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# Axon pathfinding proceeds normally despite disrupted growth cone decisions at CNS midline

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## SUMMARY

**Axons in the bilateral brain of *Drosophila* decide whether or not to cross the midline before following their specific subsequent pathways. In *commissureless* mutants, the RP3 and V motoneuron axons often fail to cross the midline but subsequently follow the mirror-image pathways and innervate corresponding muscle targets on the ipsilateral side. Conversely, in *roundabout* mutants, the RP2 and aCC motoneuron axons sometimes cross the midline abnormally but their subsequent pathways and synaptic targeting are the perfect mirror images of those seen in wild type. Furthermore, within a single segment of these mutants, bilateral pairs of motoneuron axons can make their midline decisions independently of each other. Thus, neither the growth cones' particular molecular experience nor the decision at the midline caused by these mutations affects their ability to respond normally to subsequently presented cues.**

Key words: *commissureless*, *Drosophila*, Motoneuron, *roundabout*, Preprogramming, Synaptic targeting

## INTRODUCTION

Inside a developing brain, neuronal growth cones navigate through constantly changing microenvironments. In vitro studies show that the responsiveness of growth cones to extrinsic cues can be adjusted interactively during the course of their extension (Condic and Letourneau, 1997; Murakami and Shirasaki, 1997; Song et al., 1997). Such an ability is a potentially powerful means of controlling axon pathfinding in diverse situations that occur in vivo. There are two models that illustrate how growth cones may utilize interactive readjustments during their complex navigation inside the brain. In a 'continual experience-dependent reprogramming' model, a certain cue is an essential early experience that is directly responsible for the ability of growth cones to respond to cues encountered later. In an 'experience-independent preprogramming' or 'selective desensitization' model, the growth cone is merely desensitized towards an initially overwhelmingly attractive cue and thereby revealing its innate responsiveness to other cues that has been masked previously. The two models depict fundamentally distinct developmental strategies, one that maximizes cellular adaptability to its environment (reprogram model) versus the other that achieves time efficient execution of events (preprogram model). It is generally thought that neural development takes advantage of both (Goodman and Shatz, 1993; McConnell, 1995). What is not clear is to what extent the experience-dependent reprogramming of growth cones underscores their highly reproducible pathway selections.

The CNS midline presents a universal paradigm in which a growth cone's context-dependent response adjustments can be studied. The majority of axons in a bilateral brain decide whether or not to cross the midline before following their subsequent pathways (Stoeckli and Landmesser, 1998; Tessier-Lavigne and Goodman, 1996; Thomas, 1998). Despite extensive molecular symmetry across the midline, certain axons in each half of the brain ignore guidance cues from the ipsilateral half prior to crossing the midline. After crossing, these neurons then respond to presumably the same cues on the contralateral half without re-crossing. While multiple midline molecules are likely to be involved (Chan et al., 1998; Harris and Holt, 1999; Hummel et al., 1999; Shirasaki et al., 1998; Thomas, 1998), either of the two general models outlined above could explain this midline paradox. According to the 'continual reprogramming' model, midline crossing is an essential early experience directly responsible for a gain of growth cones' later responsiveness to cues on the contralateral side. The model predicts that if growth cones fail to cross the midline, or alternatively are made to abnormally cross the midline, they will not be able to respond normally to cues beyond the midline even when they have a chance to encounter them. According to the 'experience-independent preprogramming' model, on the other hand, the midline, initially overwhelmingly attractive, quickly desensitizes growth cones towards it and thereby unmasks their innate responsiveness to cues on the contralateral side. In this case, the midline experience merely readjusts growth cones' responsiveness to the midline signals, rather than priming them for successful subsequent pathfinding. Distinguishing between the two models requires in vivo tests with specific

manipulations to the endogenous environment that growth cones experience early on at the midline and analysis of growth cones' subsequent behaviors.

Whereas many model animal systems provide examples of axons crossing the CNS midline (Stoeckli and Landmesser, 1998; Tessier-Lavigne and Goodman, 1996; Wang et al., 1995), *Drosophila* offers the genetic means to specifically disrupt midline signaling as well as single cell resolution of growth cones. In each half-segment of a wild-type embryo, five motoneuron axons (e.g. RP3, V) extend across the midline, while the other 27 motoneuron axons (e.g. RP2, aCC) grow away from it (Fig. 1A) (Landgraf et al., 1997; Schmid et al., 1999). Following a specific midline decision, each axon continues through a unique series of subsequent pathways and innervates specific muscle cell(s) (Fig. 1A). The molecules involved in midline signaling include the transmembrane protein Commissureless and the extracellular matrix component molecules, Slit and Netrins, which are provided by the midline glia cells, and the growth cone receptors Roundabout and Frazzled, which are responsible for directing at least some axonal growth cones to grow either away from or towards the midline (Harris et al., 1996; Kidd et al., 1999, 1998a; Kolodziej et al., 1996; Mitchell et al., 1996; Tear et al., 1996). Mutations in these genes cause reduced midline crossings, as with the *commissureless*, *netrin* and *frazzled* knockouts, as well as extra midline crossings, as with the *roundabout* and *slit* knockouts (Battye et al., 1999; Brose et al., 1999; Harris et al., 1996; Kidd et al., 1999, 1998b; Kolodziej et al., 1996; Mitchell et al., 1996; Seeger et al., 1993; Zallen et al., 1998).

Here we show that *commissureless* and *roundabout* mutations (Kidd et al., 1998a,b; Seeger et al., 1993; Tear et al., 1996) specifically disrupt the midline growth cone decisions of RP3, V, RP2 and aCC motoneurons, causing each either to fail to cross or to abnormally cross the midline. Furthermore, we show that, despite a disrupted midline decision, each axon retains normal responsiveness to all subsequent cues, selecting the mirror-image pathways and target muscle(s) on the other side of the midline. Our results suggest a reliance of a growth cone on an experience-independent preprogramming for its accurate pathfinding beyond the CNS midline.

## MATERIALS AND METHODS

### *Drosophila* stocks

We used the null alleles of two midline mutants, *comm*$^{5}$ (source: Mark Seeger, Ohio State University) and *robo*$^{Z570}$ (source: Corey Goodman, University of California) (Kidd et al., 1998a; Tear et al., 1996). Although an earlier paper cites unpublished data that the RP3 axon in the *comm* mutants innervates muscles 6 and 7 (Seeger et al., 1993), we could not reproduce this. We found that the Commissureless protein is normally expressed by all muscles and that its absence will prevent initiation of neuromuscular synaptogenesis by stalling motoneuron growth cones short of target muscles (Wolf et al., 1998). This synaptogenesis defect can be specifically rescued, independent of the CNS midline defects, by resupplying the wild-type gene to muscles: *GAL4*$^{005}$ */ UAS-comm*$^{wt}$*; comm*$^{5}$ */ comm*$^{5}$ (Wolf et al., 1998). When scored using mAb 1D4 immunocytochemistry, this 'muscle rescued' line exhibits 90% innervation at muscles 6 and 7 ($n$=62 half-segments in 7 embryos, not shown). The transgenic line *lim3A′-lacZ* (source: John Thomas and Stefen Thor, Salk Institute) was crossed to

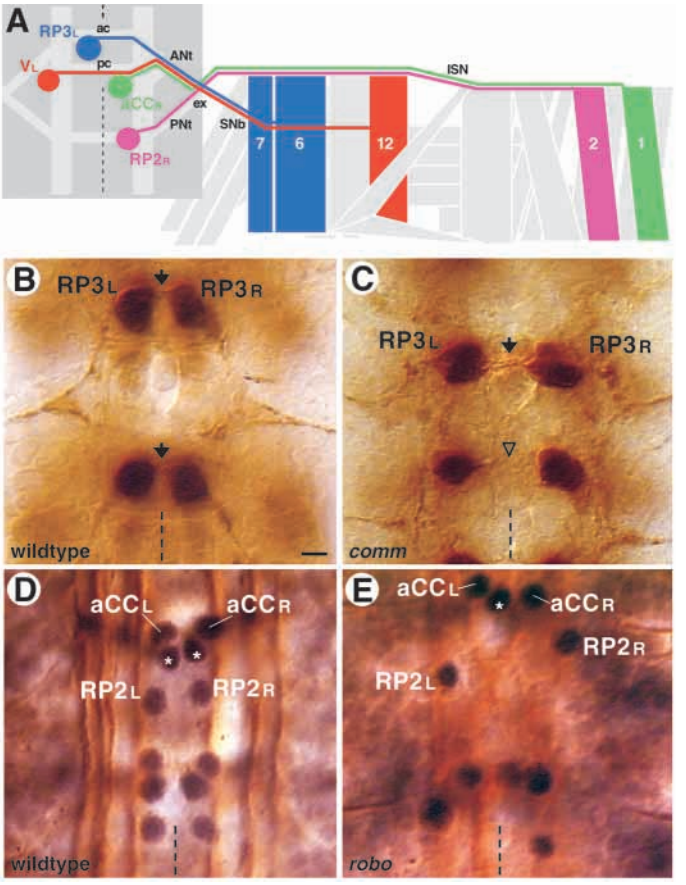


**Fig. 1.** *Drosophila* embryonic motoneurons RP3, V, RP2 and aCC, and their axon pathways. (A) The axons from RP3 (blue) and V (orange) cross the midline (broken line) via ac and pc, respectively, before joining the aCC (green) axon in ANt. The RP2 (pink) axon extends through PNt, and merges with the other axons at ex. The RP3 and V axons continue through SNb, while the RP2 and aCC axons take ISN. RP3 innervates muscles 6 and 7, V targets muscle 12, RP2 and aCC synapse with muscles 2 and 1, respectively. The cell body positions, axon pathways, and synaptic targets of these motoneurons are reiterated in every half-segment. (B,C) Immunovisualization of RP3 motoneurons in hour-17 embryos, following genetic crossing to the *lim3A′-lacZ* transgenic line (see Materials and Methods). Each panel shows two CNS segments. In wild type (B), lacZ positive axons (arrows) from bilateral pairs of RP3s ($RP3_R$, $RP3_L$) are detected at the midline (broken line) in every segment (100%, $n$=49 in eight embryos). In *comm* mutants (C), some segments (17%, $n$=59 in eight embryos) still have lacZ positive axons (arrow) crossing the midline, other segments (83%) show no lacZ positive axons (open arrowhead) at the midline. The RP3 cell bodies are laterally dislocated in the mutants. (D,E) Immunovisualization of RP2 and aCC cell bodies in hour-17 embryos, with Even-skipped antibodies (black histochemical product). Each panel shows two CNS segments. In wild type (D), RP2, aCC, as well as pCC (asterisk) cell bodies in each segment exhibit consistent positions near the midline (broken line), and inside the space between the bilateral sets of longitudinal axon fascicles (brown histochemical products, with mAb 1D4). In *robo* mutants (E), these cell body positions are variable. ac, anterior commissure; pc, posterior commissure; ANt, anterior nerve tract; PNt, posterior nerve tract; ISN, intersegmental nerve; SNb, segmental nerve branch b; ex, lateral nerve exit; $aCC_R$, $RP2_R$, $RP3_R$, $V_R$ (respectively, aCC, RP2, RP3 and V, arising from the right side), $aCC_L$, $RP2_L$, $RP3_L$, $V_L$ (respectively, aCC, RP2, RP3 and V, arising from the left side). These abbreviations also apply to Figs 2-5. Bar, 2.5 μm.

wild type and mutants for immunological cell labeling. The *lim3A′-lacZ* transgene labels the RP3 cell bodies strongly and their axons weakly; in addition, at least two other motoneurons (RP1, RP5) located closely to RP3 are labeled weakly (Thor et al., 1999; Wolf et al., 1998). Detection of *lacZ* positive axons at the CNS midline provides an estimate for the frequency of RP3's midline crossing (Fig. 1B,C).

### Retrograde labeling of motoneurons with DiI

We adopted the procedure of Landgraf et al. (1997) and used either the fixable or bright but non-fixable version of DiI (Cell Tracker CM-DiI and $DiI_{C18}$, Molecular Probes, Oregon, USA). To improve solubility, the fixable DiI (5 mg/ml) was heated to 60°C in a 1:10 mixture of ethanol:vegetable oil before use. Prior to applying DiI on a particular axon terminal, we confirmed the overall motoneuron innervation patterns by incubating dissected and fixed embryos overnight at 5°C with FITC-conjugated anti-HRP antibodies (Jackson Immuno Research Laboratories, Pennsylvania, USA; 1:100 dilution). We examined embryos at hour 17 of embryogenesis, since the muscles we studied are known to receive their first innervation by this time (B. Wolf and A. Chiba, unpublished data). Occasionally we labeled, in addition to RP3 or V, a specific later innervating motoneuron, MN6/7b or RP5, respectively. DiI labeling was performed on either the right or left half of a given abdominal (A2-A6) segment, but for clarity all figures are shown as if the dye had been applied on the right side.

### Immunocytochemistry

Immunocytochemistry with mAb 1D4 (source: Corey Goodman, University of California), anti-β-galactosidase (Promega), anti-Even-skipped (source: Chris Doe, University of Oregon), and anti-Fasciclin III (mAb 7G8, source: Developmental Studies Hybridoma Bank, University of Iowa) was performed as described (Broadus et al., 1995; Kose et al., 1997; Wolf et al., 1998). Double labeling with DiI and cell-specific antibodies (Fasciclin III or Even-skipped antibodies) was performed as follows. After DiI application (see above), the specimen was fixed with a standard 4% paraformaldehyde fixative for 1 hour, treated with 1 mM Triton X-100 for 10-20 minutes, and subjected to fluorescence immunocytochemistry.

## RESULTS

### *commissureless* mutation disrupts midline crossing by the RP3 and V motoneuron growth cones but their subsequent pathways are the mirror image of wild type

To examine specific cases in which those axons that normally cross the midline fail to do so, we chose to focus on the previously described *commissureless* (*comm*) mutant embryos (Seeger et al., 1993; Tear et al., 1996). The transmembrane protein Commissureless is a midline molecule, whose presence in midline glia normally allows certain axons to cross the midline through the commissures (Tear et al., 1996). In *comm* null mutants, immunovisualization reveals vastly reduced commissures (Seeger et al., 1993). It is thought that axons from neurons such as RP3 and V, which normally cross the midline, fail to cross in the absence of normal midline Commissureless expression. However, subsequent pathfinding by the affected axons has not been investigated.

We examined the pathfinding of RP3 and V in mutants lacking Commissureless expression at the CNS midline (see Materials and Methods). If normal midline decision-making were to play an essential role in priming growth cones for later navigation, as predicted by the 'continual reprogramming' model, we would expect axons to exhibit anomalous pathfinding following a disrupted midline decision.

As shown previously (Wolf et al., 1998), the overall organization of the nervous system outside the midline region is surprisingly similar to wild type and normal motoneuron innervation occurs on virtually all muscles (Table 1). However, we found that, contrary to earlier claims (Kidd et al., 1998b; Seeger et al., 1993; Tear et al., 1996), several axons can cross the midline in *comm* mutants (Fig. 1C). Moreover, cells near the midline are laterally dislocated (compare Fig. 1B,C), making it difficult to reliably identify any neurons based solely on their expected cell body positions. Therefore, to visualize RP3 and V, we adopted a retrograde labeling method in which lipophilic DiI is applied at the innervation site of each motoneuron (see Materials and Methods).

In wild type, DiI placed between muscles 6 and 7, the normal innervation site of the RP3 axon (Chiba and Rose, 1998), always reveals RP3, whose cell body arises across the midline (Figs 2A, 3A; Table 2). We could confirm the molecular identity of DiI-labeled neurons by counter-labeling with Fasciclin III antibodies (see Materials and Methods), which have been previously used as a marker for RP3 motoneurons (Halpern et al., 1991; Kose et al., 1997) (Fig. 2A).

In contrast, DiI applied at the same site in *comm* mutants frequently (75%) revealed one motoneuron from the ipsilateral side (Figs 2B, 3C; Table 2). Counter-labeling with Fasciclin III antibodies revealed that these DiI-labeled neurons were indeed

**Table 1. Immunovisualization of the motoneurons and their innervation**

| | | % of half-segments showing each aspect | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Genotype | *n* | Midline[a] | RP3[b] | RP2[c] | aCC[c] | 6/7[d] | 12[d] | 2[d] | 1[d] |
| Wild type | 55 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| *comm* [e] | 62 | 0 | 100[f] | 100[f] | 100[f] | 90 | 89 | 100 | 95 |
| *robo* | 51-89 | 100[h] | 100[g] | 100 | 100[g] | 73 | 76 | 90 | 98 |

Using various antibodies, we examined the presence of commissural tracts (Midline), cell bodies of RP3, RP2 and aCC, and the presence of axon terminals on muscles 6 and 7 (6/7) and 12 via SNb, and 2 and 1 via ISN (see Fig. 1A). Genetic background controls (heterozygotes) were essentially the same as wild type (not shown).

*n*, the number of half-segments examined for each aspect listed.

Antibodies used: [a]anti-HRP. Robust formation of commissures was detected in both wild type and *robo* mutants but was absent in *comm* mutants. There was evidence for occasional midline crossing by a few axons (see Fig. 1C). [b]mAb 7G8 (anti-Fasciclin III); [c]anti-Even-skipped; [d]mAb 1D4.

[e]Using a muscle specific genetic rescue (see Materials and Methods).

[f]Laterally displaced cell bodies.

[g]Medially displaced cell bodies.

[h]Partially fused midline.

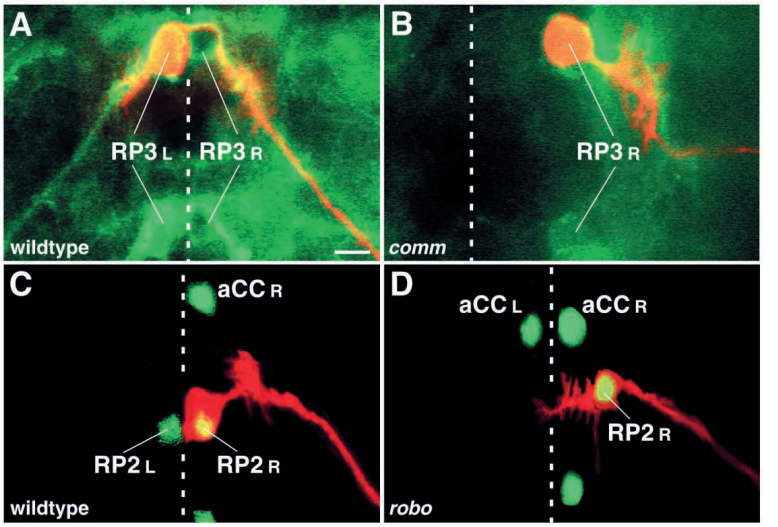


**Fig. 2.** Identities of DiI-labeled motoneurons as confirmed through counter-labeling with cell specific antibodies. (A,B) The immunovisualization of the cell surface protein Fasciclin III (green channel), normally expressed at high levels by the RP3 motoneurons, coincided with the motoneuron profile revealed by placing the fixable DiI (red channel) on RP3's normal targets, muscles 6 and 7 (see Materials and Methods for double labeling methods), in both (A) wild type ($n$=10 half-segments in four embryos) as well as (B) *comm* null mutant ($n$=12 half-segments in three embryos) embryos at hour 17. Double labeling demonstrates that the Fasciclin III positive RP3 axons in the mutants can either cross the midline similar to wild type (not shown) or stay ipsilateral (B). (C,D) The immunovisualization of the nuclear protein Even-skipped (green channel), normally expressed by the RP2 motoneurons, coincided with the motoneuron profile revealed by placing DiI (red channel) on RP2's normal target, muscle 2, in both (C) wild type ($n$=8 half-segments in three embryos) as well as (D) *robo* null mutant ($n$=16 half-segments in six embryos) embryos at hour 17. The antibodies also label the nuclei of the aCC motoneruons at this focal plane. Double labeling demonstrates that the Even-skipped positive RP2 axons in the mutants can either stay ipsilateral to the midline similar to wild type (D) or cross the midline and extend contralaterally (not shown). Bar, 2.5 μm.

ipsilateral RP3s (Fig. 2B). In every other case where counter-labeling was not possible, the cell body position of the DiI-labeled ipsilaterally projecting neuron is consistent with that of RP3, as revealed in embryos of the same genotype with known molecular markers, the *lim3A′-lacZ* strain or Fasciclin III antibodies (for example, compare to Fig. 1C). Having failed to cross the midline, these RP3 growth cones subsequently take the mirrored pathways normal for RP3 in wild type, continuing through the anterior nerve tract (ANt), lateral nerve exit point (ex) in the immediately posterior segment, the segmental nerve branch b (SNb), and onto muscles 6 and 7 (Fig. 3C). This suggests that RP3's failure to cross the midline does not alter its subsequent decisions. In the remaining 25% of *comm* mutants, DiI labeled the RP3 motoneuron across the midline (Fig. 2B; Table 2). Being slightly dislocated laterally, the cell body is still medial to the longitudinal connective and immediately beneath the perineural sheath. Similar to ipsilateral RP3 axons, subsequent pathways selected by the contralateral RP3 axons in *comm* mutants remained the same as wild type (compare Fig. 3A-C).

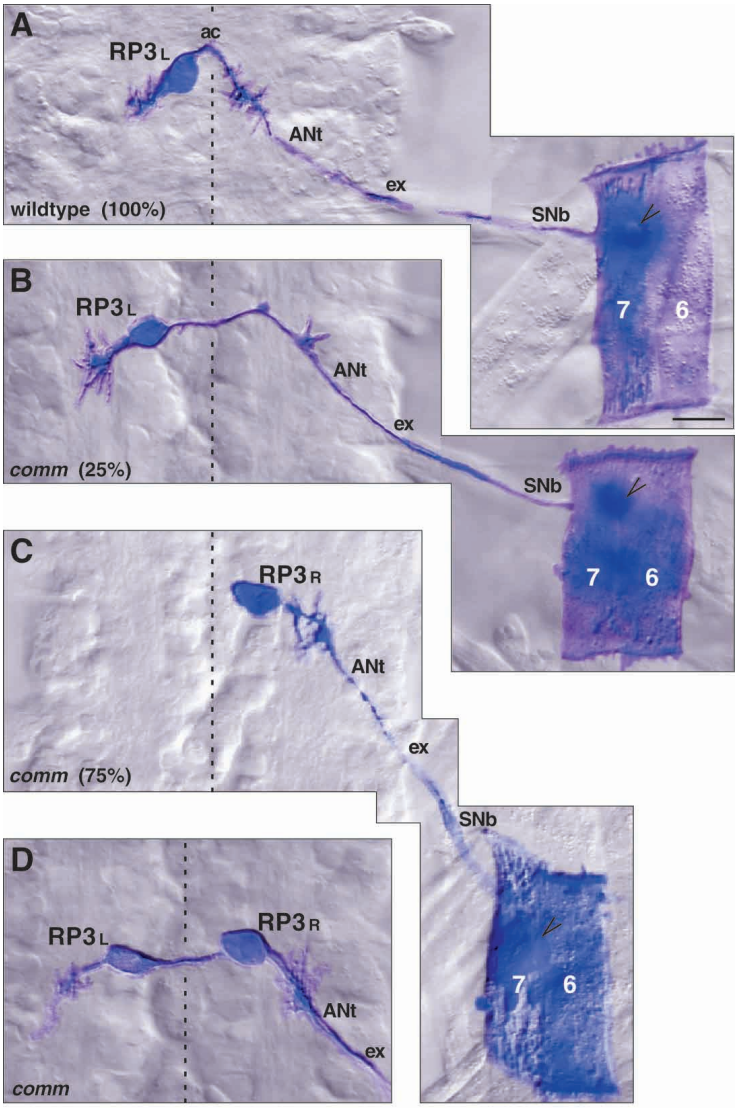


**Fig. 3.** RP3 axon pathfinding as revealed with DiI retrograde labeling in hour-17 embryos. (A) In wild type ($n$=37 half-segments in 14 embryos), the RP3 axon always follows consistent pathways: crossing the midline (broken line) through ac, proceeding along ANt, ex, SNb, and innervating muscles 6 and 7. In this and the following panels, the site of DiI application is indicated with a 'pipette tip', and the fluorescent (pseudo-colored) images revealing DiI-labeled motoneurons and the corresponding DIC (black and white) images showing axon pathways and surrounding muscles are digitally superimposed. (B-D) In *comm* mutants ($n$=55 half-segments in 15 embryos), the RP3 axon crosses the midline in 25% of the cases (B) but fails to do so in 75% (C). Regardless of its midline decision, in all cases the subsequent axon pathways remain the same as in wild type. DiI applied at one side of the body occasionally (five times) labeled the bilateral pair of RP3s, whose axons take the same pathways after the midline within a segment (D). Bar, 10 μm.

**Table 2. Single cell visualization of motoneuron growth cone pathways**

| | Route of motoneuron pathways (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RP3 | | | V | | | RP2 | | | aCC | | |
| Genotype | *n* | cross | guide | *n* | cross | guide | *n* | cross | guide | *n* | cross | guide |
| Wild type | 37 | 100 | 100 | 12 | 100 | 100 | 26 | 0 | 100 | 33 | 0 | 100 |
| *comm*[a] | 55 | 25 | 100 | 19 | 47 | 100 | n/a | n/a | n/a | 50 | 0 | 100 |
| *robo* | 18 | 100 | 100 | n/a | n/a | n/a | 43 | 19 | 100 | 38 | 3 | 100 |

Using DiI retrograde labeling methods, we scored both midline crossing (cross) and subsequent growth cone guidance accuracy (guide). The normal pathways for each motoneuron axons are: (RP3) ANt, lateral exit, SNb, muscle cleft 6/7; (V) ANt, lateral exit, SNb, muscle 12; (RP2) PNt, lateral exit, ISN, muscle 2; (aCC) ANt, lateral exit, ISN, muscle 1 (see Fig. 1A).

*n*, the number of half-segments examined.

[a]Using a muscle specific genetic rescue (see Materials and Methods).

Within the same genetic background, RP3's decision whether to cross the midline apparently is stochastic and mutually independent. We occasionally observed DiI placed on one side of the body labeling a bilateral pair of RP3s (Fig. 3D). These observations argue that RP3's ability to navigate specific pathways is not affected by the absence of normal midline experience and/or behavior.

The V motoneuron offers another example of an axon that normally crosses the midline (Fig. 1A). In 47% of *comm* mutants, DiI applied on muscle 12, V's normal innervation site, revealed a single motoneuron whose cell body position and axon pathways are virtually indistinguishable from that of wild-type V (compare Fig. 4A,B; Table 2). We also encountered cases (53%) in which one motoneuron arising from the ipsilateral but otherwise typical location for V was labeled (Fig. 4C; Table 2). As with RP3, subsequent pathways of this motoneuron were unaffected despite midline decisions, extending through ANt, ex, ISN and ending on muscle 12 (compare Fig. 4A-C). Thus, in *comm* mutants, both RP3 and V support the conclusion that neither the absence of a specific midline experience nor a failed midline decision alters subsequent pathfinding and targeting of growth cones.

### *roundabout* mutation causes abnormal midline-crossing of the RP2 and aCC motoneuron growth cones but their subsequent pathways are the mirror image of wild type

We next examined complementary situations in the *roundabout* (*robo*) mutants, in which axons that would normally extend away from the midline cross it abnormally (Kidd et al., 1998b; Seeger et al., 1993). The *robo* gene encodes a widely expressed growth cone receptor that not only interacts with Commissureless signaling (Kidd et al., 1998b; Seeger et al., 1993) but also responds negatively to another midline molecule, Slit (Brose et al., 1999; Kidd et al., 1999). We reasoned that the RP2 and aCC axons, which normally grow away from the midline (Fig. 1A), might abnormally cross it.

In *robo* mutants, we noted that RP2 and aCC cell bodies are dislocated (compare Fig. 1D,E). Despite that,

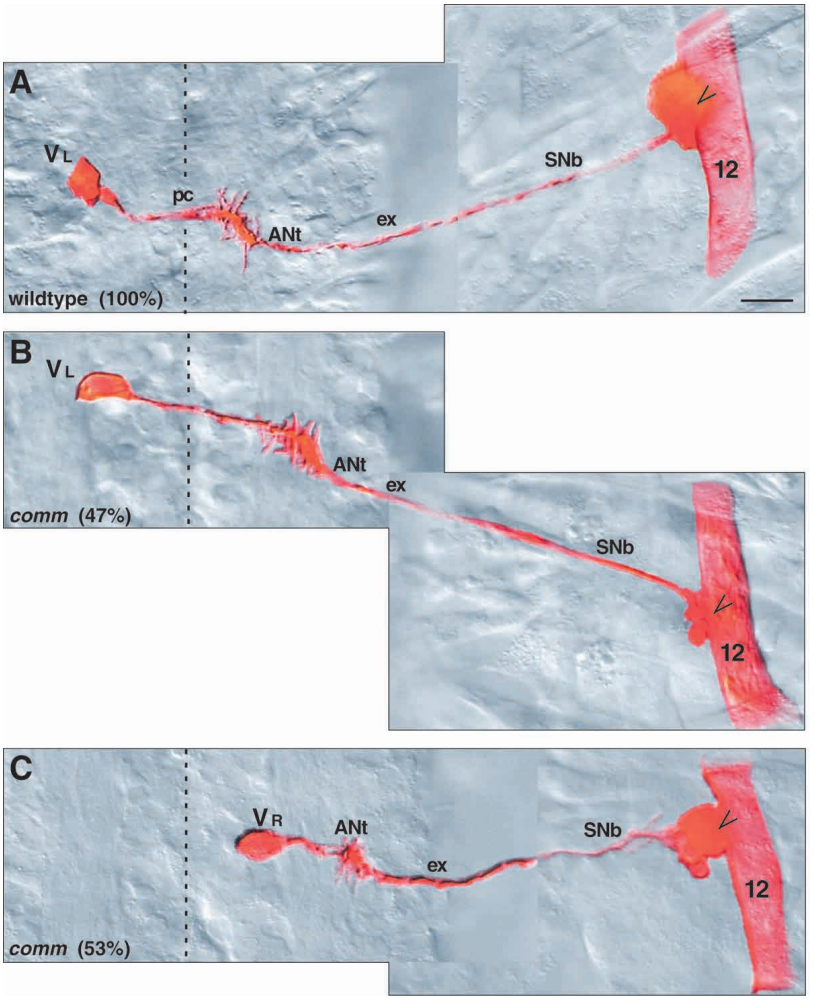


**Fig. 4.** V axon pathfinding as revealed with DiI retrograde labeling in hour-17 embryos. (A) In wild type (*n*=12 half-segments in nine embryos), the V axon always follows consistent pathways: crossing the midline (broken line) through pc, proceeding along ANt, ex, SNb and innervating muscle 12. (B,C) In *comm* mutants (*n*=19 half-segments in 13 embryos), the V axon crosses the midline in 47% of the cases (B) but fails to do so in 53% (C). Regardless of its midline decision, in all cases, the subsequent axon pathways remain the same as in wild type. Note that dissection as well as the body segment can influence an apparent positioning of the musculature with respect to the CNS. Bar, 10 μm.

immunovisualization with mAb 1D4 showed orderly motor nerves and, more specifically, wild type-like innervation on muscles 1 (98%) and 2 (90%) (Table 1).

Are muscles 1 and 2 innervated by the aCC and RP2 motoneurons in these mutants? And if so, do the axons come from ipsilateral or contralateral side? To directly examine the motoneurons that innervate these muscles in the mutants, we once again used the DiI retrograde labeling method. In wild type, both the RP2 and aCC motoneurons extend their axons away from their medially located cell bodies into the ipsilateral side of the CNS (Figs 2C, 5A; Table 2). In all cases examined, the RP2 axon, while staying entirely ipsilateral, first grows along the posterior nerve tract (PNt), and the aCC axon along ANt (Table 2). After joining at ex, the RP2 and aCC axons continue through the intersegmental nerve (ISN) and innervate muscles 2 and 1, respectively.

In *robo* mutants, we anticipated that if normal midline decisions were required for RP2 and aCC axons to correctly navigate their subsequent pathways, they would either fail to target muscles 2 and 1 or take abnormal paths to reach these muscles. DiI applied on muscle 2 in *robo* mutants labeled one motoneuron (Fig. 5B,C). Subsequent counterlabeling with Even-skipped antibodies, which label the RP2 cell bodies (Broadus et al., 1995), confirmed the DiI-labeled neurons as RP2s (Fig. 2D). In 81% of the cases, the RP2 axon did not cross the midline, just as with wild-type RP2 (compare Fig. 5A,B; Table 2). In the remaining 19%, the axon extended from the contralateral side (Fig. 5C; Table 2). In all cases, the subsequent axon pathfinding was exactly that expected for a normal RP2, growing through PNt, ex, ISN and onto muscle 2 (compare Fig. 5A-C). As with RP3 in *comm* mutants, RP2 in *robo* mutants showed cell autonomous midline decisions (Fig. 5D).

In the same *robo* background, we also observed a similar scenario with aCC, with one case of abnormal midline crossing but with unaltered subsequent pathfindings in all cases (*n*=38 DiI-labeled aCCs, Table 2).

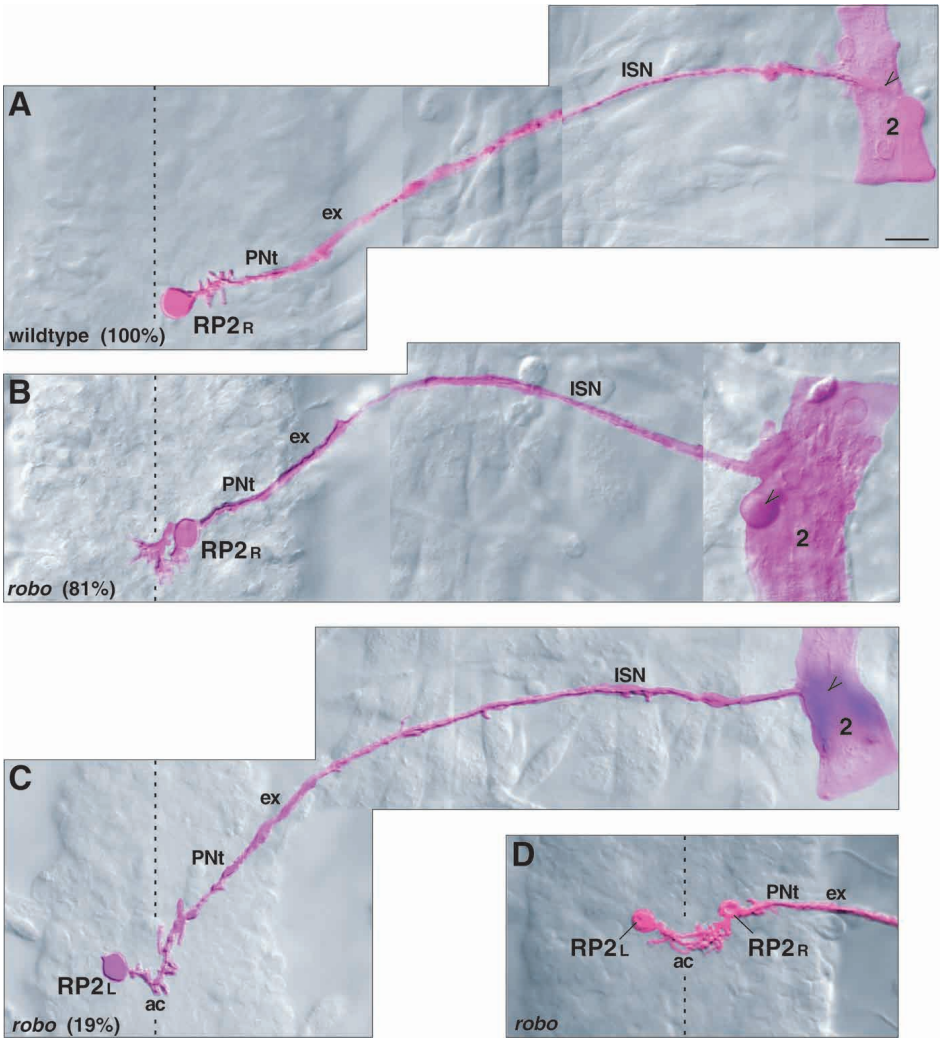


**Fig. 5.** RP2 axon pathfinding as revealed with DiI retrograde labeling in hour-17 embryos. (A) In wild type (*n*=26 half-segments in 18 embryos), the RP2 axon always follows consistent pathways: growing away from the midline (broken line), proceeding along PNt, ex, ISN and innervating muscle 2. (B-D) In *robo* mutants (*n*=43 half-segments in 24 embryos), the RP2 axon stays ipsilateral in 81% of the cases (B) but abnormally crosses the midline in 19% (C). Regardless of its midline decision, in all cases the subsequent axon pathways remain the same as in wild type. DiI applied on one side of the body occasionally (four times) labeled the bilateral pairs of RP2s, whose axons take the same pathways after the midline within a segment (D). Bar, 10 μm.

## DISCUSSION

### Growth cones are guided by an experience-independent preprogramming

In this study, we evaluated the relative importance of growth cones having a normal experience at the CNS midline in order to succeed in navigating beyond that point and following complex subsequent pathways. We analyzed the consequences of disrupting a specific midline signaling and/or growth cone decision at the midline. Our logic was that the growth cones would retain their ability to respond normally to all subsequent cues if they were relying on an experience-independent preprogramming (Fig. 6, Preprogram model). But, if their normal mode of operation were a continual reprogramming, and the final axon pathways were a result of a specific sequence of interactive reprogramming, missing the normal cues at the midline would lead to a subsequent deviation from the normal pathways at one point or another (Fig. 6, Reprogram model). We reasoned that by following the axon pathways of individual

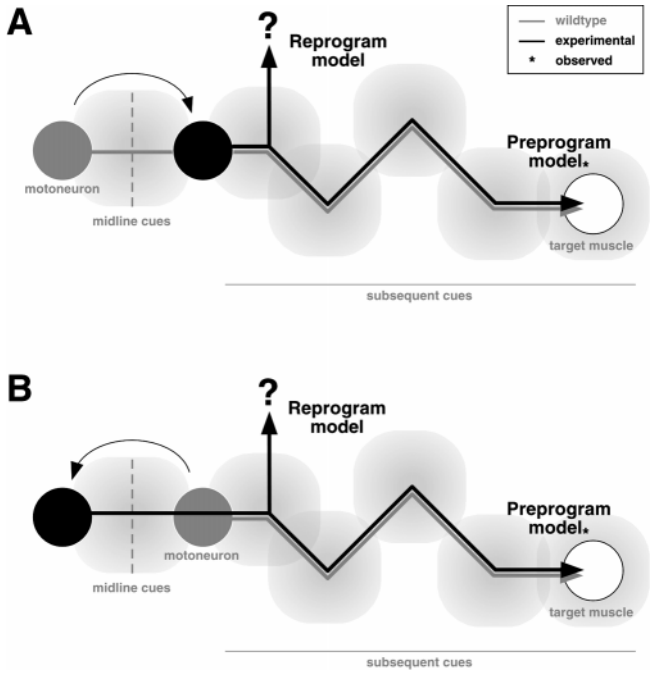


**Fig. 6.** Two models of growth cone re-adjustment in vivo and their different predicted outcomes in the experiments performed in this study. (A) In the first set of experiments, the *comm* mutation frequently prevented the RP3 and V motoneuron axons from crossing the midline. The 'experience-dependent continual reprogramming' model (Reprogram model) would predict that, after missing the normal midline cues and/or experience, the axon growth cone cannot exhibit the normal responsiveness towards the subsequent cues, eventually deviating from the normal pathways at some point later, if not immediately following the midline decision. In contrast, the 'experience-independent preprogramming' model (Preprogram model) would anticipate that the growth cone, despite the altered experience at the midline early on, remains perfectly normal following the rest of the subsequent cues. In all cases in which the midline crossing of the RP3 and V growth cones failed to occur, we observed that their subsequent pathways are the perfect mirror-images of those in wild type, the scenario predicted by the 'preprogram' model (asterisk). (B) In the second set of experiments, the *robo* mutation occasionally caused the RP2 and aCC motoneuron axons to abnormally cross the midline. This introduces the extra experience of growing through the midline, while the subsequent microenvironment remains the same as when the growth cone stays ipsilaterally. Again, the 'experience-dependent continual reprogramming' model (Reprogram model) would predict that, after abnormally crossing the midline and/or failing to have a normal experience at the midline, the axon growth cone cannot respond normally to subsequent cues, eventually deviating from the normal pathways at some point later, whereas the 'experience-independent preprogramming' model (Preprogram model) would anticipate that the growth cone still remains perfectly normal in responding to the subsequent cues. In all cases in which abnormal midline crossing of the RP2 and aCC growth cones occurred, we observed that their subsequent pathways were the perfect mirror images of those in wild type. The data add further support to the 'Preprogram' model (asterisk).

neurons affected by midline mutations, we would be able to start teasing apart these scenarios experimentally.

The first set of experiments with *comm* mutants provide cases in which growth cones are prevented from crossing the midline (Fig. 6A). However, due to the bilateral symmetry of the molecular and cellular organizations across the midline, the RP3 and V motoneuron growth cones, when they fail to cross the midline, still find themselves surrounded by the same microenvironment that they would normally experience after crossing the midline. The net effect is essentially equivalent to a cellular transplant across the midline (Fig. 6A, arched arrow). Without experiencing the Commissureless protein on the midline glia (Tear et al., 1996), and despite the abnormal decision to not cross the midline, these growth cones are nevertheless perfectly capable of responding normally to all subsequent cues, allowing them to follow the mirror-image peripheral pathways all the way to their respective target muscles and to initiate synaptogenesis there (Table 2).

The second set of experiments with *robo* mutants provide complementary cases in which growth cones are made to abnormally cross the midline (Fig. 6B), presumably due to either full or partial loss of growth cones' ability to respond to midline repulsion signals (Kidd et al., 1998a). The Roundabout protein is a widely expressed neuronal growth cone receptor (Kidd et al., 1998a), and its deletion offers a means to disrupt the midline decisions of growth cones independently of *comm* mutations. In all cases, when they cross the midline abnormally, the RP2 and aCC motoneuron growth cones retain normal responsiveness to all subsequent cues, selecting the mirror-image pathways and muscles on the other side of the midline (Table 2).

Our results clearly demonstrate that neither disrupted midline decisions nor a lack of midline signaling molecules affect the motoneuron growth cones' ability to respond normally to cues encountered beyond the midline. We conclude that, under the situations examined, the growth cones rely on an experience-independent preprogramming for their navigation through complex in vivo environments.

### Lack of extensive reprogramming may be common outside the midline

The midline of the CNS is one of the first microenvironments, and therefore often the first 'choice point', for axonal growth cones in the CNS. Subsequent to the midline, typical growth cone pathways include a number of distinct molecular microenvironments. Unfortunately, our knowledge is limited concerning the molecular guidance mechanisms in most other choice points, except for those for synaptic targeting in the periphery by motoneurons (Keshishian et al., 1996). Is the midline unique in that growth cones' particular experience there has very little impact on how they behave later at other choice points? Or, do choice points in general operate independently of each other?

Previous studies on the synaptic targeting of the RP3 motoneuron provide some hints about whether or not growth cones reprogram extensively when dealing with cues outside the CNS (Chiba and Rose, 1998). RP3 is known to respond positively to muscles 6 and 7, which express the cell surface molecule Fasciclin III, and forms synapses with these muscles (Chiba et al., 1995; Kose et al., 1997). Before reaching the muscles, the RP3 growth cone encounters proximal muscles that express another transmembrane molecule, Toll, which is thought to be an inhibitory molecule for RP3's synaptogenesis initiation (Rose et al., 1997). Under normal conditions, the growth cone would experience first Toll and then Fasciclin III.

However, when Fasciclin III is ectopically expressed by the proximal muscles, out of context and slightly out of timing, the RP3 growth cone can misinnervate those muscles which it normally ignores (Chiba et al., 1995). Conversely, when Toll is ectopically expressed on muscles 6 and 7, again out of context and off the normal timing, the growth cone is inhibited from initiating synaptogenesis just short of the normal innervation site (Rose et al., 1997). These data suggest that the exact order of experiencing Toll and Fasciclin III does not dictate the response which the RP3 growth cone exhibits towards these molecules in the musculature.

### Bilaterally paired neurons exhibit independent midline decisions

The four motoneurons studied here all come in bilateral pairs. In wild type the probability of each motoneuron sending its axon across the midline is either 100% (RP3 and V) or 0% (RP2 and aCC) (Table 2). In the mutants, the overall probability can vary considerably among the neurons, ranging between 3% (aCC in *robo* mutants) to 47% (V in *comm* mutants) (Table 2). This provides an interesting opportunity to ask whether a bilateral pair of axons in a given segment make concerted decisions regarding their midline crossing.

RP3s in *comm* mutants send their axons across the midline at an overall rate of 25%, as compared to 100% in wild type. We found a total of five cases in which an RP3 axon from one side of the CNS crosses the midline while its bilateral counterpart axon stays ipsilateral (Fig. 3D). Clearly it is not the case that when an RP3 axon on one side of the CNS decides to cross, its bilateral counterpart will also cross, or vice versa. Considering that DiI application methods do not always successfully label all neurons that innervate particular muscles, such observations are consistent with the conclusion that bilaterally paired neurons make their individual midline decisions stochastically and independently of each other.

Further support comes from the additional observation that bilaterally paired RP2 axons in *robo* mutants also are seen (four times in total) to co-innervate the same muscle 2 on one side of the body (Fig. 5D). Thus, the situation is not unique to either RP3 or the *comm* mutation.

### Why does growth cone guidance depend on preprogramming?

Our study showed that a growth cone can proceed with appropriate responsiveness toward subsequently presented guidance cues even after there are specific distractions in the normal molecular cues or a correct decision at the midline. Such results surprised us since cell-cell interactions and experience-dependent plasticity are common during neural development (Bhat, 1998; Chen and Tonegawa, 1997; Katz and Shatz, 1996; McConnell, 1995). Is growth cone guidance one exceptional period during which little experience-dependent genetic programming is at work?

Most examples of known neuronal fate readjustments come from either earlier periods of precursor differentiation or later periods of synaptic remodeling (Bhat, 1998; Chen and Tonegawa, 1997; Katz and Shatz, 1996; McConnell, 1995). In contrast, during axogenesis, in vivo evidence so far is scarce for experience-dependent growth cone reprogramming being the essential mechanism of defining any specific axon pathway. There are demonstrations that transplanted motoneurons can still seek out appropriate muscle tissues even when they initially make de novo pathway decisions (Eisen et al., 1986; Landmesser, 1992), generally consistent with our study. Thus, the type of growth cone readjustments supported by the available data is limited. In bilateral animals, a growth cone's initial encounter with the midline cues somehow desensitizes its attraction towards the midline (Shirasaki et al., 1998). However, such a desensitization has only the limited effect of selectively readjusting growth cone responsiveness against specific cues already encountered but not against other cues that are seen later.

What could be the advantage of genetic preprogramming of growth cone responsiveness? Is controlling axon guidance not a challenge best dealt with by continual genetic reprogramming in order to respond interactively to a constantly changing cellular environment? The reason may have to do with uncompromising speed and also the high degree of accuracy required for axon pathfinding in vivo.

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