (**C**) Quantification of the number of midline crosses of Fasciclin II-positive axon bundles. The number of embryos counted, the average number of ectopic crosses per embryo and the standard deviation for each genotype is indicated.

signaling pathway (Fig. 5). Similar results were obtained for the *kus*^{G113} mutant, in which a truncated β-Spectrin protein was expressed (Fig. 2; data not shown).

β-spectrin^{kus} mutations affect *α-spectrin* expression

In addition to affecting *β-spectrin* expression, all of the *β-spectrin*^{kus} alleles affect *α-spectrin* protein levels (Fig. 2C). Interestingly, the effects on α-Spectrin levels do not correlate with the extent of the C-terminal β-Spectrin deletions. This is in agreement with the notion that the C-terminal domain of β-Spectrin is required to bind, and thus presumably stabilize, α-Spectrin (Deng et al., 1995; Yan et al., 1993). To determine the subcellular localization of α-Spectrin and the truncated β-Spectrin proteins we stained whole-mount *β-spectrin*^{kus}-mutant embryos and, in addition, generated homozygous-mutant *β-spectrin*^{kus} tissue in the eye antennal imaginal discs using the Flp/FRT system. When we generated mutant clones using the two hypomorphic *β-spectrin*^{kus} alleles *G113* or *E292*, we could detect reduced levels of β-Spectrin expression that was still correctly localized at the cell membrane and a concomitant reduction in α-Spectrin protein levels (Fig. 7A,B). To test whether α-Spectrin is similarly required for the stability of β-Spectrin protein, we generated homozygous-mutant eye discs lacking *α-spectrin* expression. In these experiments we observed the loss of α-Spectrin in the mutant clone but did not detect any change in the level of β-Spectrin that was still correctly localized to the subcortical region of the cell (Fig. 7C). Thus, in contrast to α-Spectrin, β-Spectrin can localize independently to the cell membrane. To further analyze the close regulatory interaction between these spectrin genes, we performed gain-of-function studies and expressed full-length β-Spectrin in wild-type wing imaginal discs. This not only led to a clear up-regulation of β-Spectrin protein expression but also to a concomitant increase in the levels of α-

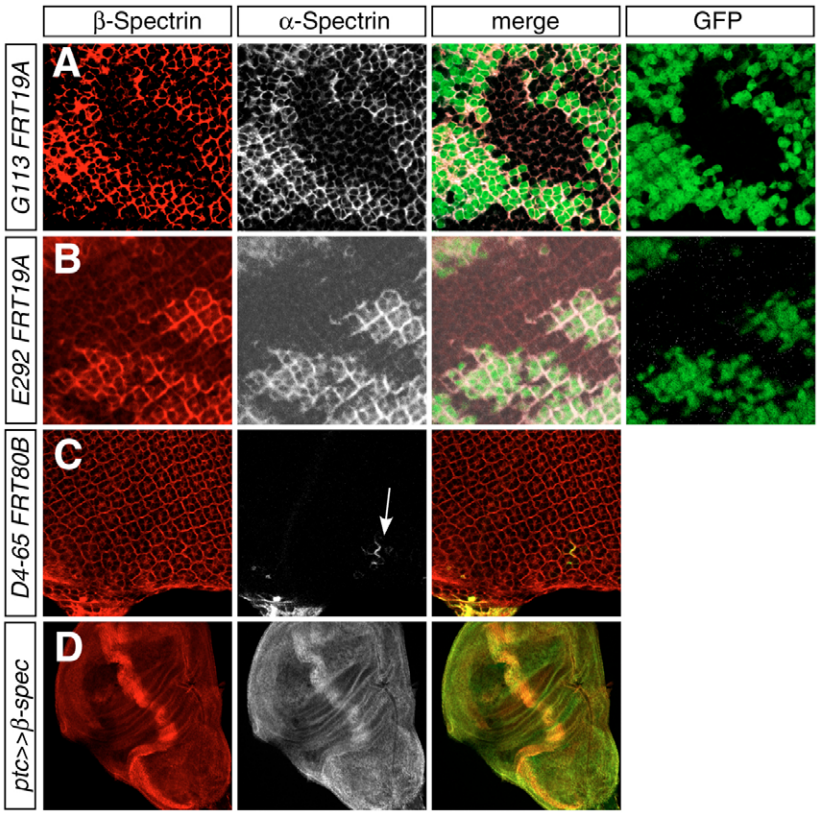

Fig. 7. β-Spectrin regulates α-Spectrin stability. Top three panels show eye imaginal discs with homozygous-mutant cell clones lacking β-Spectrin (red) or α-Spectrin (white), as indicated. Single confocal planes are shown. Clones were induced by expressing *flp* under the *eyeless* promoter and are marked by either the lack of GFP expression (green) or the loss of Spectrin expression. (**A**) In cell clones that are homozygous-mutant for the strong hypomorphic allele *β-spectrin*^{G113}, significantly reduced levels of *β-spectrin* can be detected that appear to localize normally at the cell cortex. Concomitant to the reduction of β-Spectrin expression we noticed a reduction in the level of α-Spectrin. (**B**) Similar results were obtained for cells homozygous for the *β-spectrin*^{E292} mutation. (**C**) Clones homozygous for the strong *α-spectrin* allele *D4-65* lack α-Spectrin protein expression but β-Spectrin expression is not affected. Almost the entire eye is mutant for *α-spectrin*, except for a small area (arrow). (**D**) Wing imaginal disc expressing the UAS-*β-spectrin* construct under the control of the *patched*::Gal4 driver. Elevated levels of β-Spectrin result in a concomitant up-regulation in the level of α-Spectrin.

Spectrin protein (Fig. 7D). As RNA levels were not affected (data not shown), we conclude that there exists a post-translational mechanism stabilizing α-Spectrin protein. Together, these experiments demonstrate an intimate regulation of α- and β-Spectrin, and show that *β-spectrin*^{kus} mutants are functional *α-* and *β-spectrin* double mutants.

α-spectrin is not required for axon pattern formation

Our data demonstrate that *β-spectrin*^{kus}-mutant embryos are impaired in both α-Spectrin and β-Spectrin expression. In our recent large-scale EMS mutagenesis that led to the identification of *β-spectrin*^{kus} mutants, we identified several other mutations affecting the formation of the segmental commissures (Hummel et al., 1999a; Hummel et al., 1999b). One of the complementation groups identified was *klötzchen*, whose members show a commissural phenotype distinct to that of *kus* mutants (Hummel et al., 1999a) (Fig. 8B). Using standard genetic-mapping techniques, we localized the common lethality of five independent alleles to the chromosomal interval 61F-62A and subsequently showed that these mutants affect the *α-spectrin* locus. Sequence analysis of the *klötzchen* allele *E2-26* revealed a A→T mutation at position 250 of the *α-spectrin* ORF that leads to the termination of translation after 83 amino acids (instead of 2416 amino acids for the full-length α-Spectrin protein). Thus, *E2-26* probably represents a null allele. Interestingly, heterozygous *α-spectrin*-null mutations have relatively normal levels of α-Spectrin protein, indicating a strict regulation of the expression levels (Fig. 8G, compare lanes *w*^{1118} and *Df(3L)aprt/+*).

klötzchen mutants were initially isolated based on their embryonic CNS phenotype; however, following the removal of all lethal background mutations on the *klötzchen*$^{E2–26}$ chromosome by meiotic recombination (see Materials and methods), no obvious axonal phenotypes were detected (Fig. 8C). Similarly, the previously described *α-spectrin*-null mutation *rg41* (Lee et al., 1993) does not lead to an embryonic CNS phenotype. In addition, we did not see any Fasciclin II-positive axonal tracts crossing the CNS midline in homozygous *E2-26* mutants (40 embryos scored, Fig. 6C). The distribution of β-Spectrin protein in the longitudinal axon tracts did appear slightly altered, which might be due to fasciculation defects (Fig. 3A; Fig. 8E,F). Thus, zygotic α-Spectrin is not required for normal axonal patterning, but loss of *α-spectrin* renders neurons sensitive to background mutations. In agreement with this notion, we noted ectopic midline-crossing of Fasciclin II-positive axons when we removed one copy of *slit* in a homozygous *α-spectrin*-mutant background (Fig. 6C).

In an independent experiment that aimed to clarify the genetic organization at the genomic interval spanning the *α-spectrin-discs lost* region, we generated 21 additional EMS induced *α-spectrin* alleles. All mutants were lethal in trans to deficiencies of the region (*Df(3L)Aprt32* or *Df(3L)My10*) and most animals died during the first-larval-instar stage. Two alleles, *P-2* and *1.3*, appeared to have normal levels of α-Spectrin protein (Fig. 8G) and were lethal during the early third-larval-instar stage, indicating that they are hypomorphic alleles. Interestingly, some of these *α-spectrin* mutants showed temperature-dependent intragenic complementation (see Table 1). Similarly, the axonal phenotype of *α-spectrin* alleles appeared temperature-sensitive (Fig. 8D). As the temperature sensitivity is not due to an altered stability of the α-Spectrin protein in these animals (see Fig. S2 in the supplementary material), the Spectrin network itself might be temperature labile.

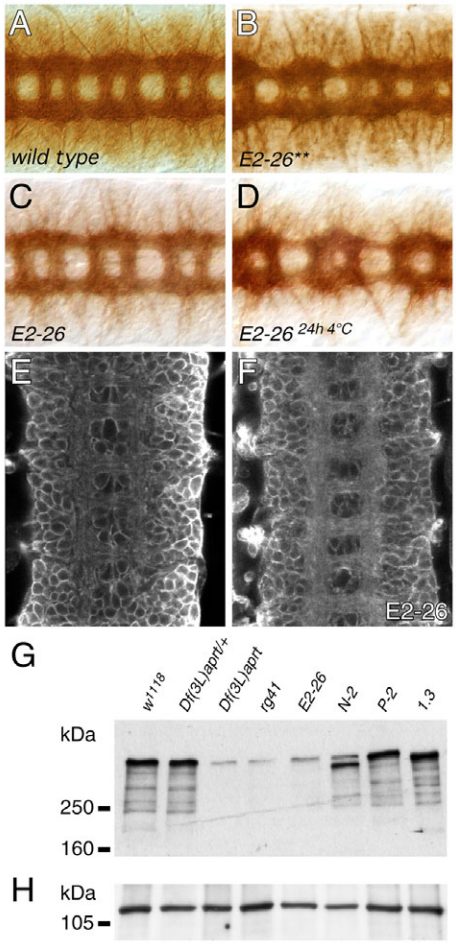

Fig. 8. The *α-spectrin* gene is affected in *klötzchen* mutants. (**A-F**) CNS preparations of stage-16 embryos stained for BP102 (A-D) or β-Spectrin (E,F). (A) Wild-type embryos are characterized by a regular axonal pattern with separated segmental commissures and longitudinal connectives. (B) Homozygous *E2-26*-mutant embryos show partially fused commissures. (C) A homozygous *E2-26* mutant with no further background mutations shows no axonal phenotype. (D) When such embryos are allowed to develop at 4°C for 1 day, a fused-commissure phenotype develops. (E) β-Spectrin protein in the ventral nerve cord of wild-type embryos. (F) In homozygous *E2-26* mutants, β-Spectrin expression in the neuropil appears altered. (**G**) α-Spectrin protein expression in some of the *α-spectrin* mutants. Proteins were isolated from ten stage 16/17 embryos and were separated on a 6% SDS gel. Following western blotting, α-Spectrin expression was detected using the monoclonal antibody 3A9. *w*^{1118} embryos were used as a wild-type control. If not otherwise indicated, homozygous mutants were used. Genotypes are as indicated. The *P-2* mutation does not lead to a detectable truncation of α-Spectrin. In *N-2* and, possibly, *1.3* mutants, a slight reduction of the α-Spectrin protein size is noted. In the deficiency *Aprt32*, and in the mutants *rg41* and *E2-26* all zygotic α-Spectrin expression is removed. Only maternal α-Spectrin expression is visible. (**H**) To control for equal loading, the same membrane was probed with anti-Kette antibodies.

DISCUSSION

The formation of the neuronal network requires a multitude of cellular interactions for precise axonal pathfinding and the establishment of specific synaptic connections (Dickson, 2002). In an attempt to identify some of the key regulators that control axonal

Table 1. Temperature-dependent complementation of some of the weak *α-spectrin* alleles

17°C

	α-spectrin allele					
Female genotype	1.2.1	1.3	N-2	P-2	S-1	N2-141
1.2.1		+	–	–	–	–
1.3	n.d.		+	+	–	–
N-2	–	Esc.		Esc.	–	–
P-2	Esc.	Esc.	+		–	–
S-1	–	–	–	Esc.		–
N2-141	–	–	–	n.d.	–	

25°C

	α-spectrin allele					
Female genotype	1.2.1	1.3	N-2	P-2	S-1	N2-141
1.2.1		+	–	–	n.d.	–
1.3	+		+	+	n.d.	n.d.
N-2	–	**+**		Esc.	–	–
P-2	**+**	**+**	+		**Esc.**	**Esc.**
S-1	–	–	–	Esc.		–
N2-141	–	–	–	Esc.	–	

All EMS-induced mutations are lethal in homozygosity or in trans to *α-spectrin* deficiencies. More than 100 progeny were analyzed in each cross. *P-2* shows a maternal effect in trans to *1.2.1*. Bold indicates differences between the two temperatures. +, full complementation; –, no complementation; Esc., few surviving adults; n.d., not determined.

pattern formation, we have isolated mutants that have specific defects in the developing embryonic nervous system of *Drosophila* (Hummel et al., 1999a; Hummel et al., 1999b). Here, we present the identification and further characterization of two of these mutations, *kus* and *klötzchen*.

Rescue experiments and direct sequence analysis demonstrate that *kus* encodes the *Drosophila* β-Spectrin protein. *kus* mutants were initially isolated due to a distinct axonal phenotype, including ectopic CNS-midline crossing of Fasciclin II-positive axons. To further analyze this phenotype, we labeled the progeny of single neuroblasts in *kus*-mutant embryos but, despite the large number of labeled clones, we were unable to detect any aberrant midline crossings. Similarly, when we employed cell-type-specific Gal4 drivers, we could not see clear pathfinding defects across the midline. It is therefore likely that the observed phenotype is a result of inappropriate contact between medial Fasciclin II-expressing axons from both sides of the midline mimicking ectopic midline crossings. Because wild-type Slit levels are required to position the longitudinal fascicles, a reduction of *slit* gene dosage results, as expected, in a further medial positioning of the longitudinal connectives, explaining the increase in the number of ectopic midline crosses in these animals. Similar phenotypes were also observed by Garbe et al. (Garbe et al., 2007).

Interestingly, we found defects in the architecture of the neuronal growth cones in *β-spectrin*^{kus}-mutant animals, which may explain the general sensitivity of *β-spectrin*^{kus}-mutant neurons to guidance signals such as Slit. The enlarged growth cones detected in *β-spectrin*^{kus} mutants correlate nicely with data on growth cone formation after axotomy (Gitler and Spira, 1998); axonal injury leads to an increased activity of the protease calpain, which cleaves Spectrin and results in the removal of the submembranous Spectrin meshwork prior to the regeneration and growth of the growth cone (Gitler and Spira, 1998). In secretory cells, the submembranous Spectrin cytoskeleton prevents the premature fusion of vesicles with the plasma membrane (Aunis and Bader, 1988; Perrin et al., 1992). Similarly, Spectrins may function to regulate the fusion of intracellular membrane vesicles needed to enlarge and advance the growth cone (Gitler and Spira, 1998), which could explain the enlarged growth cones that we detected in *β-spectrin* mutants (Fig. 4).

Within the *Drosophila* nervous system, α- and β-Spectrin are the only Spectrins that are expressed. These two proteins form a heterodimer in which β-Spectrin appears to be the key determinant, because α-Spectrin protein is only stable in the presence of β-Spectrin and ectopic expression of β-Spectrin leads to a concomitant increase in the level of α-Spectrin protein. To test whether this regulation occurs at the level of RNA or protein, we determined the expression of the corresponding transcripts, but noted no alteration (data not shown). It is possible that the association of α- and β-Spectrin blocks ubiquitination of α-Spectrin and its subsequent degradation via the proteasome. Ubiquitination has been previously reported for α-Spectrin (Corsi et al., 1995; Galluzzi et al., 2001) and may thus help to define the correct protein-expression levels.

Despite the intimate coupling of the two expression profiles, it has been demonstrated that *β-spectrin* can function independently of α-Spectrin. During the development of the midgut, the correct localization of the Na^+/K^+-ATPase requires only *β-spectrin*, but not *α-spectrin*, function (Dubreuil et al., 2000). Similarly, the phenotypes associated with the different spectrin mutants isolated in this study are distinct. Whereas *β-spectrin* leads to a typical looping of CNS axons during stage 13, no abnormal axonal phenotypes could be detected for *α-spectrin* alleles. Similarly, we failed to detect any midline phenotypes for Fasciclin II-positive axons in *α-spectrin*$^{E2\text{-}26}$-null mutants. A possible explanation to this phenotypic discrepancy may be the maternal contribution of *α-spectrin*; however, a similarly strong maternal component has been described for *β-spectrin*. Attempts to generate *α-spectrin* germline clones using the null allele *rg41* failed because of an essential function of *α-spectrin* during oogenesis (de Cuevas et al., 1996). To circumvent this maternal *α-spectrin* function, we employed the hypomorphic *α-spectrin*-mutant alleles *α-spec*$^{N\text{-}2}$, *α-spec*$^{P\text{-}2}$ or a-spec$^{1.3}$ to generate germline clones using the *ovo*^{D}/FRT system. However, embryos with both impaired maternal and zygotic *α-spectrin* expression displayed no nervous system phenotype, supporting the notion that *β-spectrin* acts independent of α-Spectrin protein.

α-spectrin mutations turned out to be sensitive to background mutations and temperature effects. In addition to the phenotypic effects of uncharacterized background mutations, we detected a temperature dependence of the spectrin-mutant phenotypes and temperature-dependent intragenic complementation of hypomorphic

α-spectrin alleles. It is well-known that microtubule dynamics depend on temperature and that microtubules depolymerize in the cold (Osborn and Weber, 1976). As microtubule stability is already compromised in *α-spectrin* mutants (Pielage et al., 2005), any further destabilization might have significant effects on (neuronal) development (Dent and Gertler, 2003). Alternatively, temperature sensitivity might reflect differences in the efficacy of endocytosis. Although we cannot pinpoint the molecular mechanism underlying the temperature sensitivity of *spectrin* mutants, we can conclude that Spectrins act as a global stabilizing protein network that coordinates a large variety of membrane receptors, including the Robo receptor that is needed to sense the Slit protein.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/4/713/DC1

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