Integrins Regulate Responsiveness to Slit Repellent Signals

Adrienne Stevens and J. Roger Jacobs

Department of Biology, McMaster University, Hamilton, Ontario, L8S 4K1, Canada

Integrins are concentrated within growth cones, but their contribution to axon extension and pathfinding is unclear. Genetic lesion of individual integrins does not stop growth cone extension or motility, but does increase axon defasciculation and axon tract displacement. In this study, we document a dosage-dependent phenotypic interaction between genes for the integrins, their ligands, and the midline growth cone repellent, Slit, but not for the midline attractant, Netrin. Longitudinal tract axons in *Drosophila* embryos doubly heterozygous for *slit* and an integrin gene, encoding αPS1, αPS2, αPS3, or βPS1, take ectopic trajectories across the midline of the CNS. *Drosophila* doubly heterozygous for *slit* and the genes encoding the integrin ligands Laminin A and Tiggrin reveal similar errors in midline axon guidance. We propose that the strength of adhesive signaling from integrins influences the threshold of response by growth cones to repellent axon guidance cues.

Key words: axon guidance; adhesion; extracellular matrix; Drosophila; *midline; commissure*

Integrin receptors provide a primary means of cell interaction with the extracellular matrix (ECM). Integrins bind adhesive molecules of the ECM, such as Laminin and Fibronectin, which communicate information for cell adhesion and movement (for review, see Cheresh and Mecham, 1994; Brown, 2000). Intracellularly, integrins provide the major linkage to the actin cytoskeleton, which mediates cell motility (for review, see Critchley et al., 1999). Integrin receptors are concentrated at the tips of filopodia in neuronal growth cones, where regulation of actin-based motility mediates axonal growth and pathfinding in development and regeneration (Grabham and Goldberg, 1997; Takagi et al., 1998).

Association of integrin receptors with ECM ligands is modeled to provide the physical linkage and adhesive signaling that stabilizes the actin cytoskeleton and enables growth cone extension. Integrin function is hypothesized to regulate the rate of axon growth (Letourneau, 1992; McKerracher et al., 1996; Condic and Letourneau, 1997). Axon growth may be facilitated by a low density of integrin adhesive contacts that can be formed and broken easily. In contrast, a high density or high binding affinity of integrin adhesive contacts could slow axon growth (Palecek et al., 1997; Maheshwari et al., 2000).

The large number of integrins in the mammalian CNS has hampered a genetic analysis of function in axonogenesis (Fassler et al., 1996; Hynes, 1996). Genetic data from mutations in the integrin genes of *Caenorhabditis elegans* and *Drosophila* do not suggest that integrins facilitate axon growth. Axon growth in these mutants is not noticeably slower, nor is pathfinding significantly altered (Baum and Garriga, 1997; Hoang and Chiba, 1998). Errors in passing innervation targets and axon defasciculation characterize the neuronal phenotype of these mutants. These phenotypes suggest a role for integrins in modulating adhesive signals, perhaps through regulated changes in adhesive affinity, and second messenger signaling to the cytoskeleton and other adhesion molecules (Hoang and Chiba, 1998).

In *Drosophila* integrin mutants, axon bypass errors occur at pathfinding checkpoints. Integrin adhesion or signaling may intersect with growth cone guidance signals at pathfinding choice points. These may be revealed by a genetic analysis of phenotypic interaction of mutations in the integrin genes and genes encoding growth cone attractants or repellents.

We have previously characterized the role of the growth cone repellent signal, Slit, in repressing the choice of commissural axon projection in axons of the longitudinal tracts (LTs) of the *Drosophila* embryo (Battye et al., 1999; Jacobs, 2000). Axons in embryos lacking *slit* function are attracted to the midline and do not leave. Increases or decreases in the level of *slit* expression in the midline of the *Drosophila* CNS correspondingly alter the number of axons that approach the midline (Battye et al., 2001). Midline repellent signaling is sensitive to gene dosage. For example, LT axons misproject across the midline in embryos with a single copy of the *slit* gene and a single copy of the gene encoding one of the receptors, Robo (Battye et al., 1999; Kidd et al., 1999).

If *slit* function is dose sensitive, a reduction in expression of other genes that affect midline axon guidance will reveal a phenotype in a heterozygous *slit* background. With this perspective, we have generated *Drosophila* doubly heterozygous for known mutations and *slit* and screened for errors in midline axon guidance. In this study, we provide genetic data to demonstrate that all integrins known to be expressed in the CNS are required to suppress errors in axon guidance. Furthermore, a penetrant dosage-dependent interaction of the genes for integrins and their ligands, with the midline repellent *slit*, suggests a role for integrins in regulating responsiveness to midline axon guidance signals.

MATERIALS AND METHODS

Drosophila *strains. slit²* (formerly *slit^{IG107}*) was isolated by Nüsslein-Volhard et al. (1984), and *slit^{2990}* was isolated by Seeger et al. (1993). A wild-type genetic background was restored and then maintained as a *yw*, balanced strain. Loss of function alleles of *dock^{04723}* and integrins *mys¹*, *if^{k27c}*, *mew^{M6}*, *scb²*, and hypomorphic *scb^{01288}* were obtained from the Indiana Stock Centre. The Netrin deficiency, *NP5*, *robo¹*, and *robo³* were provided by G. Tear (King's College, London, UK) (Mitchell et al., 1996), *lanA^{9-32}* was provided by C. Goodman (University of California,

Berkeley, CA) (Garcia-Alonso et al., 1996), and tig^x was provided by T. Bunch (University of Arizona, Tucson, AZ) (Bunch et al., 1998).

Immunocytochemistry. Immunocytochemistry was adapted from Patel (1994). Embryos were collected at 22°C and fixed at 16 hr intervals. Monoclonal antibody 1D4 [provided by N. Patel (University of Chicago, Chicago, IL) and C. Goodman] was diluted 1:4 in PBS with 0.1% Triton X-100 and incubated at room temperature for 6 hr followed by 2 hr incubation in goat anti-mouse conjugated with HRP (Jackson ImmunoResearch, West Grove, PA) at a 1:1000 dilution. The presence of $P[lacZ]$ balancer chromosomes was assessed with rabbit β-galactosidase antibody, 1:200 dilution (Cappel). Reactions with most embryos were performed in the presence of 0.03% cobalt chloride. Nerve cords were dissected in methyl salicylate before mounting in DPX (Sigma 31761–6) and visualized on a Zeiss Axiophot microscope. Phenotypes scored as percentages were determined from 90–200 segments scored in 12–25 embryos for each phenotype. The variance in the number of affected segments per embryo was comparable when the number of midline guidance errors was high (e.g., sli^2/scb^2 was 5 ± 2.1) or low (e.g., $mew/+$; $sli^2/+$ was 0.7 ± 1.6).

RESULTS

slit function is sensitive to dosage of interacting genes

Previous studies have demonstrated that mutations in *slit* interact phenotypically with mutations in *robo*, a gene encoding one of the Slit receptors (Battye et al., 1999; Kidd et al., 1999). The interaction is manifested as a reduction in midline repellent signaling, resulting in the misprojection of LT axons across the midline. These contralateral projections can be detected by assessing the distribution of Fasciclin II (Fas II)-labeled axons in the CNS at late embryogenesis. In wild-type embryos, Fas II is restricted to a bilateral trio of axon fascicles that maintain a constant distance from the midline (Fig. 1*A*). In the absence of midline repellent signals, as in *slit* loss-of-function mutants, all Fas II axons fuse into a single tract at the midline (Fig. 1*B*).

Three characteristic features of reduced midline signaling are evident in loss-of-function mutants for *robo*. Axons of the most medial Fas II fascicle cross and recross the midline, whereas lateral tracts are less affected (Fig. 1*C, arrowhead*). The intensity of Fas II labeling drops in LT axons between segments (Fig. 1*C, arrow*). Finally, the nervous system is narrower. Dock, a *Drosophila* ortholog of Nck, is an SH2/SH3 adapter protein implicated in axon guidance signaling (Garrity et al., 1996). Embryos lacking *dock* function have a subtle Fas II phenotype, characterized by wavy LT fascicles, and interruptions in Fas II labeling in the lateral axon tracts (Fig. 1*D*). Although *Drosophila* with a single working copy of the *slit*, *robo*, or *dock* genes (heterozygous for a loss-of-function mutant) appear normal, novel axon guidance phenotypes emerge in embryos that have a single copy of two or more of these genes. Midline guidance errors are seen in 44% of segments in embryos with a single copy of the *slit* and *robo* genes (Fig. 1*E*) (all counts are summarized in Table 1). Embryos with a single copy each of the *slit* and *dock* genes have midline guidance errors in 77% of segments, sometimes involving all axon fascicles (Fig. 1*F*).

Changes in phenotype consistent with a change in gene copy number suggest gene function in a dosage-dependent event, such as signaling positional information in a gradient. Because Robo is a Slit receptor and Dock is a second messenger in the same or a parallel pathway, we hypothesize that the intensity of signaling by these molecules communicates positional information. Therefore, other genes that are required directly or indirectly to deliver this signal should also have a dosage-dependent phenotypic interaction with *slit*. We were interested in identifying other proteins on the cell surface or ECM that may interact with Slit and play a role in midline guidance. We therefore examined a number of muta-

tions in genes encoding ECM proteins to determine whether they had a dosage-dependent phenotypic interaction with *slit* (Stevens, 2000). Mutations affecting integrin function revealed a dosage-dependent interaction with *slit*, which is reported further here.

Integrin mutations affect axon tracts

We examined axon fasciculation and guidance in the CNS of embryos mutant for the β integrin gene myospheroid (*mys*) and three α integrins, αPS1 (*mew*), αPS2 (*if*), and αPS3/4 (*scb*). [It is not known whether the *scb* locus affects αPS3 or αPS4 or both, because the αPS4 locus is separated from αPS3 by 259 bp; Brown et al. (2000)]. Variation in penetrance of mutant phenotype was observed with all integrin alleles; representative examples of the more severe phenotypes are pictured here (Fig. 2*A–D*). The three longitudinal fascicles are intact in loss-of-function mutations in *mys*, which encodes the only β integrin expressed in the CNS (Fig. 2*A*); however, midline fusions of the most medial tract are seen in some segments. Axon tract structure was least disrupted in *mew* mutant embryos. No midline guidance errors were seen; however, the longitudinal fascicles appeared to be thinner, with occasional defasciculation (Fig. 2*B, arrow*). *if* mutant embryos were similar in phenotype to *mew*, but also revealed a low frequency of midline crossover errors (Fig. 2*C, arrowhead*). Midline fusions as well as transient merging of lateral fascicles were seen in *scb* mutant embryos (Fig. 2*D, arrow*).

Integrin mutants interact with *slit*

Axon tract fasciculation appears normal in embryos heterozygous for mutations in one integrin gene and also in embryos heterozygous for mutations in two integrin genes (data not shown). However, all four integrin mutations reveal a semidominant phenotype when doubly heterozygous with *slit*. In all instances, the frequency of midline guidance errors is increased over the levels seen in homozygous integrin mutants. Apart from midline axon crossings in one-third of the segments, the LT appeared normal in $mys/+;sli/+$ embryos (Fig. 2*E, arrowhead*; Table 1). Less than 10% of segments revealed midline guidance errors in $mew/+$; $sli/+$ embryos (Fig. 2*F*). In contrast, 40 and 58% of segments had midline guidance errors in $if/+;sli/+$ and scb/sli embryos (Fig. 2*G,H*, respectively). Only in scb/sli double heterozygotes were the middle and most lateral axon tracts affected, indicating a strong phenotypic interaction between *scab* and *slit*.

Midline guidance phenotypes were observed in integrin homozygotes that were also haplosufficient for *slit*. In particular, the frequency of midline crossing is much higher in $mew/Y;sli/+$ and $if/Y;sli/+$ mutants and also involves more lateral axon tracts (Fig. 2*J,K*, respectively).

We were not able to generate an *scb,sli* recombinant to assess the *scb,sli/scb* phenotype. These genes would be expected to recombine in 1 of 25 chromosomes; however, we isolated no recombinants in 200 chromosomes screened. It was possible to investigate the interaction of these genes by using overlapping deficiencies. Midline guidance was assessed in *scb* and *sli* heterozygotes, in *trans* to a deficiency that uncovers either *scb* or *sli* or both genes (Fig. 3, *schema*). Consistent with our characterization of the *sli* and *scb* phenotypes, *sli* in *trans* to a deficiency uncovering *sli* has complete midline fusion of all axon tracts (Fig. 3*E,F*), and *scb* in *trans* to a deficiency uncovering only *scb* has an integrin mutant phenotype (Fig. 3*A*). The semidominant interaction of *sli* and *scb* is also confirmed when *scb* is *trans* to a deficiency uncovering only *slit* (Fig. 3*B*) or when *sli* is *trans* to a deficiency uncovering only *scb* (Fig. 3*D*). A synthetic *scb* homozy-
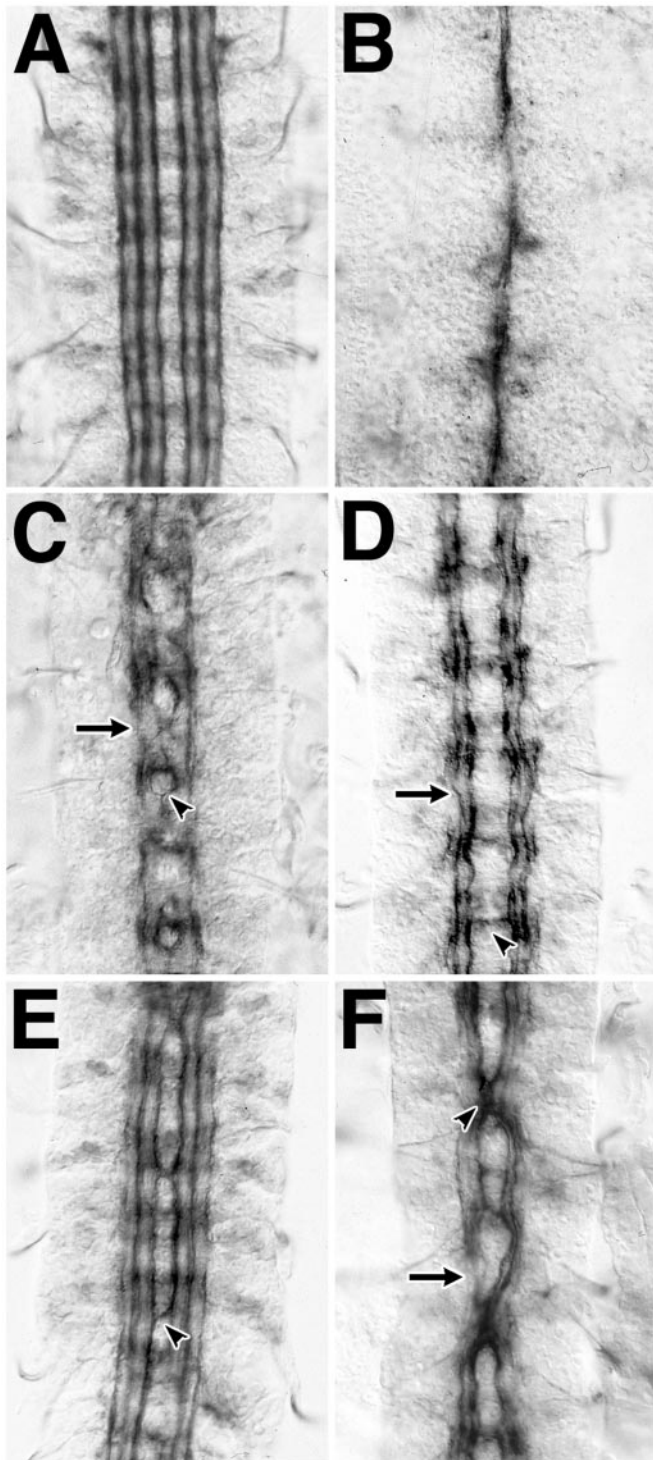
Figure 1. *slit* interacts genetically with *robo* and *dock*. A bilateral set of three distinct axon fascicles are labeled with antibody to Fasciclin II in stage 17 embryos of wild-type *Drosophila* (*A*). All axons fuse at the midline in embryos mutant for *slit* (*B*), whereas medial axons recross the midline in embryos mutant for a Slit receptor gene, *robo* (*C*, *arrowhead*). Lateral axons do not make midline guidance errors, but occasional gaps in Fasciclin II labeling are seen (*C*, *arrow*). Midline guidance errors are very rare in embryos mutant for *dock* (*D*, *arrowhead*); however, gaps in Fasciclin II labeling in the lateral axon fascicles are common (*D*, *arrow*). Midline guidance errors in the most medial axon tract are common in embryos that have a single wild-type and a single mutant copy of both the *slit* and *robo* genes (*E*, *arrowhead*). Embryos similarly heterozygous for both *slit* and *dock* have more frequent and profound midline guidance

gote and *sli* heterozygote phenotype was generated in embryos with a *scb* mutant allele in *trans* to a deficiency uncovering both *scb* and *sli*. This phenotype is qualitatively similar to the *scb/sli* phenotype, revealing frequent midline crossing or fusion of the two most medial but not the most lateral axon fascicles (Fig. 3*C*).

Genes for integrin ligands interact with *slit*

The midline axon phenotype of integrin mutants is part of a more complex phenotype involving defasciculation, irregular fascicle position, and "wavy" axon trajectories. This phenotype emerges even when *slit* function is normal and may reflect axon guidance functions of known integrin ligands in the nervous system. Two integrin ligands have been identified within the LT, Laminin, and Tiggrin (Montell and Goodman, 1989; Fogerty et al., 1994). We have characterized the Fas II phenotypes of embryos mutant for these ECM proteins to clarify their possible contribution to axon guidance.

Tiggrin is a secreted glycoprotein that contains an RGD motif and is considered to be a ligand of the PS2 integrin (Fogerty et al., 1994; Bunch et al., 1998). Embryos homozygous for a loss of function allele of Tiggrin have a subtle Fas II phenotype reminiscent of integrin mutants. CNS axon tracts are wavy, and no midline axon guidance errors are seen. Labeling of the most lateral axon tract is interrupted between segments (Fig. 4*A*, *arrow*). Like the integrin genes, *tig* also has a semidominant interaction with *slit*. Fas II labeling of fascicles between segments is reduced (Fig. 4*B*, *arrow*). Midline guidance errors are seen in one in three segments (Fig. 4*B*, *arrowhead*).

Drosophila Laminin is a trimer of three proteins, Laminin A, B1, and B2 (Montell and Goodman, 1989). Laminin is known to be a ligand of PS1 integrin and possibly other integrins as well (Gotwals et al., 1994). Mutants have not been isolated for the B1 and B2 chains; however, a loss of function allele for *lanA* encoding the A chain has been characterized (Garcia-Alonso et al., 1996). The Fas II phenotype of the *lanA* mutant is nearly wild type, revealing midline guidance errors in 4% of segments (Fig. 4*C*) ($n = 145$ segments). When doubly heterozygous with *sli*, in *sli/+;lanA/+* embryos, the frequency of midline crossovers is >30% (Fig. 4*D*).

Does a change in *lanA* function also affect integrin function in CNS axon tract formation? We chose to examine *lanA* interaction with *scb* because Laminin is not known to be a ligand of αPS3/4 (encoded by *scb*), and *scb* has a strong semidominant interaction with *slit*. Both *lanA* and *scb* reveal midline guidance errors when homozygous (Figs. 2*D*, 4*C*). However, in the *scb/+;lanA/+* double heterozygote, midline guidance errors are not seen (Fig. 4*E*). Nevertheless, this genotype shares aspects of the integrin CNS phenotype: defasciculation and interruptions in Fas II labeling of the most lateral fascicle (Fig. 4*E*, *arrow*). This suggests function of both genes in a common or parallel pathway. If the interaction of *scb* and *lanA* is independent of the interaction of either gene with *sli*, then the phenotype of the triple heterozygote *scb/sli; lanA/+* would reflect the addition of the *scb/sli*, *scb/+;lanA/+*, and *sli/+;lanA/+* phenotypes. The degree of defasciculation and midline guidance errors in all axon tracts of the triple heterozygote (Fig. 4*F*) appears to be additive. However, a narrowing of the CNS and the medial displacement of all axon tracts are also seen

←

errors, involving all labeled axon fascicles (*F*). In this and subsequent figures, *arrowheads* indicate midline guidance errors, and *arrows* indicate interruptions in the longitudinal tracts.

Table 1. Frequency of midline guidance errors in semidominant interactions with _sli²_ heterozygotes

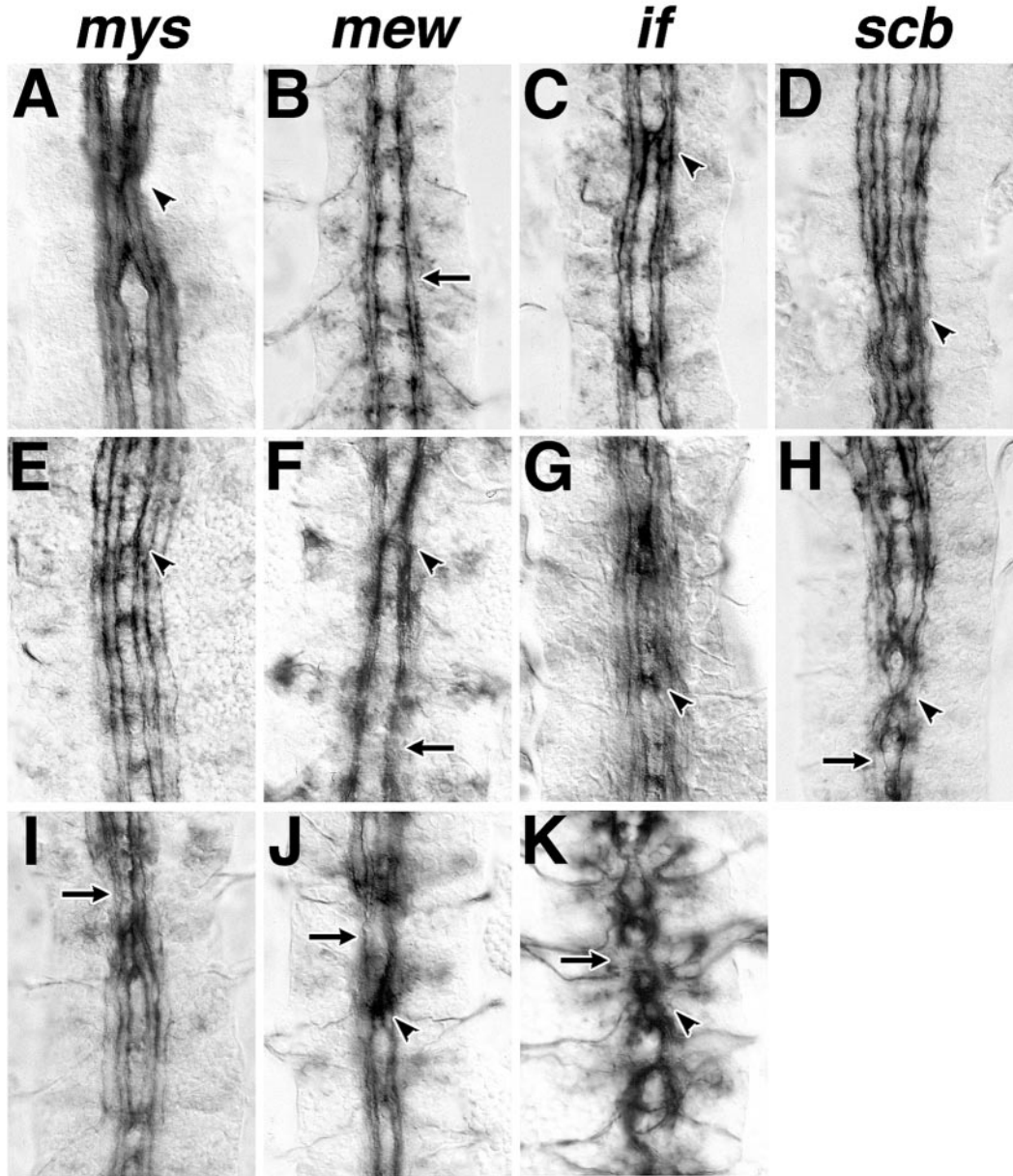| Genotype	sli/+	sli/robo	sli/dock	mys/+; sli/+	mew/+; sli/+	if/+; sli/+	sli/scb	sli/+; tig/+	sli/+; lanA/+	NP5/+	NP5/+; sli/+	NP5/+; robo/+	NP5/+; scb/+
% Midline errors	3.4	44	77	31	8.3	40	58	38	31	2.2	28	26	3.2
Number of segments	146	127	110	90	96	104	108	92	99	138	198	120	92

Figure 2. _slit_ interacts genetically with integrin mutations. Embryos mutant for β-integrin (_A, mys_), αPS1 integrin (_B, mew_), αPS2 integrin (_C, if_), and αPS3 integrin (_D, scab_) have a range of axon guidance phenotypes; more severe examples are represented here. Midline errors (_arrowheads_) can be found in all integrin mutants; however, they occur more frequently and involve more axons in _scab_ and progressively fewer in _if, mys,_ and _mew_ mutant embryos. Axon guidance errors are seen in embryos heterozygous for both an integrin gene and _slit_ (_E, mys; F, mew; G, if;_ and _H, scb_) and in embryos homozygous mutant for an integrin gene and also heterozygous for _slit_ (_I, mys; J, mew;_ and _K, if_). Midline axon crossover is most frequent in _scb/sli_ heterozygotes and then less often in _if, mys,_ and _mew_ double heterozygotes, respectively.

in the triple heterozygote. This phenotype is typical of mutants in genes required for midline guidance and is not a component of the integrin mutant phenotype. The synergistic interaction of these three genes suggests dosage-dependent function for each gene in common or parallel pathways.

Netrin interacts weakly with midline repellent genes

Given that genes which function in cell to ECM adhesion interact with axon repellent signals, we wondered whether adhesion gene phenotypes interact similarly with midline attractant signals. We therefore explored whether embryos homozygous or heterozy-
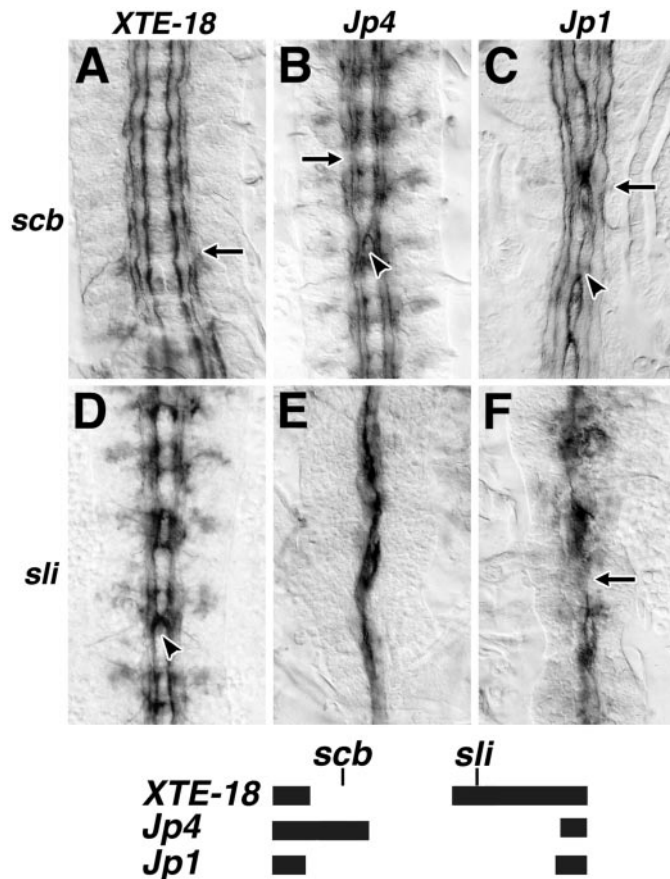
Figure 3. Phenotypic interaction of *scab* and *sli*. Midline guidance errors were assessed in embryos *trans*-heterozygous for either *scab* or *sli* and an overlapping deficiency. The extent of each deficiency is shown graphically below. Embryos heterozygous for *scab* and a deficiency that does not uncover *slit* have a *scab*-like phenotype (*A*, *scb/Df(2R)XTE-18*), and embryos heterozygous for *slit* and a deficiency that does uncover *slit* have a *slit*-like phenotype (*E*, *sli/Df(2R)Jp4*; *F*, *sli/Df(2R)Jp1*). The semidominant genetic interaction between *sli* and *scab* is regenerated in *scb/Df(2R)Jp4* and *sli/Df(2R)XTE-18* embryos (*B* and *D*, respectively). The medial Fascicilin II fascicles in *scb/Df(2R)Jp1* embryos, which lack all *scab* function and are heterozygous for *sli*, make midline guidance errors in all segments (*C*, *arrowhead*). More lateral fasicles are affected also (*C*, *arrow*).

gous for a deficiency that uncovers both Netrin genes, *netA* and *netB*, affected midline guidance in a gene interaction assay. Embryos that lack *netrin* function have few commissural axons, and most commissures are missing (Mitchell et al., 1996). Embryos with one copy of each *netrin* gene (*NP5*/+) have normal commissures; however, the LTs, visualized with BP102, are thinner between segments and thicker within segments. Embryos that are heterozygous for mutations in both an integrin gene and the netrins show no enhancement or suppression of this phenotype (data not shown).

These data suggest that there may be a dosage-sensitive function of netrins in the organization of the LT. We chose to examine the morphology of Fasciclin II axon bundles more closely. Embryos homozygous or heterozygous for the netrin deficiency reveal irregularity and interruptions in longitudinal Fasciclin II bundles (Fig. 5*A*,*B*, respectively). The heterozygote netrin phenotype was not enhanced in embryos also heterozygous for *slit*, *robo*, or *scab* function (Fig. 5*D–F*, respectively). In contrast, the frequency of midline guidance errors was increased in *slit*/+ or *robo*/+ but not *scb*/+ embryos when netrin function was
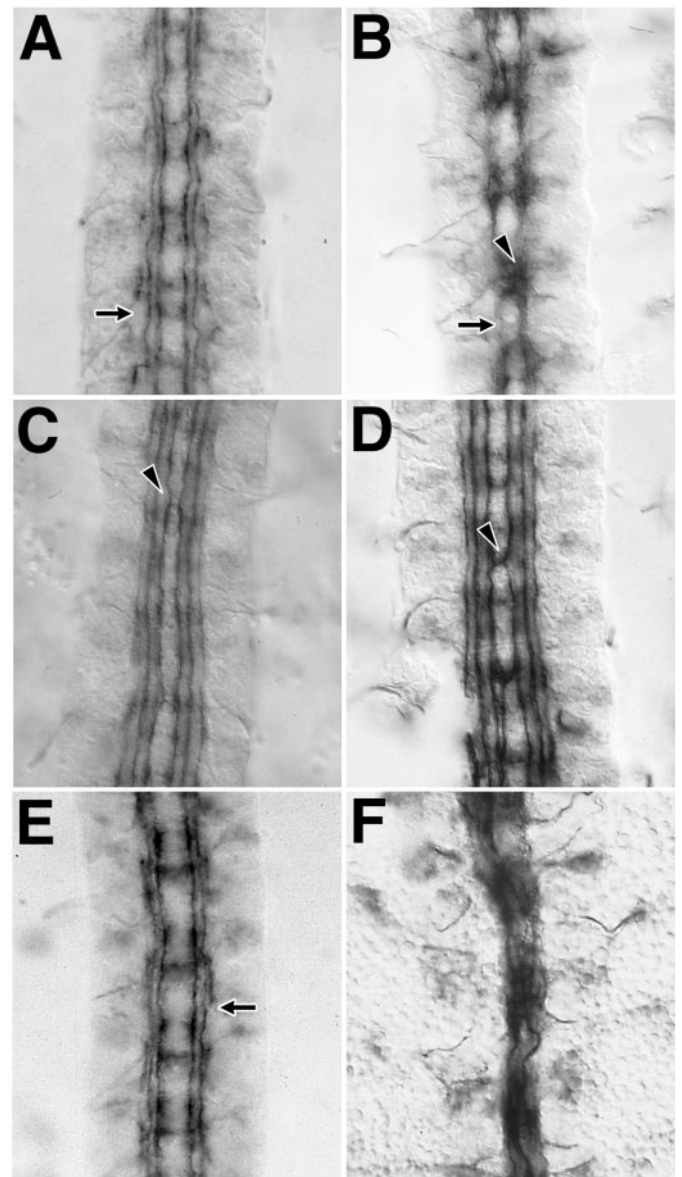

Figure 4. Integrin ligands interact genetically with *scab* and *slit*. Embryos homozygous for *tig* have normal midline axon guidance but have wavy and undulating longitudinal tract fasicles (*A*, *arrow*). In embryos that are heterozygous for both *tig* and *slit*, fasciculation within the longitudinal tracts is abnormal (*B*, *arrow*), and many Fas II-labeled axons project toward the midline (*B*, *arrowhead*). Embryos homozygous mutant for *lanA* have a nearly normal Fas II labeling pattern, with rare midline guidance errors (*C*, *arrowhead*). Embryos heterozygous for both *lanA* and *slit* are similar, but the frequency of midline guidance errors is higher (*D*, *arrowhead*). In embryos doubly heterozygous for both *lanA* and *scb*, defasciculations and gaps occur in the longitudinal tracts (*E*, *arrow*). An embryo with a single copy of *sli*, *scb*, and *lanA* has pronounced medial displacement of all longitudinal tracts and midline fusion of medial axon tracts (*F*).

also reduced or removed (Fig. 5*D–F*, *arrowheads*; Table 1). These data indicate that repellent signaling is significantly more dosage sensitive than attraction in midline phenotypes.

DISCUSSION

In this study, we have shown that a reduced level of expression of the genes for four integrins (αPS1, αPS2, αPS3/4, and βPS1) or two integrin ligands (Tiggrin and Laminin) increases the probability that CNS axons make pathfinding errors when *slit* expres-
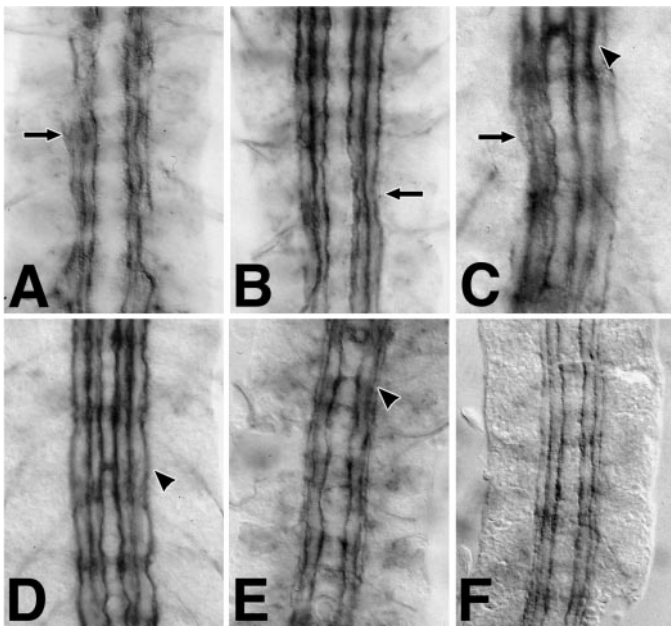
Figure 5. The *netrin* phenotype interacts weakly with *slit*. Fasciclin II labeling reveals defasciculation and gaps (*arrow*) in embryos homozygous for a small deficiency uncovering both *netA* and *netB* (*A*, *NP5/Y*). Embryos heterozygous for the Netrin deficiency have similar but less severe irregularities in Fasciclin II distribution (*B*, *NP5/+*). Embryos lacking *netrin* function and heterozygous for *slit* reveal a *netrin* phenotype, with an increase in the frequency of midline guidance errors (*C*, *NP5/Y; sli/+*). Similarly, embryos doubly heterozygous for *netrin* and for *slit* (*D*, *NP5/+; sli/+*) or *robo* (*E*, *NP5/+; robo/+*) share the NP5/+ fasciclin II phenotype and a minor increase in the frequency of midline guidance errors (*D*, *E*, *arrowheads*). Midline guidance errors are not seen in netrin, *scab* double heterozygotes (*F*, *NP5/+; scb/+*).

sion is reduced. The *netrin* mutant phenotype was not affected by reduced integrin function.

Axon tract phenotypes of integrins and their ligands

Expression of the integrins Tiggrin and Laminin A has been demonstrated previously in the CNS (Montell and Goodman, 1989; Fogerty et al., 1994; Hoang and Chiba, 1998; Rohrbough et al., 2000). Integrin expression is not localized and may be expressed in both glia and neurons. Overexpression of αPS3 or Laminin A in motoneurons affects axon guidance (Kraut et al., 2001). Loss of function of the integrins disrupts axon fasciculation and longitudinal axon fascicle placement in the embryonic nerve cord but does not clearly affect axon guidance (Hoang and Chiba, 1998). In this study, we extended these observations with different alleles of the integrins, demonstrated a similar function for αPS3/4, and revealed axon fascicle phenotypes for loss of function of integrin ligands Tiggrin and Laminin A. The mutant phenotypes share common elements: mild phenotypes show wavy axon tracts and reduced Fas II labeling between segments, whereas severe phenotypes include defasciculation and fascicle displacement, including midline axon guidance errors. The integrins have different extracellular ligands. Therefore, the integrins contribute similarly to axon tract integrity, independent of the ligand that they bind.

Integrin phenotypes in the CNS do not demonstrate a direct role for integrins in growth cone guidance. In contrast, perturbation of midline growth cone repellent signals results in a medial narrowing of the CNS and ectopic midline crossing of longitudinally projecting axons, rather than defasciculation and displacement of axon tracts. One feature of integrin and *tiggrin* phenotypes shared with *robo* and *dock* mutant phenotypes is a thinning or loss of Fas II labeling in the most lateral axon fascicle. This fascicle expresses Fas II late in embryogenesis. This phenotype may reflect impaired or delayed development of independent fascicles in the nerve cord, as implicated by other studies of *robo* function (Rajagopalan et al., 2000; Simpson et al., 2000).

The nature and significance of genetic interaction with axon guidance signals

Axon guidance cues such as Netrin or Slit are secreted proteins that associate with the ECM. Vertebrate Slit, for instance, binds to Laminin, Netrin, and Glypican (Brose et al., 1999; Liang et al., 1999). These cues also act at a distance from the cells that synthesize them. Whether or not Slit forms a detectable gradient in the ECM, the amount of protein alters the potency of repellent signaling. A *robo*-like phenotype is seen in hypomorphs of *slit* that produce less protein, and overproduction of *slit* reduces the number of commissural axons (Battye et al., 1999, 2001); therefore, Slit signaling is dosage sensitive. If reduced expression of another gene enhances the midline guidance phenotype of *slit*, then the normal function of that gene contributes functionally to inhibit axons from crossing the midline. This may reflect function in the production or transduction of the repellent signal or another function in the growth cone that reduces the probability of a growth cone approaching the midline.

The semidominant interaction of all integrins, Tiggrin, and Laminin A with *slit* is more prevalent than might be expected if the integrins play a specialized role in Slit signaling. *scab* also has a dramatic semidominant interaction with *dock* (Nck), which functions in diverse axon guidance events (Garrity et al., 1996; Hing et al., 1999). *scb/dock* double heterozygotes have disrupted longitudinal, commissural, and peripheral axon tracts (J. R. Jacobs, unpublished observations). A similar genetic test suggests that βPS integrin modulates RhoA activity and axon stability in the mushroom body (Billuart et al., 2001). These diverse phenotypes reflect an adhesive function of the integrins that reduces the responsiveness of growth cones to guidance signals. Independent evidence suggests that this occurs in the growth cone, but a role for integrin in the glia that emit guidance signals cannot be discounted.

Mutations in the *netrinA* and *netrinB* genes did not reveal semidominant interactions with genes for integrin function. Therefore Netrin signaling is not dosage sensitive in this genetic assay. Although Netrin might form a gradient *in vivo*, these data suggest that Slit may more effectively communicate positional information than does Netrin. Double mutants of *netrin* and *slit* have a *slit* phenotype, indicating that Netrin signaling acts genetically upstream of repulsion and also that attraction to the midline persists in the absence of Netrin (Jacobs, unpublished observations). These data suggest that Netrin is not the sole midline attractant in *Drosophila*. More axons approach the midline in a *netrin, slit* double heterozygote than would if only *slit* function is reduced. Therefore Slit and Netrin do not generate independent, additive guidance signals. Netrin can bind to Slit (Brose et al., 1999). Furthermore, the Slit receptor may silence attractant signaling by the Netrin receptor (Stein and Tessier-Lavigne, 2001). Copresentation of Slit and Netrin to the receptors on the growth cone may enhance the repellent signal. Attraction to the midline requires silencing of Slit signaling. In *Drosophila,* repellent signals can be silenced by Comm, which facilitates internalization of the Robo receptor (Kidd et al., 1998).

How do integrins contribute to axon guidance?

Integrins are concentrated in the growth cones of *Drosophila* axons (Takagi et al., 1998; Takagi et al., 2000), and their ligands are uniformly distributed over pathways of axon extension, (Montell and Goodman, 1989; Fogerty et al., 1994). Integrins facilitate the growth of axons by providing a link between the ECM and the cytoskeleton of the growth cone. Defasciculation and guidance errors seen in integrin mutants reflect decreased adhesion to the ECM and a lower threshold to errors in guidance. Axon extension is not impaired in *Drosophila* integrin mutants, although it is possible that a maternal contribution of integrin may mask this requirement.

Part of the adhesive function of integrins is to activate intracellular signals that alter motility and axon outgrowth. During ligand binding, β integrins may activate Focal Adhesion Kinase and Rho, which stabilize actin structures, permit actin filament growth, and facilitate the formation of focal adhesions (Clark et al., 1998; Bishop and Hall, 2000). Intracellular signals can also modify adhesiveness by altering the affinity of integrins for their ligands. These signals can combine to cluster integrins and strengthen their attachment to the ECM. This increased adhesiveness can act in opposition to factors that decrease adhesion and axon extension, such as myelin or aggrecan (David et al., 1995). Furthermore, integrin signaling can influence other adhesion systems active in the growth cone (Hemler, 1998; Arregui et al., 2000; Iba et al., 2000).

Similarly, integrin function alters the sensitivity of growth cones to repellent signaling by Slit. When *slit* expression and integrin function are both reduced, growth cones are more likely to respond to attractive guidance from the midline. Signals from axon guidance receptors promote growth cone reorientation and remodeling of the growth cone cytoskeleton. Integrin adhesion promotes local stabilization of cytoskeletal links to the ECM, which antagonizes growth cone reorientation. The threshold of response of growth cones to axon guidance signals is therefore regulated by the ability of guidance signals to reduce the stability of ECM to cytoskeletal linkages. This threshold may be reached at axon guidance choice points, including the segment boundary and the commissures, where clustering of errors in axon guidance occur. The efficacy of guidance signals may be reduced between choice points by local increases in ECM affinity.

Integrin–ligand affinity and integrin–cytoskeletal linkages are logical targets of axon guidance signals. Further studies of the targets of integrin and guidance signals should reveal how growth cones integrate information from the ECM.

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