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The Development and Subsequent Elimination of Aberrant Peripheral Axon Projections in *Semaphorin3A* Null Mutant Mice

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Semaphorin3A (previously known as Semaphorin III, Semaphorin D, or collapsin-1) is a member of the semaphorin gene family, many of which have been shown to guide axons during nervous system development. Semaphorin3A has been demonstrated to be a diffusible chemorepulsive molecule for axons of selected neuronal populations *in vitro*. Analysis of embryogenesis in two independent lines of *Semaphorin3A* knockout mice support the hypothesis that this molecule is an important guidance signal for neurons of the peripheral nervous system (M. Taniguchi *et al.*, 1997, *Neuron* 19, 519–530; E. Ulupinar *et al.*, 1999, *Mol. Cell. Neurosci.* 13, 281–292). Surprisingly, newborn *Semaphorin3A* null mutant mice exhibit no significant abnormalities (O. Behar *et al.*, 1996, *Nature* 383, 525–528). In this study we have tested the hypothesis that guidance abnormalities that occurred during early stages of *Semaphorin3A* null mice development are corrected later in development. We have found that the extensive abnormalities formed during early developmental stages in the peripheral nervous system are largely eliminated by embryonic day 15.5. We demonstrate further that at least in one distinct anatomical location these abnormalities are mainly the result of aberrant projections. In conclusion, these findings suggest the existence of correction mechanisms that eliminate most sensory axon pathfinding errors early in development. **© 2000 Academic Press**

Key Words: **DRG; axon guidance; Semaphorin3A.**

INTRODUCTION

Neuronal growth cones navigate along specific pathways to find their correct targets. The behavior of the growth cones is believed to be guided by attraction and repulsion mechanisms (Tessier-Lavigne and Goodman, 1996). The development of *in vitro* assays led to the identification of a large number of guidance molecules (Muller *et al.*, 1996). One such example of an axon guidance molecule is Semaphorin3A (Sema3A) (Kolodkin *et al.*, 1993; Luo *et al.*, 1993). Sema3A, a member of the type 3 secreted semaphorins, selectively repels the growth cones of different populations of neurons including subsets of dorsal root ganglia (DRG) cells and spinal motor axons *in vitro* (Luo *et al.*, 1993; Messersmith *et al.*, 1995; Puschel *et al.*, 1996; Varela-Echavarria *et al.*, 1997). The peripheral expression of *Sema3A* mRNA combined with its *in vitro* inhibitory activity suggests that the *in vivo* actions may produce an axon exclusion zone. This zone may channel peripheral axon projections to their topographically correct targets (Giger *et al.*, 1996; Wright *et al.*, 1995).

The role of Sema3A *in vivo* has been studied using two independent *Sema3A* null mouse lines (Behar *et al.*, 1996; Taniguchi *et al.*, 1997). Consistent with the mRNA distribution of *Sema3A* and its activity *in vitro*, Taniguchi and colleagues found that in the absence of *Sema3A*, severe abnormal peripheral projections are found at early stages of embryonic development (E12) (Taniguchi *et al.*, 1997). In sharp contrast, no peripheral projection abnormalities were detected in the postnatal peripheral ner-

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vous system of the line of *Sema3A* null mice analyzed by Behar and colleagues (Behar *et al.*, 1996). Based on these apparent differences, and the fact that both lines of *Sema3A* null mice have similar target deletions, we have hypothesized that the aberrant axonal projections in *Sema3A* null mice are generated during early development, but are eliminated in later stages of development. In this study we have tested our hypothesis by studying the degree of peripheral nervous system abnormalities in different stages of *Sema3A* mice development. Using retrograde-labeling and immunocytochemical analysis, our results demonstrate that many pathfinding errors in *Sema3A* null mutant mice at E11.5 are eliminated by E15.5. These results suggest the existence of an as yet unknown "oversight" mechanism capable of correcting early errors in axonal pathfinding.

METHODS

Animals

The procedures for the generation and genotyping of the *Sema3A* null mutants used in the present study have been described elsewhere (Catalano *et al.*, 1998). Timed embryos were obtained by overnight mating of heterozygotes. The morning on which the vaginal plug was observed has been considered embryonic day 0.5. Pregnant females at different stages of gestation (E11.5, 13.5, 15.5) were sacrificed by cervical dislocation and embryos (n = at least 6 per embryonic day) were processed for immunocytochemistry; immersion-fixed in 3% paraformaldehyde/15% picric acid in 0.1 M phosphate buffer, pH 7.4, for 2 h; or fixed with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for lipophilic tracer labeling. Embryos destined for immunocytochemistry were then placed in 30% sucrose overnight and frozen in OCT embedding medium on dry ice. Embedded embryos were serially sectioned transversely at 20 μm through the hindlimb region, mounted on SuperFrost/Plus slides (Fisher), and allowed to air-dry.

Lipophilic Tracer Labeling

Following fixation, small crystals of DiI (1,1′-dioctodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR) were placed into either lumbar DRGs or distal hindlimb (n = 3) with the aid of a dissecting microscope. Labeled embryos were then stored in 10% formalin at 37°C for 1–5 weeks. Embryos were then embedded in agar, and transverse sections (125 mm) were obtained using a Vibratome. Sections were mounted on slides in 0.05 M phosphate buffer, viewed with a rhodamine filter, and photographed with a Nikon Eclipse800 photomicroscope. The protocol for photoconversion of DiI signal has been previously described (Ozaki and Snider, 1997).

Immunohistochemistry

Tissue sections were incubated for 1 h in a blocking solution consisting of SuperBlock buffer (Pierce Chemical Company, Rockford, IL), 0.3% Triton X-100, 1.0% porcine gelatin, and 1.5% normal serum. This solution was used for the dilution of both primary and secondary antibodies. The polyclonal antibody against protein gene-related peptide 9.5 (PGP9.5; Accurate Science, Westbury, NY) was used at a dilution of 1:1000. Rtk-Ex polyclonal antibodies generated against the extracellular domain of TrkA (Clary *et al.*, 1994) were generously provided by Dr. Louis F. Reichardt and used at a dilution of 1:5000. Sections were incubated in the primary antibody overnight (12–16 h). Slides were then washed three times (5 min each) with PBS and visualized with a Cy3-labeled secondary antibody (goat-anti-rabbit IgG; Amersham) and coverslipped.

Quantification of Lateral Projection Axon Defects

Two blinded observers performed the analysis of the age-dependent elimination of aberrant lumbar DRG projections in *Sema3A* null mutant embryos. Embryos from two time points (E11.5, n = 4; and E15.5, n = 5) were used in this analysis. For quantification of lateral projection defects, entire L4 and L5 DRGs of E11.5 and E15.5 null mutant embryos were sectioned and the number of aberrant DRG projections per DRG was determined. L4/L5 DRGs were serially sectioned in a coronal plane at 20 μm, stained with the antibody against PGP9.5, and analyzed for abnormal lateral axon projections (see inset of Table 1). A score of (+) or (−) was assigned to each section of DRG. In addition to an assigned score of (+) or (−), the number of defects seen observed per DRG was also tallied. Only sections with both a clear DRG profile and a lateral projection from the DRG were scored as abnormal. When abnormal axons appeared in the same general location on adjacent serial sections of tissue, the axons were scored as one lateral projection defect. We attempted to control for the variation of DRG size differences at E11.5 and E15.5 by first measuring the overall size of the DRGs present in the lumbar region. It was determined that at E11.5, 20 sections per DRG are sufficient, and 50 sections per DRG are sufficient at E15.5.

RESULTS

Development of DRG Aberrant Axons in Sema3A Null Mutants

In a previous study of *Sema3A* null mice it has been shown that peripheral axon projection abnormalities are detectable in E12 embryos (Taniguchi *et al.*, 1997). Since no abnormalities were found in our line of *Sema3A* null mice analyzed after birth (Behar *et al.*, 1996), we set out to determine whether peripheral axon projection abnormalities are also found in early developmental stages in this line of *Sema3A* null mice. The projection of lumbar DRG primary afferent axons in both wild-type (+/+) and *Sema3A* null mutant (−/−) littermates was examined at E11.5, E13.5, and E15.5. DRG axons were visualized in both mutant and wild-type embryos by means of DiI labeling and immunocytochemistry using polyclonal antibodies against the neurotrophin receptor TrkA and PGP9.5, which identifies neurons and associated axons. At E11.5, wild-type embryos labeled with either DiI (Fig. 1A) or TrkA immunoreactivity (Fig. 1D) exhibited a characteristic peripheral nerve trajectory, with dorsal, ventral, and splanchnic rami extending from the nerve. DRG axons also extended to the dorsal root entry zone of the spinal cord but did not yet penetrate spinal gray matter (Fig. 1D, arrowhead).

The corresponding analysis of *Sema3A* null mutants at

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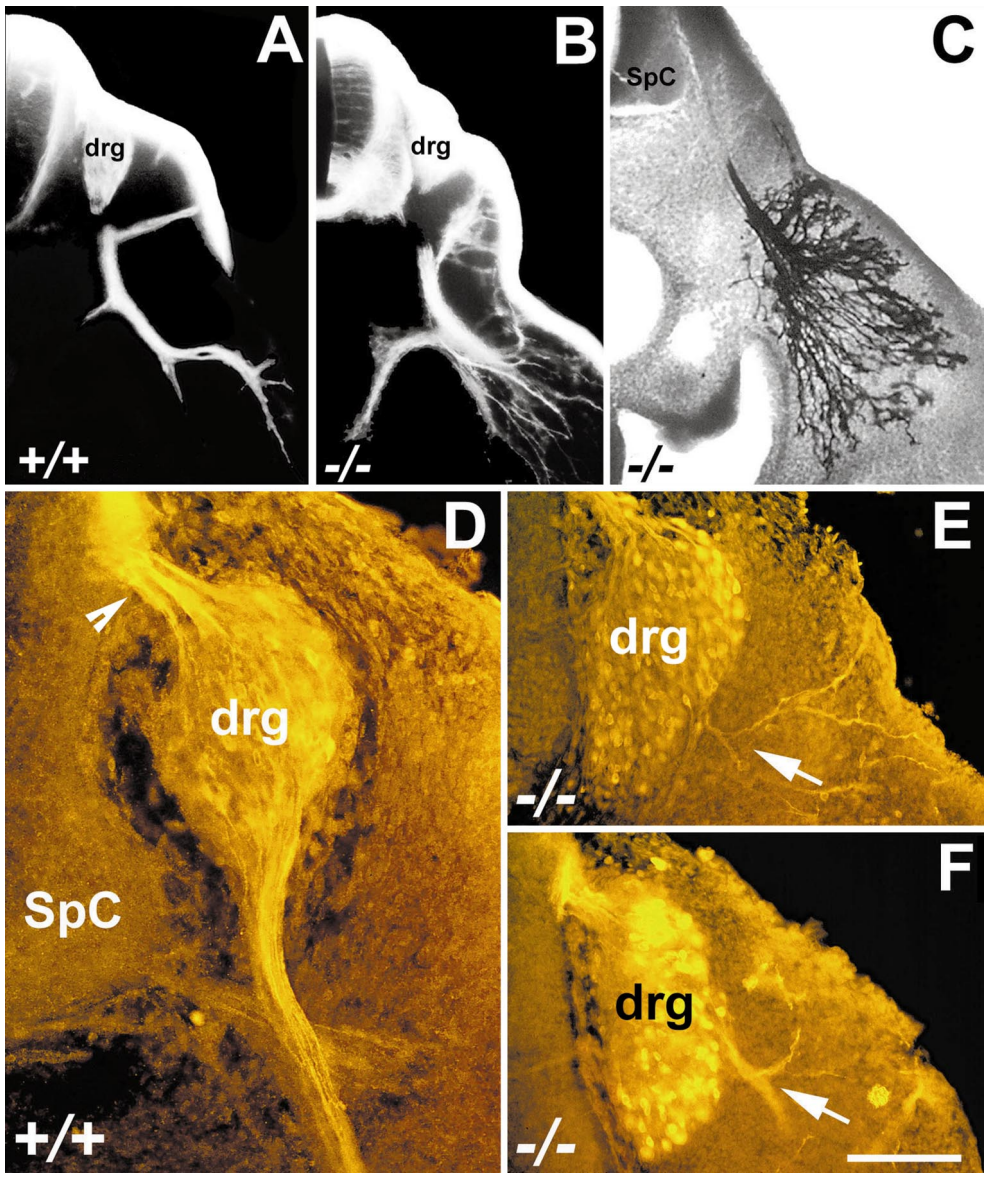

FIG. 1. Photomicrographs of DRG axon projections in E11.5 *Sema3A*+/+ and *Sema3A*−/− mouse embryos using both DiI and an antibody against TrkA. (A) *Sema3A*+/+ embryos exhibit specific peripheral spinal nerve branches that include dorsal, ventral, and autonomic rami of spinal neurons. (B) *Sema3A*−/− embryos exhibit multiple defasciculations and spurious branching of both dorsal and ventral rami of spinal nerves. It is of interest that the splanchnic branch projecting to visceral targets does not appear to exhibit aberrant axons. (C) Photoconversion of DiI-positive axons in a *Sema3A*−/− embryo. Note the degree of distal axon defasciculation present. (D–F) Axons of *Sema3A*+/+ and *Sema3A*−/− embryos immunoreactive for antibody against TrkA and labeled with the fluorochrome Cy3. This antibody labels large numbers of cells in the DRG in addition to both peripheral and central axon projections. (D) Sema3A+/+ embryos exhibit tightly fasciculated peripheral processes and central processes extending to the dorsal root entry zone (arrowhead). (E and F) *Sema3A*−/− embryos labeled with antibody against TrkA display abnormal lateral projections (arrows) extending through the developing vertebral arch and somitic tissue toward the skin. The dorsal root entry zone, in contrast, appears normal. Structures are labeled as follows: spinal cord (SpC), dorsal root ganglion (drg). Scale bar, 2 mm in A and B, 3 mm in C, and 300 μm in D–F.

E11.5 displayed a major segmental projection of axons ventrally from the DRG into a common spinal nerve, but with multiple defasciculations in the regions of the dorsal and ventral rami (Figs. 1B and 1C). DiI-positive axons at E11.5 (Figs. 1B and 1C) exhibit a large degree of axon defasciculation with multiple aberrant axon projec-

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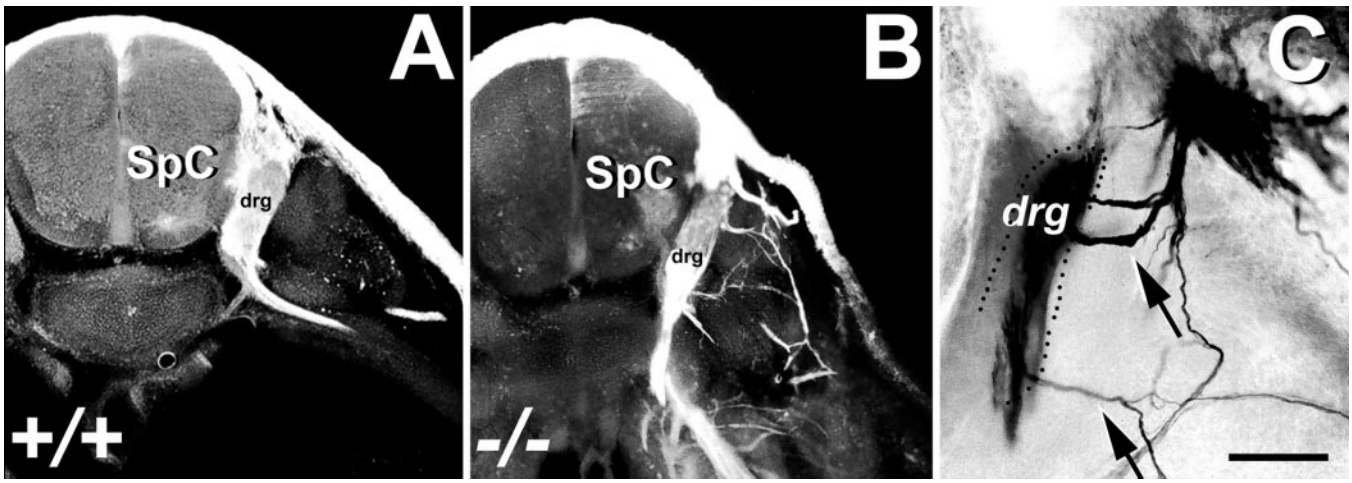

FIG. 2. Photomicrographs of DiI-labeled DRG axon projections present in E13.5 *Sema3A*+/+ and *Sema3A*−/− embryos. (A) *Sema3A*+/+ embryo exhibits a discrete spinal nerve projecting from DRG. (B) *Sema3A*−/− embryos exhibit multiple fascicles extending from the spinal nerve, in addition to lateral DRG projections through somitic tissues. (C) Photoconversion of DiI-labeled axons (arrows) in a *Sema3A*−/− embryo projecting through prevertebral arch (scale bar, 1 mm in A and B and 400 μm in C).

tions into the surrounding dermamyotome in null mutant mice. Most defasciculated axons of the dorsal rami extend in a fan-like projection pattern toward the skin (Fig. 1C). Many aberrant projections that were observed in *Sema3A* null mutants were restricted to territory distal to the junction of ventral root and spinal nerve. In many sections, TrkA-immunoreactive axons (Figs. 1E and 1F) were observed projecting through the precartilage primordium of the vertebral arch immediately lateral to the DRG. These fiber bundles represent a minority of aberrant projections.

In E13.5 *Sema3A* null embryos, multiple nerve branches of varying sizes are seen throughout somite-derived tissues (Fig. 2B). These projections differ considerably from the individual, discrete rami seen in wild-type embryos (Fig. 2A). Multiple and often sizable fascicles of DRG axons are seen to project laterally from the DRG through the developing bone of the vertebral arch (Figs. 2B and 2C). These distinct DRG axon projections appear to observe a series of conserved exit points (Fig. 2C, arrows). In addition, the E13.5 null mutant mice (Fig 2B) also exhibit the massive defasciculation in the distal spinal nerve similar to the E11.5 Sema3A null.

Elimination of Aberrant Axon Projections in Sema3A Null Mutants

In order to test whether misguided axons found at E11.5–E13.5 in *Sema3A* null mice are eliminated in later stages of development, we have compared the projection pattern of the latter with those of E15.5 *Sema3* null mutant embryos. When the *Sema3A*+/+ embryo is compared with *Sema3A*−/− littermates, it was noted that peripheral projections of DRG neurons at E15.5 in *Sema3A* null embryos (Figs. 3B–3E) appear to lack many of the aberrant trajectories present at earlier time points. Few axon abnormalities were noted in embryos sectioned through thorax and lumbar regions (Fig. 3B). A subsequent semiquantitative analysis of aberrant lumbar DRG axons present in *Sema3A* null embryos determined that there is a 2.8-fold reduction in the number of lateral projection defects in lumbar DRGs between E11.5 (n = 4) and E15.5 (n = 5). In addition, there is a 6.1-fold reduction in the number of lateral projections defects per lumbar DRG between E11.5 and E15.5 (see Table 1).

Collateral Branching or Abnormal Axonal Projections?

Using DiI injections applied directly to DRG of *Sema3A* null embryos we documented dramatic defasciculations of distal axons at E11.5–E13.5 (see Figs. 1 and 2). These defasciculations may be the result of collateral axon branching or the result of premature defasciculation of axon bundles. To assess which of the two possibilities is the cause of the defasciculations detected, we used a DiI bulk-labeling strategy in the distal hindlimb of both wild-type and null mutant embryos at E13.5. This method was successful at labeling the majority of distal sciatic nerve axon projections, associated cell bodies in DRG and ventral spinal cord, central axon projections, and presumably the presence of any collateral branches.

At E13.5 DiI-labeled hindlimb axons of wild-type embryos exhibited patterns of projections similar to those documented in embryos (E11.5 and 13.5) with DiI-labeled DRGs (Figs. 4A and 4B; also see Figs. 1A and 1D). The

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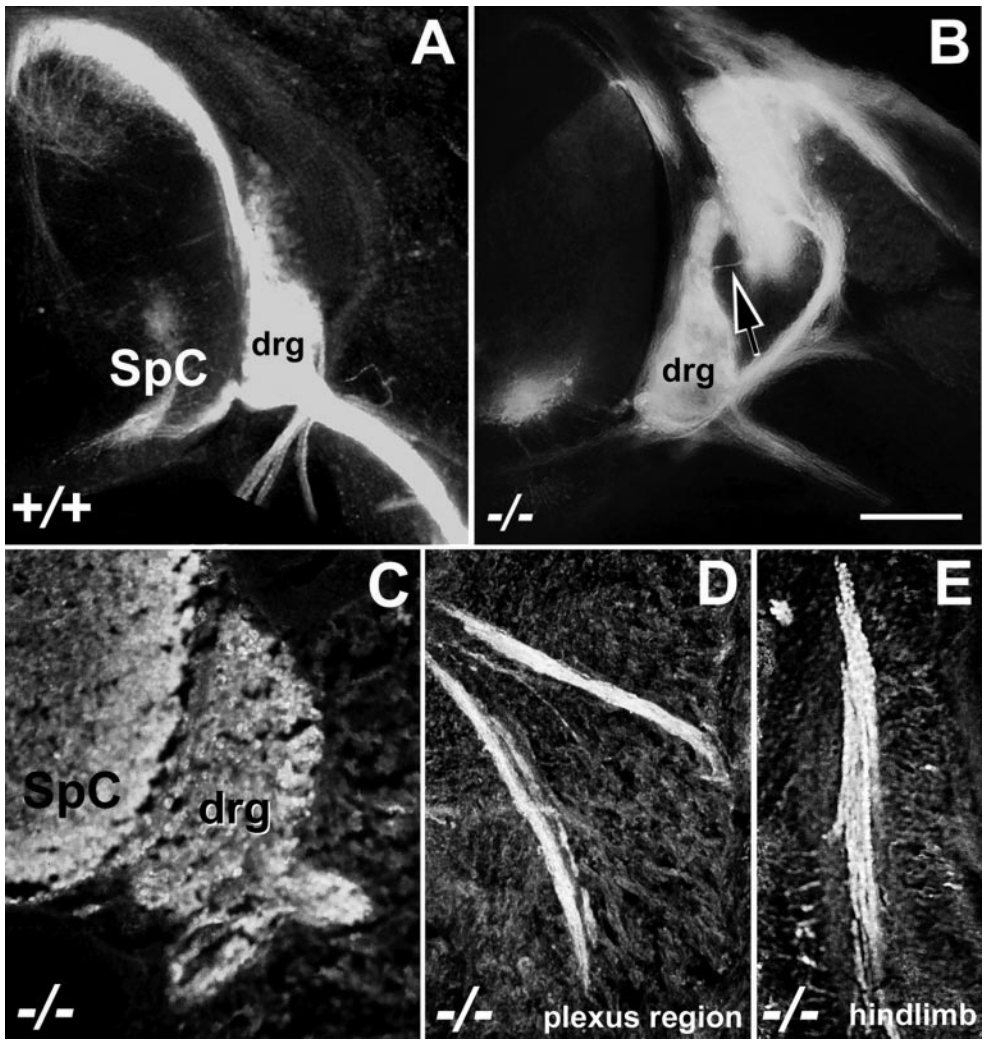

FIG. 3. Photomicrographs of peripheral nerve and associated axons present in E15.5 *Sema3A*+/+ and *Sema3A*−/− embryos. (A) DiI-labeled DRG axon projections in *Sema3A*+/+ and (B) *Sema3A*−/− embryos. Note the reduced number of aberrant lateral projections emerging from DRG (arrow). (C) Photomicrographs of lumbar DRG axon projections in E15.5 *Sema3A*−/− mouse immunostained with anti-PGP9.5 to identify neurons and associated axons. E15.5 *Sema3A*−/− mouse exhibits normal spinal and splanchnic nerve branches. Distal projections present in (D) lumbar plexus and (E) hindlimb exhibit tightly fasciculated axon bundles that lack aberrant axon projections. This pattern of peripheral axon distribution does not differ from that of wild-type littermates (scale bar, 400 μm A and B, 200 μm in C–E).

spinal nerve in *Sema3A* null mutant mice appears largely similar to those present in the wild-type embryos (Figs. 4C and 4D), although rare instances of proximal axon branches were observed (data not shown). These findings are in sharp contrast to the abnormal organization of the spinal nerve as documented in embryos (E11.5 and 13.5) with DiI-labeled DRGs (Figs. 1B, 1C, and 2B). The absence of proximal axon branches in this location suggests that the abnormal defasciculation in these locations is not a result of collateral sprouting. However, labeled sciatic nerve axons in the proximal hindlimb may represent potential branching (Fig. 4C).

DISCUSSION

In this study we found that at early stages of peripheral axon outgrowth, there is a high degree of guidance abnormalities in *Sema3A* null mutant mice. The sites of pathfinding errors are consistent with the peripheral mRNA distribution of *Sema3A* (Giger *et al.*, 1996; Wright *et al.*, 1995). Despite the numerous axon projection errors, there is a significant population of peripheral axons that exhibit normal projection patterns. The abnormal anatomy of the spinal nerve that was revealed in this study is mainly the result of true pathfinding errors. Surprisingly, aberrant

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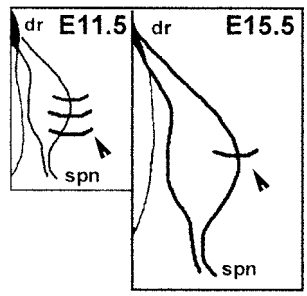
TABLE 1
Percentage and Average Number of Lateral Axon Projection Defects Exhibited by *Sema3A*−/− Lumbar DRGs at E11.5 and E15.5

Percentage of lumbar DRGs with lateral projection defects	E11.5 −/−	E15.5 −/−
Number of lumbar DRGs	16	18
Number of DRGs with defects	15	6
Percentage of DRGs with defect	93.75%	33.33%

Average number of lateral projection defects per lumbar DRG	E11.5 −/−	E15.5 −/−
Number of lumbar DRGs	16	18
Average number of defects per DRG (±SEM)	3.375 ± 1.45	0.55 ± 0.74



Note. dr—dorsal root; spn—spinal nerve; arrowhead indicates lateral projection defect.

projections present in the *Sema3A* null mutant at E11.5 and E13.5 are largely eliminated by E15.5. The transient nature of the majority of abnormal projections implies the existence or the activation of compensatory mechanisms capable of eliminating many of the sensory axon pathfinding errors occurring during development.

Correction of Abnormal Sensory Projections

During development, many central and peripheral nervous system axons extend to the vicinity of their appropriate target region in a highly stereotypic and directed manner, making very few navigation errors albeit lacking the topographic precision of mature connections (Goodman and Shatz, 1993). It has been shown that as part of the normal axonal patterning in the mammalian central nervous system, there is a selective elimination of primary axon segments and collateral branches during a discrete period of development (O'Leary and Koester, 1993). This selective elimination has been shown to be the result of axon pruning and not cell death (O'Leary and Koester, 1993).

Axon pruning is not limited to the developing central nervous system. Injured adult muscle fibers induce sprouting from nearby nerve terminals of uninjured motoneurons. However, all collateral sprouting by nearby uninjured motoneuron axons is rapidly resorbed once the injured muscle fiber is reinnervated by the original injured motor axon (see review by Sanes and Lichtman, 1999). This evidence suggests that the pruning mechanism, which is in operation after muscle fiber injury, may also pervade during the development of the peripheral nervous system. In this case, it is possible that the elimination of abnormal projections in *Sema3A* null mutants is the result of this constitutive pruning mechanism. Alternatively, there may be compensatory induction of pruning in response to the *Sema3A* mutation.

Another mechanism of correction is based on cell death. During development of the peripheral nervous system, large numbers of neurons are eliminated by naturally occurring cell death (see review by Oppenheim, 1991). To survive this developmental period of apoptosis, sensory neurons depend on neurotrophins generated by peripheral targets. Subsequently, most neuronal loss during development has been attributed to a limited availability of neurotrophic factors at the target site. More recent findings revealed that DRG neurons are dependent on neurotrophins prior to final target innervation (see review by Reichardt and Fariñas, 1997). In addition, many neurons switch their neurotrophin dependency to another neurotrophin expressed at the target site once they arrive in their target field (see review by Davies, 1997). It is therefore possible that an inadequate exposure to trophic support along the growth path to the target field can lead to programmed cell death of the majority of the misguided DRG neurons.

This possibility is supported by recent findings in the central nervous system. The spinal cord's floor plate is an intermediate target for developing commissural neurons. The floor plate is known to secrete the chemoattractant molecules netrin 1 and 2 (Kennedy *et al.*, 1994). Recent findings have also demonstrated that the floor plate also contains a survival factor for commissural neurons (Wang and Tessier-Lavigne, 1999). Later in development, these neurons switch their survival requirements to a new survival factor generated by their target. Commissural neurons that were not exposed to the survival factor generated by their intermediate target did not survive (Wang and Tessier-

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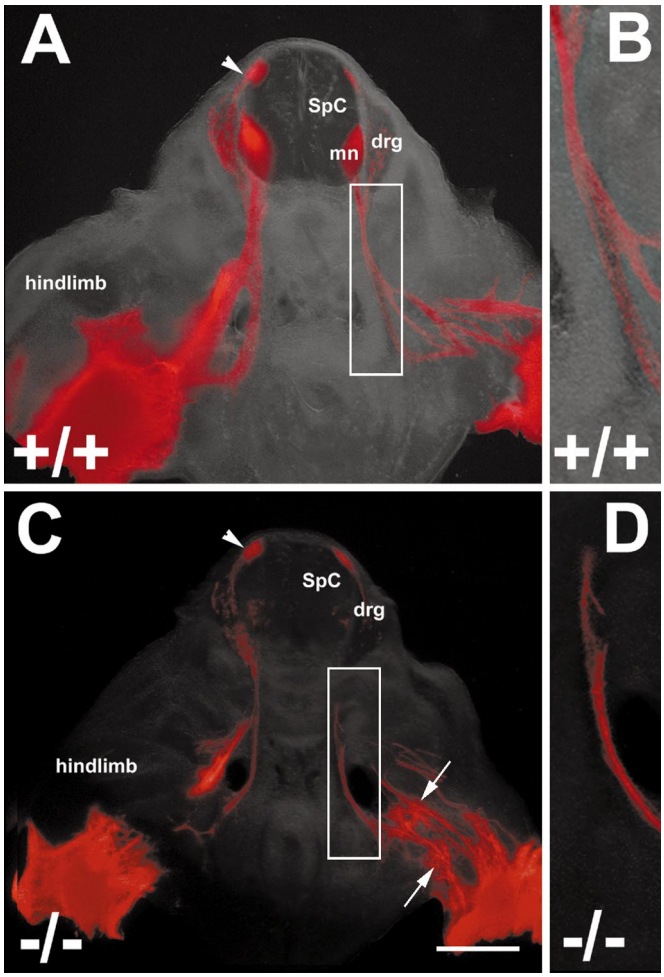

FIG. 4. Photomicrographs of DiI bulk-labeled peripheral axons, DRG, motor neurons, and central projections (arrowhead) present in (A) E13.5 *Sema3A*+/+ and (C) *Sema3A*−/− embryos. (B) Enlargement of DiI-labeled spinal nerve projections in *Sema3A*+/+, which display fasciculated axons that are similar to those seen in DRG DiI-labeled tissue in Fig. 2B. (D) *Sema3A*−/− embryos also display fasciculated spinal nerve axons. Potential collateral branching in sciatic nerve region (arrows). Structures are labeled as follows: spinal motor neurons (mn), spinal cord (SpC), dorsal root ganglion (drg). Scale bar, 400 μm in A and C, 200 μm in B and D).

Lavigne, 1999). Therefore, survival factor switching between the intermediate target and the final target has been proposed as a mechanism for eliminating misguided neurons (Wang and Tessier-Lavigne, 1999).

Considering the commissural neuron example and the neurotrophic switch of DRG neurons, it is conceivable that the pathfinding abnormalities that are corrected in the *Sema3A* null mice are based on cell death. In *Sema3A* null mice, misguided axons may not be exposed to either the appropriate amount or type of trophic support which is necessary to retain target innervation; subsequently, neurons with misguided axons would undergo apoptotic cell death. However, it is extremely difficult to prove that this is the dominant mechanism responsible for the elimination of misguided axons as there is extensive naturally occurring cell death in the DRG (E11.5–E15.5) during the period of misguided axon elimination (White *et al.*, 1998). Consistent with this view, preliminary experiments evaluating degree of DRG cell death in *Sema3A* null mutant mice did not reveal a significant increase in the numbers of apoptotic neurons (data not shown).

Pathfinding Errors or Premature Branching?

During the developmental stages by which DRG neurons send their projections to their peripheral target, it has been shown that *Sema3A* mRNA is localized around the DRG and the spinal nerve (Giger *et al.*, 1996; Wright *et al.*, 1995). Together with the inhibitory properties of Sema3A on DRG neurons *in vitro* at this stage of development (Messersmith *et al.*, 1995; Puschel *et al.*, 1996), it has been hypothesized that the role of Sema3A is to guide DRG axons to their correct targets. Studies of sensory projections in *Sema3A* null mice (Figs. 1 and 2) revealed a significant defasciculation of peripheral projections. The defasciculation of axons observed can be the result of preexposure of the developing axons to chemoattractant signals and/or branching factors. To differentiate between the two possibilities, we have tested the anatomy of the spinal nerve as it is revealed by DiI labeling of the DRG in comparison to bulk labeling of the sciatic nerve itself. If the massive defasciculation detected by labeling the DRG is a result of collateral branching, labeling of axons at the periphery should label abnormal branches in the proximal spinal nerve region. In contrast, if the defasciculation is a result of true abnormal guidance errors, DiI labeling of the spinal nerve from the periphery should show a normal spinal nerve. Using this approach we were able to demonstrate that the majority of projection abnormalities detected in the spinal nerve are the result of abnormal projections of the main axon branch. However, other anatomical abnormalities that were detected in this study may still be a result of abnormal branching.

In summary, the present study has provided direct *in vivo* evidence that Sema3A is initially important for the appropriate guidance of many axons in the peripheral nervous system. The observed axon abnormalities in *Sema3A* null mutant mice are transient as evidenced by the dramatic elimination of aberrant projections early in development. Elimination of these aberrant projections is most probably due to mechanisms based on programmed cell death and/or selective axonal pruning. Our findings have revealed an important property of the developing peripheral nervous system: the existence of a constitutive or inducible "oversight" mechanism capable of correcting early errors in axonal pathfinding.

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