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Disruption of *Semaphorin III/D* Gene Causes Severe Abnormality in Peripheral Nerve Projection

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Summary

The molecules of the *collapsin/semaphorin* gene family have been thought to play an essential role in axon guidance during development. Semaphorin III/D is a member of this family, has been shown to repel dorsal root ganglion (DRG) axons in vitro, and has been implicated in the patterning of sensory afferents in the spinal cord. Although *semaphorin III/D* mRNA is expressed in a wide variety of neural and nonneural tissues in vivo, the role played by semaphorin III/D in regions other than the spinal cord is not known. Here, we show that mice homozygous for a targeted mutation in *semaphorin III/D* show severe abnormality in peripheral nerve projection. This abnormality is seen in the trigeminal, facial, vagus, accessory, and glossopharyngeal nerves but not in the oculomotor nerve. These results suggest that semaphorin III/D functions as a selective repellent in vivo.

Introduction

During embryogenesis, axons reach their targets correctly to form the complex neural network found in the mature functional nervous system. The tip of a growing axon, the growth cone, is specialized for reacting to environmental cues during navigation. Many cell adhesion molecules and extracellular matrix molecules have been thought to play an essential role as attractive cues for such growth cone guidance. Over the past decade, however, several studies have indicated the importance of repulsive guidance cues, e.g., formation of separate axon territories in cocultures of central and peripheral nervous tissues (Kapfhammer and Raper, 1987a, 1987b), avoidance of posterior tectum by temporal retinal ganglion cell axons (Walter et al., 1987; Cox et al., 1990), avoidance of posterior-half somites by spinal axons (Davies et al., 1990), and repulsion of olfactory tract axons by the septum (Pini, 1993; also reviewed by Goodman, 1996; Tessier-Lavigne and Goodman, 1996). So far, three families are regarded as repulsive cues; netrin-1 (which is also known as a chemoattractant; Colamarino and Tessier-Lavigne, 1995), the Eph receptor-ligand family (reviewed by Tessier-Lavigne, 1995; Drescher et al., 1997), and the collapsin/semaphorin family (reviewed by Kolodkin, 1996; Püschel, 1996). Recently, mice deficient in one Eph family member, *Nuk*, were generated and showed defects in axonal guidance (Henkemeyer et al., 1996). Also, another Eph family member, ELF-1, was shown to act as a repulsive guidance cue for temporal retinal axons but not for nasal axons in vivo and in vitro (Nakamoto et al., 1996). These results support the importance of repulsive guidance cues in vivo.

Semaphorin III/D is a member of the collapsin/semaphorin family. This family of molecules is found in both vertebrates and invertebrates and includes secreted and transmembrane molecules (Kolodkin et al., 1993; Inagaki et al., 1995; Luo et al., 1995; Püschel et al., 1995; Adams et al., 1996; Furuyama et al., 1996; Hall et al., 1996; Roche et al., 1996; Sekido et al., 1996; Xiang et al., 1996). This gene family is characterized by a conserved domain of ~500 amino acids (the semaphorin domain) and several conserved cysteine residues. Each member of the *collapsin/semaphorin* family is expressed in a specific pattern within developing embryos (Inagaki et al., 1995; Luo et al., 1995; Püschel et al., 1995; Adams et al., 1996; Püschel et al., 1996). Among them, grasshopper semaphorin I (formerly fasciclin IV) is the founding member of this family and has been implicated in Ti1 axon guidance in developing limb buds (Kolodkin et al., 1992), and Drosophila semaphorin II has been implicated in confining synaptic arborization to specific muscles in vivo (Matthes et al., 1995). These results suggest the importance of this gene family in axonal guidance.

Collapsin-1 (formerly collapsin) was originally identified on the basis of its ability to induce collapse of dorsal root ganglion (DRG) growth cones in vitro (Luo et al., 1993). Additionally, semaphorin III (the collapsin-1 homolog in mammals, also known as semaphorin D) can repel DRG axons in collagen-stabilized culture (Messersmith et al., 1995). Moreover, it has been reported that collapsin-1-coated beads can steer growth cones away from the beads (Fan and Raper, 1995). These results show that collapsin-1/semaphorin III/D not only induces growth cone collapse but can guide neurite outgrowth. Recent studies in vitro and in situ hybridization analyses

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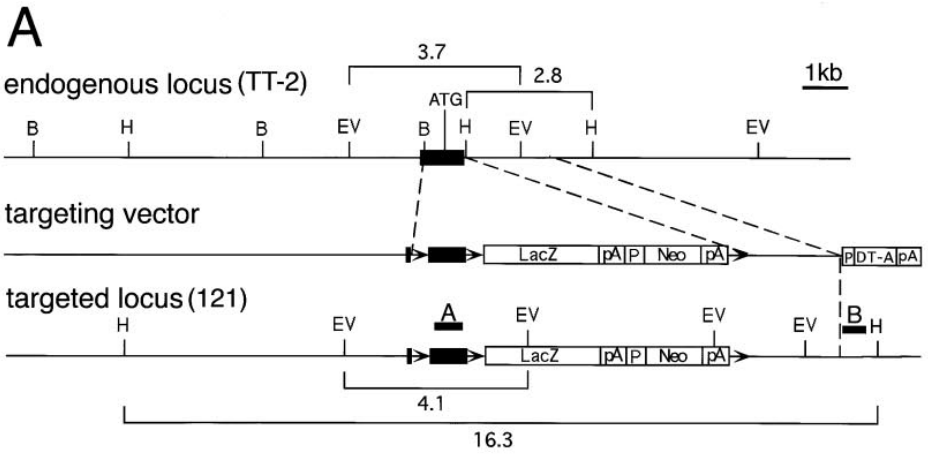
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2.8
1kb
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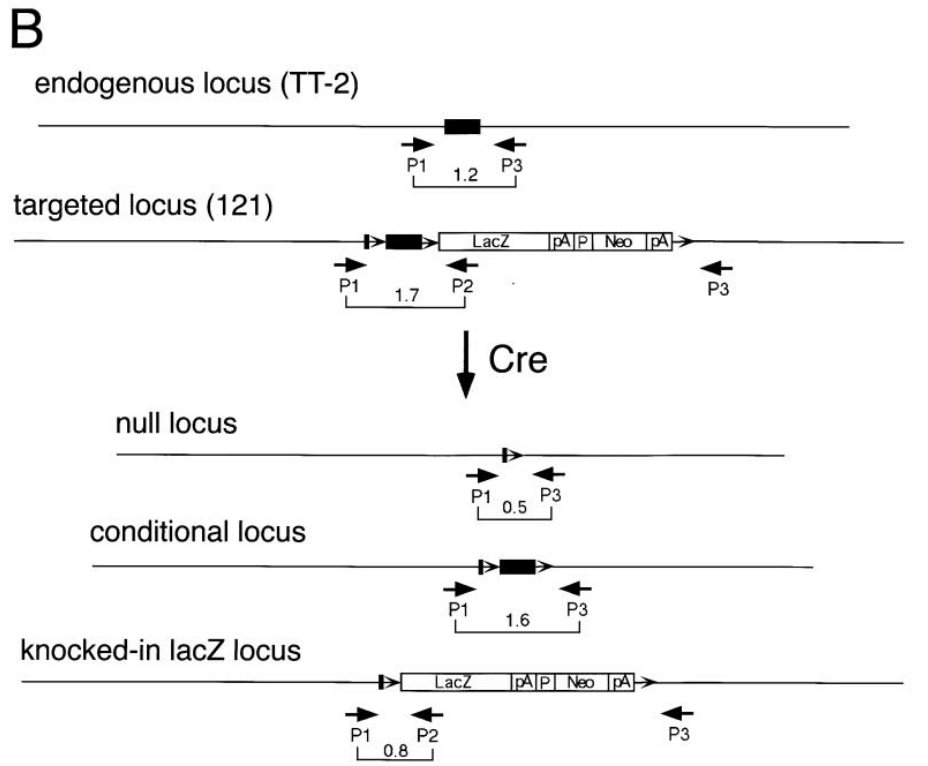
targeting vector
LacZ pA P Neo pA
P DT-A pA
targeted locus (121)
H EV A EV EV EV B H
LacZ pA P Neo pA
4.1
16.3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targeted locus (121)
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P1 1.7 P2 P3
Cre
null locus
P1 0.5 P3
conditional locus
P1 1.6 P3
knocked-in lacZ locus
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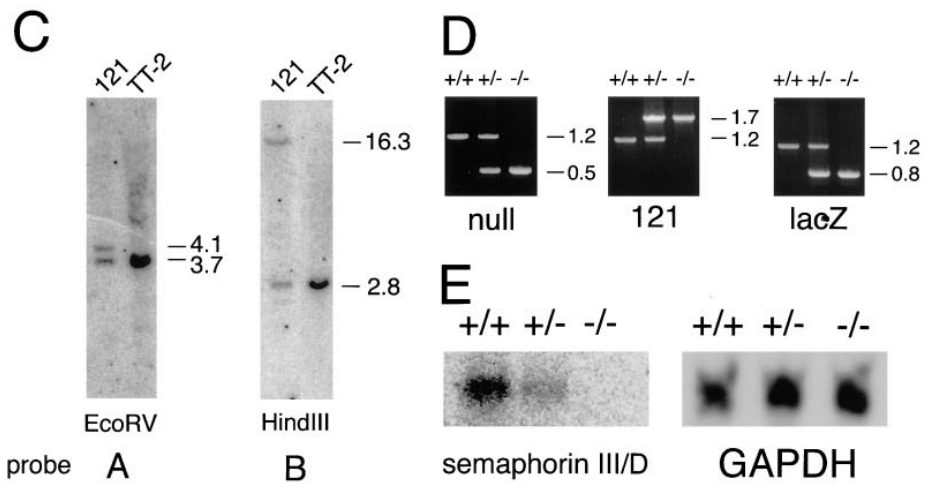
P1 P2 P3
0.8


C
121 TT-2
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16.3
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1.2
0.5
null
1.7
1.2
121
1.2
0.8
lacZ
E
+/+ +/- -/-
+/+ +/- -/-
semaphorin III/D
GAPDH

have suggested a role for semaphorin III/D in the patterning of sensory afferents in the spinal cord (Messersmith et al., 1995). Semaphorin III/D expressed in the ventral part of the spinal cord is thought to repel the cutaneous afferents, restricting their terminals to the dorsal part of the spinal cord, while allowing semaphorin III/D-insensitive Ia afferents to penetrate into the ventral cord. Additionally, detailed in situ hybridization studies have revealed expression of *collapsin-1/semaphorin III/D* in a variety of regions in developing embryos outside the spinal cord (Wright et al., 1995; Giger et al., 1996; Shepherd et al., 1996). However, the in vivo function of semaphorin III/D in these other regions remains largely unknown.

In order to determine the role of axonal guidance by semaphorin III/D, we generated semaphorin III/D-deficient mice. These mice were viable into adulthood. Analysis of peripheral nerves in homozygous embryos showed significant abnormalities in the trajectory of several cranial nerves, including the trigeminal, facial, glossopharyngeal, vagus, and accessory nerves, but showed no effects on the oculomotor nerve. Semaphorin III/D heterozygotes carrying a *lacZ* gene inserted into the *semaphorin III/D* locus showed that axons normally avoid *lacZ*-expressing areas, confirming the importance of semaphorin III/D in restricting nerve terminations in these regions.

Results

Generation of *Semaphorin III/D* Mutants

A targeting vector construct was designed to insert a combined *loxP* site and *lacZ-Neo* cassette into the *semaphorin III/D* gene (Figure 1A). The diphtheria toxin A (DT-A) fragment gene (Yagi et al., 1993b) containing a poly A signal was used as a negative selection marker. The vector was electroporated into the TT-2 line of embryonic stem (ES) cells derived from F1 embryo between C57BL/6 and CBA (Yagi et al., 1993a), and G418 resistant clones were screened for the presence of the predicted mutant allele by Southern blot analysis (Figure 1C). The frequency of homologous recombinants was 2.1% of G418 resistant colonies. One homologous recombinant (clone 121) was further manipulated to recombine the *loxP* sites by transient expression of Cre recombinase (Figure 1B). This resulted in the generation of three different loci, depending on which *loxP* sites were used in recombination. The null locus lacks the first exon, including about 0.6 kb of 5′-untranslated region and encoding the amino-terminal 38 amino acids, and has a *loxP* site. In the knocked-in *lacZ* locus, the first exon is replaced by the *lacZ* gene. In the conditional locus, the coding region remains intact, but two *loxP* sites are incorporated on either end of the first exon, allowing for further generation of conditional gene targeting. Thus, separate clonal cell lines were generated and identified for each of these three types of recombined loci by polymerase chain reaction (PCR) and Southern blot analyses (data not shown). These three clones and the original homologous recombinant, 121 clone, were injected into mouse embryos at the eight-cell stage to produce chimeric males that transmitted the mutation through the germ line. Offspring of these founders were identified by Southern blot analysis (data not shown). Heterozygous mice were obtained by crossing the germline chimeric mouse with C57BL/6 females. Heterozygous mice used in this study were backcrossed onto C57BL/6 for at least five generations. Semaphorin III/D heterozygotes seemed to be normal and were bred to obtain homozygotes, identified by PCR (Figure 1D) and Southern blot analyses. Northern blot analysis confirmed that semaphorin III/D-deficient mutants expressed no *semaphorin III/D* mRNA (Figure 1E), even though any *semaphorin III/D* cDNA used as a probe did not detect truncated transcripts.

Semaphorin III/D homozygous mice are largely viable after birth. The distribution of genotypes obtained from crosses of heterozygous mice was counted after weaning and compared to that of embryos. The percentages of mice after weaning were 26.1% wild-type, 53.3% heterozygous, and 20.6% homozygous mice ($n = 165$); the percentages of embryos were 22.0% wild-type, 54.3%

Figure 1. Targeted Disruption of *Semaphorin III/D* Locus

(A) Exon 1 of endogenous *semaphorin III/D* locus of ES cell line TT-2 (top), targeting vector for *semaphorin III/D* (middle), and targeted *semaphorin III/D* locus after recombination (bottom). The first exon includes ~0.7 kb of 5′-untranslated region and encodes 38 amino-terminal amino acids. The 5′-*loxP* site is in the untranslated region of the first exon. The sizes of the DNA fragments are indicated in kilobase pairs (kb). Solid boxes represent *semaphorin III/D* exon, and arrowheads represent *loxP* sites. The restriction map around this locus used for Southern blots of the DNA fragments is shown. Black bars labeled "A" and "B" represent the probes used in Southern blot analysis. Restriction enzyme sites: B, BamHI; EV, EcoRV; and H, HindIII. Other abbreviations: LacZ, β-galactosidase; pA, polyadenylation signal; P, promoter; Neo, neomycin transferase; DT-A, diphtheria toxin A-fragment; and ATG, initiation codon.

(B) Recombinants obtained by the transient expression of Cre recombinase in 121 cells. Three different products were generated, depending on the *loxP* sites used for recombination. The null locus lacks the first exon, which includes the initiation codon. The conditional locus retains the first exon, but with the addition of two *loxP* sites, permitting a tissue-specific recombination event at some future time. In the knocked-in *lacZ* locus, the first exon is replaced by the *lacZ* gene for examination of expression in heterozygotes. PCR primers used in the analyses of mutant genotypes are shown by arrows (P1, P2, and P3). The sizes of the DNA fragments are indicated in kb.

(C) Southern blot analyses for disrupted *semaphorin III/D* locus. DNAs from control (TT-2) and targeted ES cells (121) were digested with EcoRV (left) and HindIII (right). Blotted DNAs were hybridized with either probe A (left column) or B (right column), which are shown in (A). Probe A is a *semaphorin III/D* genomic internal probe (exon). Probe B is a 3′ *semaphorin III/D* genomic flanking probe, not present in the targeting vector. The sizes of the DNA fragments are indicated in kb.

(D) PCR analyses for *semaphorin III/D* mutant genotypes. The genotypes of 121, null, and knocked-in *lacZ* mutants used in this study are shown. The wild-type (+/+), heterozygous (+/−), and homozygous mice (−/−) are indicated. PCR primers used here are shown in (B). The sizes of the DNA fragments are indicated in kb.

(E) Northern blot analyses for *semaphorin III/D* null mutants. Total RNAs from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice of null mutants were used. The first exon of *semaphorin III/D* cDNA was used as a probe. The same blot was rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

heterozygous, and 23.7% homozygous embryos (n = 232). Thus, there is no evidence of significant lethality in either the embryonic or early postnatal periods.

Homozygous mice are smaller than wild-type mice: at 4 weeks the body weight of homozygous mice is about half that of their wild-type littermates, but no difference of growth hormone quantity was detected between wild-type and homozygous mice (n = 17, data not shown). The cause of this decreased size of homozygous mutants is not yet known. Homozygous mice are sufficiently healthy to be able to breed and rear their offspring.

Expression Pattern of *Semaphorin III/D* Assessed by X-Gal Staining

Heterozygous knocked-in *lacZ* mice were used for the analysis of expression pattern of *semaphorin III/D* during embryonic development (Figure 2). *lacZ* expression was first detected at embryonic day 9.5 (E9.5), predominantly in the forelimb buds and somites (data not shown). At E10.5, *lacZ* expression was strongly observed in the forelimb buds, moderately in the somites (predominantly in the caudal part of each somite) and hindlimb buds, and weakly in the heart, first and second branchial arches, and tissues surrounding the eye (Figure 2A). At E12, *lacZ* expression was observed in the sclerotome, mandibular process, tongue, nasal foramen, auricle, first branchial arch, heart, and fore- and hindlimb buds, and weakly in the eye and tissues surrounding the eye (Figure 2B). In contrast to the peripheral tissue, little signal was detected in the spinal cord in E12 embryos. Postnatally, X-Gal staining is detected in many of the same tissues. Strong expression was also seen in Purkinje cells in the cerebellum of postnatal day 2 mice (data not shown).

The Trajectory of Cranial and Spinal Nerves Is Abnormal in Mutant Mice

Collapsin-1/Semaphorin III/D has been shown to be a repellent for growth cones and has been suggested to play an essential role in axon guidance. To test this idea, axon trajectories of mutant mice were visualized by performing whole-mount staining with anti-neurofilament antibody (2H3) and were compared to that of wild-type littermates. Null and 121 (targeted) mutant mice were analyzed in this study, and both mice gave the same results (described below).

Cranial Nerves

In E10.5 wild-type embryos, the ophthalmic branch of the trigeminal nerve projects to the eye, the maxillary nerve to the maxillary process, and the mandibular nerve to the first branchial arch. The facial nerve projects to the second branchial arch, and the glossopharyngeal nerve projects to the third branchial arch. The mandibular and facial nerves fasciculate tightly between the ganglia and their target tissues (Figures 3A and 3C). In contrast to this wild-type pattern, in all 14 homozygotes analyzed, these nerves tended to defasciculate (Figures 3B and 3D). While the majority of axons in the trigeminal, facial, and glossopharyngeal nerves extended toward their targets, along the way these nerves spread, and occasionally individual axons projected aberrantly. The

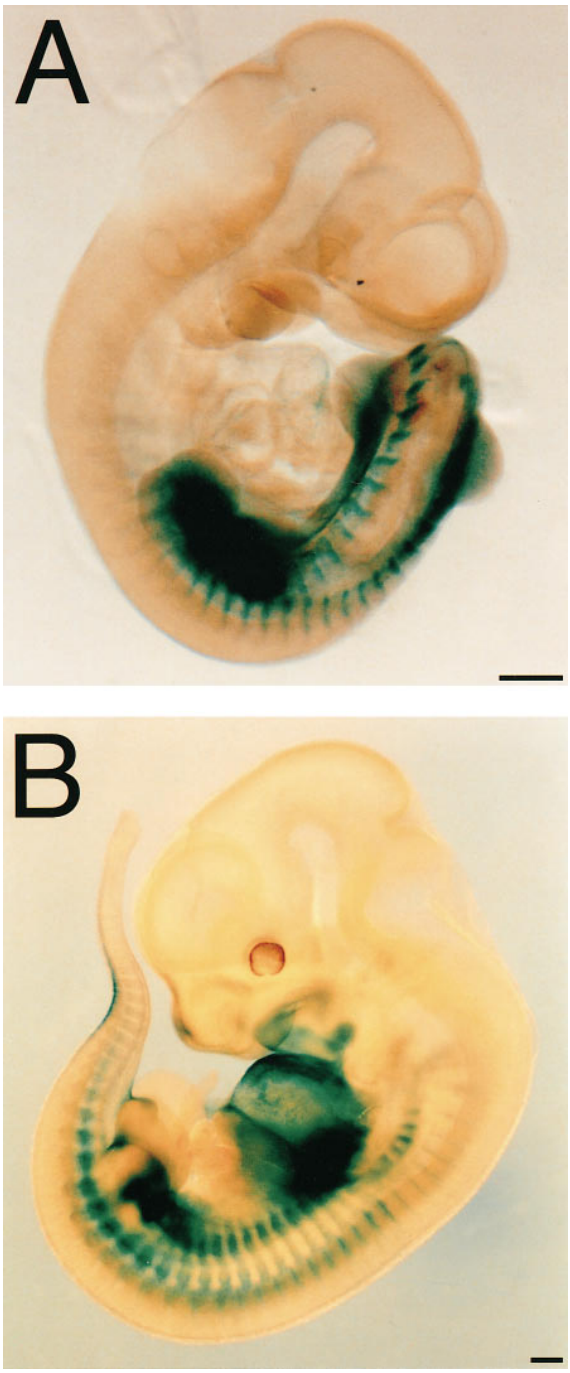

Figure 2. X-Gal Staining of *Semaphorin III/D* Knocked-In *lacZ* Mice

X-Gal staining of E (embryonic day) 10.5 (A) and E12 (B) heterozygous knocked-in *lacZ* mice.

(A) In E10.5 embryo, strong signal is seen in the forelimb buds, moderate signal in the somites and hindlimb buds, and weak signal in the heart, first and second branchial arches, and tissues surrounding the eye.

(B) In E12 embryo, staining is observed in the sclerotome, mandibular process, tongue, first branchial arch, heart, nasal foramen, auricle, eye, tissues surrounding the eye, and fore- and hindlimb buds, but little staining is observed in the brain and spinal cord.

Scale bars, 500 μm.

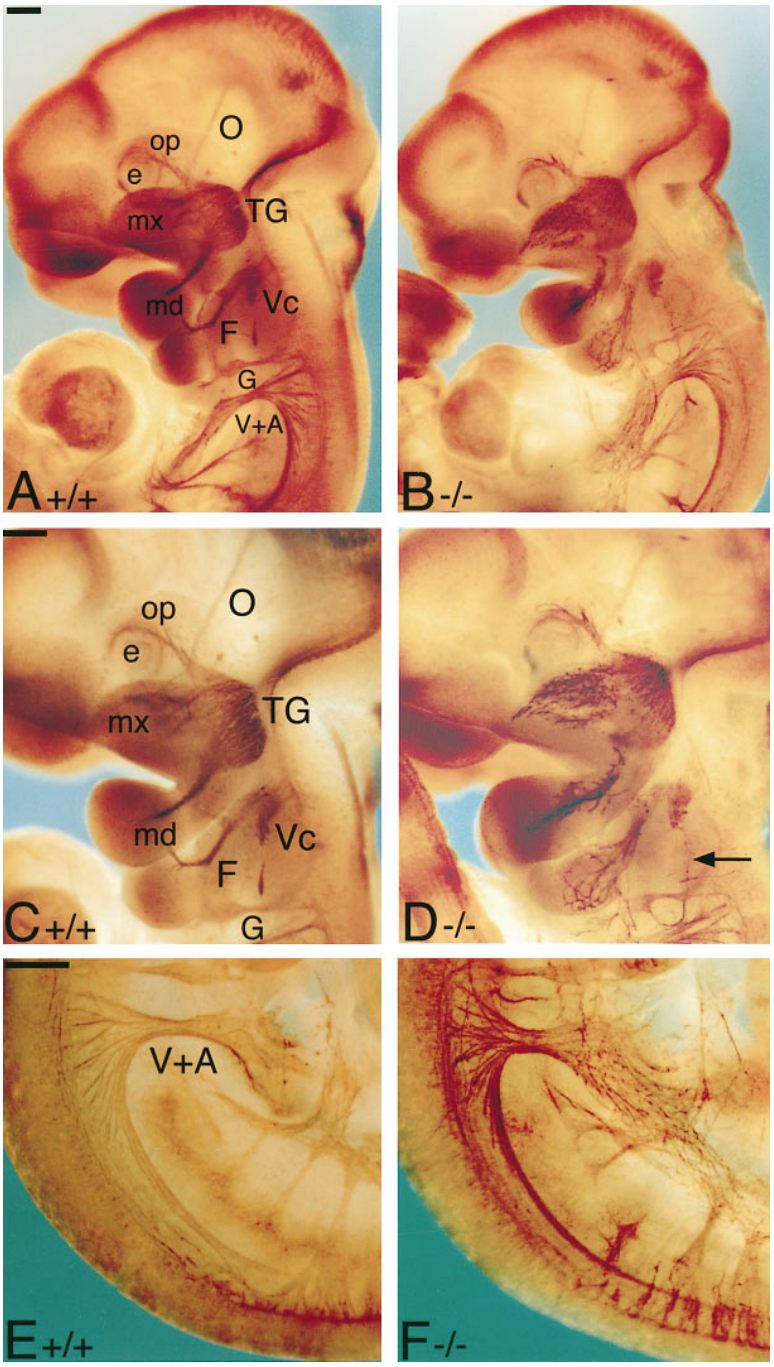

Figure 3. Abnormal Nerve Projections in Cranial Nerve Tracts for *Semaphorin III/D* Null Mutants at E10.5

Wild-type ([A], [C], and [E]; left column) and homozygous embryos of null mutants ([B], [D], and [F]; right column) at E10.5, showing whole-mount staining with antineurofilament antibody.

(A and C) Ophthalmic (op), maxillary (mx), mandibular (md), facial (F), and glossopharyngeal (G) nerves in wild-type embryo are indicated.

(B and D) In homozygous mutants, these nerves extend in the correct direction to their targets. However, on the way to the target, these nerves defasciculate and occupy a wider area. The vestibulocochlear nerve (Vc) looks normal in homozygous embryos, although it seems to be slightly thick and spread out. Aberrant projections are occasionally seen further beyond the otic vesicle (arrow in [D]). On the other hand, no abnormality is seen in the oculomotor nerve (O).

(A and E) The vagus (V) and accessory nerves (A) in a wild-type embryo are indicated.

(B and F) In homozygous embryos, terminals of the vagus and accessory nerves spread out.

Abbreviations: e, eye; TG, trigeminal ganglion. Scale bars, 250 μm.

vestibulocochlear nerve, however, looked normal in homozygous littermates, although it sometimes appeared slightly thick and spread out compared to that of wild-type littermates. Some aberrant projections, which are not seen in controls, were seen further beyond the otic vesicle (Figure 3D, arrow).

Abnormal projection was also seen in the vagus and accessory nerves. In wild-type embryos, terminals of these nerves are narrowly bundled (Figures 3A and 3E). However, in all 14 homozygous mutant littermates analyzed, terminals of these nerves spread out and occupied a wider area (Figures 3B and 3F). In contrast to these nerves, no abnormality was seen in the oculomotor nerve (Figures 3B and 3D), which correctly projected to the ciliary ganglion, forming a tightly fasciculated nerve in both wild-type and mutant mice.

Facial Innervation around the Eye

In wild-type embryos at E12.5, the trigeminal nerve innervates a region around the eye, extending into the maxillary process and muzzle (Figure 4A). Additionally, the cornea is heavily innervated by the trigeminal, sympathetic, and ciliary nerves. Examination of 121 heterozygous embryos of comparable age shows *semaphorin III/D* expression in the lens and certain areas in the

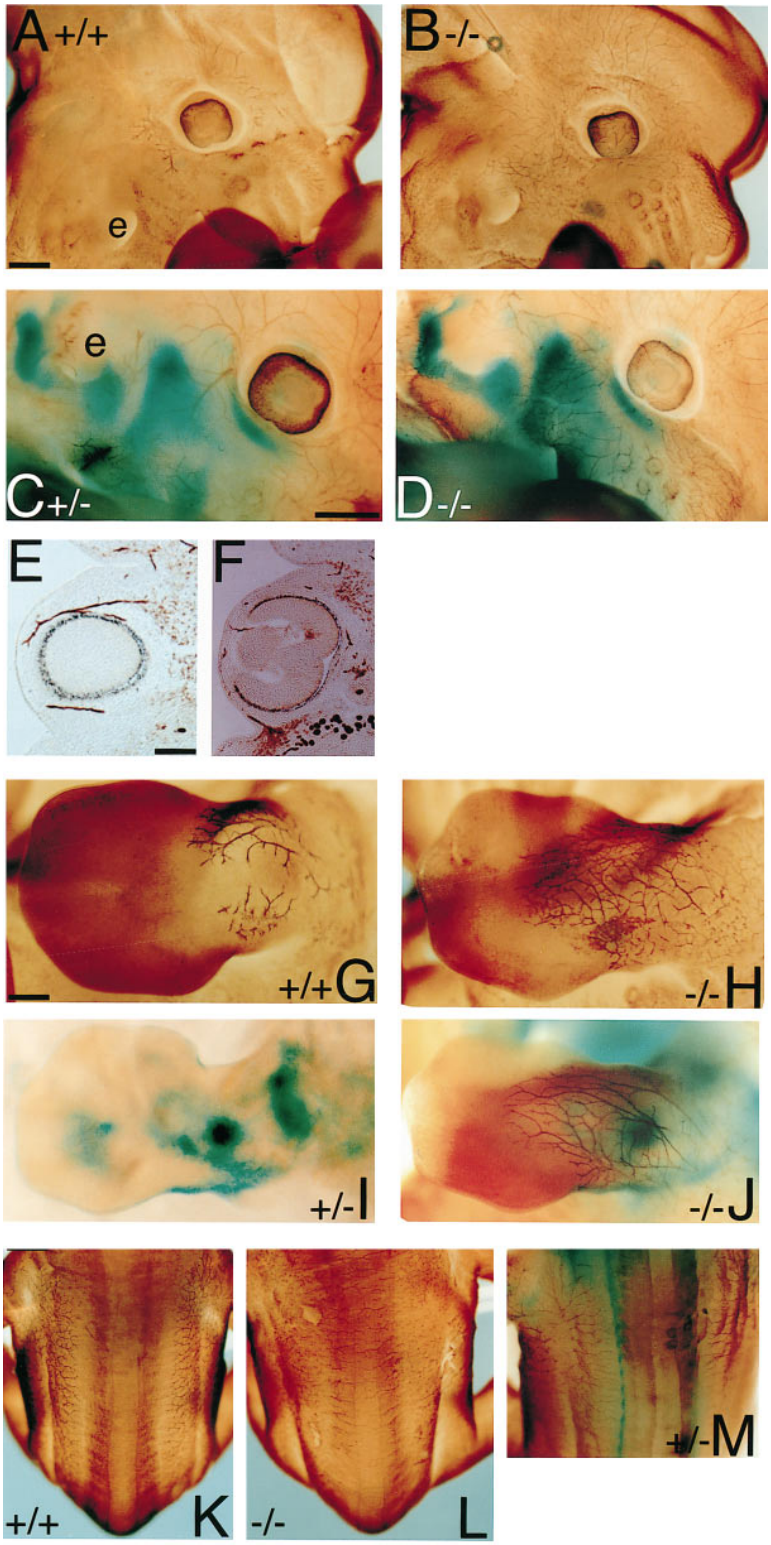

Figure 4. Abnormal Projections Observed around the Eye, in the Forelimb Bud, and on the Back Muscles of *Semaphorin III/D* Mutants

(A, B, G, H, K, and L) Whole-mount staining with antineurofilament antibody (2H3) at E12.5.

(C, D, J, and M) Double staining with 2H3 and X-Gal at E13.

(E and F) Section staining with antineurofilament antibody at E12.5.

(I) X-Gal staining at E12.5. Control animals are shown in the left column ([A], [G], and [K], wild type; [C] and [M], 121 heterozygous; [I], knocked-in *lacZ* heterozygous) and mutant homozygotes are shown in the right column ([B], [E], [F], [H], and [L], null; [D] and [J], 121).

(A and C) Trigeminal innervation around the eye in wild-type embryos. Axons seldom invade the *semaphorin III/D*-expressing area visualized by *lacZ* expression (C).

(B and D) In homozygous mutants, the trigeminal nerve spread out and occupied a wider area around the eye. Abnormal projection is also seen over the lens. Axons often invade *lacZ* positive areas (D). Abbreviation: e, ear.

(E and F) Two different examples of abnormal projection seen around the eye of homozygous mutants. Some axons enter the tunica fibrosa bulbi (E); others enter the lens vesicle (F).

(G) Normal innervation pattern in the forelimb bud of wild-type mice.

(I) Distribution of *semaphorin III/D* in wild-type mice visualized by *lacZ* expression. Semaphorin III/D expression is detected at the cartilage of the forelimb buds, handplate cartilage, humerus, radius, and ulna. Comparison of (G) and (I) shows that spinal nerves avoid the area that expresses high levels of *semaphorin III/D*.

(H and J) Abnormal projection seen in the homozygous mutants. Axons make more branches than in controls, enter the *lacZ*-positive area (J), and reach the base of the handplate.

(K) Normal pattern of spinal nerve projection in the dorsal muscles of the wild-type embryo.

(L and M) The spinal nerves stop at the sclerotome, where *semaphorin III/D* is expressed. In homozygous mutants (L), these nerves do not stop at this line, and they occasionally even cross the dorsal midline.

Scale bars, 500 μm ([A], [C], and [K]); 200 μm ([E] and [G]).

ventrocaudal quadrant of the face (Figure 4C). Regions of *lacZ* expression and the locations of axonal tracts visualized by antineurofilament staining were roughly complementary. Few axons extended into the areas where X-Gal staining suggested *semaphorin III/D* expression (Figure 4C).

In all 18 null homozygous embryos analyzed, abnormalities were seen in the projection patterns of all of

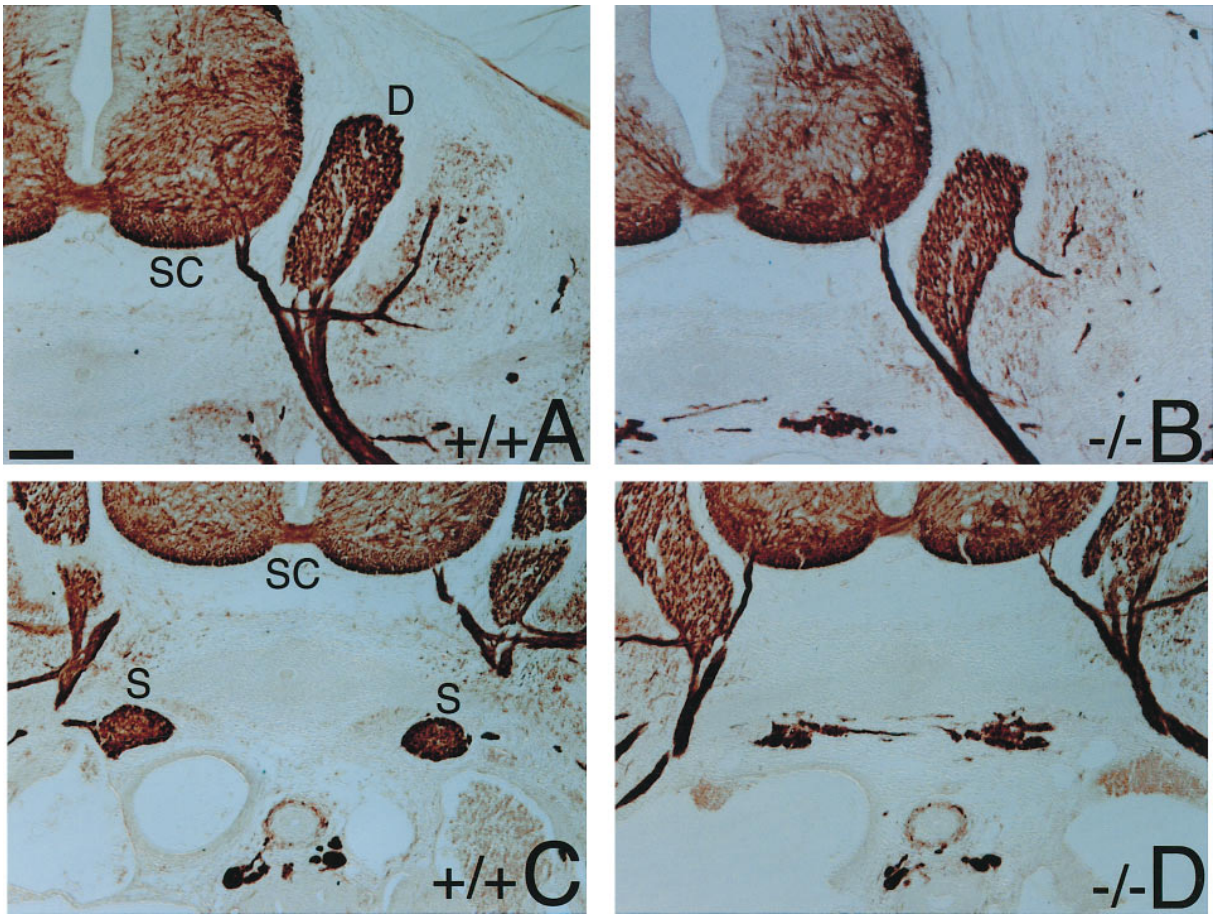

Figure 5. Projections of Sensory Ganglia Are Disrupted in *Semaphorin III/D* Mutants

Transverse sections of wild-type ([A] and [C]) and homozygous null embryos ([B] and [D]) at E12.5. Dorsal is up.
(A and B) Axons extending from the DRG.
(A) In wild-type embryos, peripheral projections of sensory neurons leave the DRG from its ventral side.
(B) In homozygous mutants, sensory axons often leave the ganglion from the lateral side.
(C and D) Comparison of the sympathetic chain in wild-type (C) and mutant (D) embryos. Normal sympathetic chains (S) are oval-shaped (C), while in homozygous embryos, sympathetic neurons and processes are more scattered (D).
Abbreviations: D, DRG; SC, spinal cord. Scale bar, 100 μm.

these nerves (Figure 4B). The most notable abnormality was in the innervation of the eye. In wild-type embryos, corneal innervation is restricted to the periphery at this stage, and axons never grow over the *semaphorin III/D*-expressing lens (Figure 4A). In contrast, in homozygous mutants, many aberrant projections were observed in the cornea extending over the lens (Figures 4B and 4D). Examination of sections through the eye demonstrate that these nerve fibers enter the tunica fibrosa bulbi and the lens vesicle of the eye in these embryos (Figures 4E and 4F, respectively). The trigeminal projection around the eye was also abnormal (Figure 4B); it extended further and occupied twice as much area as compared to wild-type littermates. Sometimes trigeminal terminals entered the area in the ventrocaudal quadrant of the face where *semaphorin III/D* is normally expressed (Figures 4C and 4D).

Spinal Nerves

Staining for *lacZ* was detected in the cartilage and bones (humerus, radius, and ulna) of developing forelimb buds in heterozygous embryos (Figure 4I). In normal E12.5 embryos, spinal nerves could be seen to avoid the cartilage where *semaphorin III/D* was heavily expressed, and they did not invade the basal part of the handplate (Figure 4G). In contrast, null homozygotes' spinal nerves grew over the cartilage to enter the basal part of the handplate (Figure 4H). These nerves also tended to be more branched compared to those of wild-type littermates. Double staining with X-Gal and 2H3 staining revealed that in 121 homozygous embryos, spinal nerves grew freely over *lacZ*-expressing regions of the limb bud (Figure 4J).

On the dorsal surface of the embryos, *lacZ* signal was detected in the sclerotome surrounding the DRGs

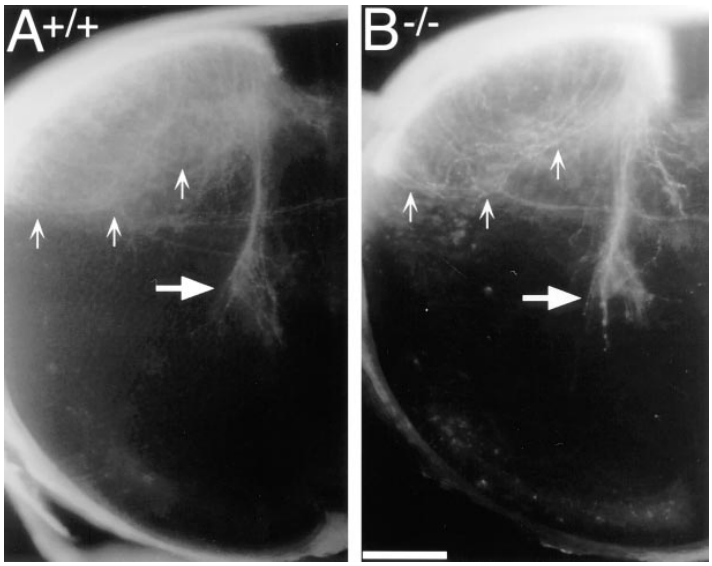

Figure 6. Projections of Dorsal Root Afferents Are Normal in *Semaphorin III/D* Mutants

Transverse section of wild-type (A) and homozygous null embryos (B) at E15.5. Dorsal is up.

(A) In wild-type embryos, putative muscle afferents project to the ventral horn (large arrow), and cutaneous afferents project to the deep dorsal horn (small arrows).

(B) In homozygous embryos, the overall pattern of the afferent projections is quite similar to that in (A), and cutaneous afferents do not project to the ventral horn (small arrows). Scale bar, 100 μm.

(Figure 4M). In wild-type embryos, the spinal nerves that project into the back normally stop near the *semaphorin III/D*-expressing sclerotome and never extend further beyond them (Figure 4K). However, in null homozygous embryos, spinal nerves did not stop at the sclerotome boundary and even crossed the dorsal midline instead (Figure 4L).

Antineurofilament antibody staining of sectioned embryos revealed abnormal axon projections from DRGs and disruptions of the sympathetic chains. In E12.5 wild-type embryos, the peripheral projections of sensory neurons leave the DRGs from their ventral sides and meet with the ventral roots to form spinal nerves. A large dorsal branch then separates from the ventral nerve and projects dorsally (Figure 5A). In all null homozygous mutants ($n = 5$), however, the majority of examined axons left the DRG from its lateral side and directly innervated the dorsal muscles (Figure 5B). The projection of dorsal root fibers did not show apparent abnormality in mutants (Figure 5B). Sympathetic chains are usually tightly fasciculated, and the shape of the ganglion is oval in wild-type embryos (Figure 5C). However, in the absence of semaphorin III/D, the shape of sympathetic chains was disrupted, with cell bodies scattered in clumps over a broader medial-lateral area (Figure 5D). Semaphorin III/D was found to be expressed immediately adjacent to both DRGs and sympathetic ganglia in control mice, suggesting that it may normally act to prevent these extensions (data not shown).

The Trajectory of Dorsal Root Afferents Is Normal in Mutant Mice

Semaphorin III/D has been suggested to play a important role in the patterning of sensory afferents within the spinal cord (Messersmith et al., 1995). In order to confirm this idea, the trajectory of dorsal root afferents in the spinal cord of mutant mice was visualized by DiI labeling and compared to that of wild-type littermates. In E15.5 *semaphorin III/D* homozygous embryos, the trajectory of dorsal root afferents was apparently normal (Figure 6B; $n = 5$). The overall pattern of the afferent trajectory in mutants is quite similar to that of wild-type embryos (Figure 6A; Ozaki and Snider, 1997); putative muscle afferents projected to the ventral horn, and cutaneous afferents were limited within the dorsal horn. Similar results were also obtained from the E16.5 homozygous embryos ($n = 4$).

Discussion

We generated targeted *semaphorin III/D* homozygous mutant mice to elucidate the in vivo functions of semaphorin III/D. We produced four kinds of targeted *semaphorin III/D* mice: targeted (121) mice, and, by use of the Cre-*loxP* system, null, conditional, and knocked-in *lacZ* mice. In this study, we used null mutant mice to analyze effects on nerve projection, knocked-in *lacZ* mice to elucidate the normal expression pattern of *semaphorin III/D*, and 121 mice for the comparison of nerve trajectory and *lacZ* expression. Our results show that semaphorin III/D functions as a repulsive guidance cue during development of the nervous system.

Expression Pattern of *Semaphorin III/D*

In this study, we investigated the expression pattern of *semaphorin III/D* through the use of knocked-in *lacZ* heterozygous mice. The pattern of *lacZ* expression in the periphery is mostly the same as the distribution pattern of *semaphorin III/D–collapsin-1* mRNA (Wright et al., 1995; Giger et al., 1996; Shepherd et al., 1996); for example, at ~E12, *lacZ* expression was seen in the sclerotome, limb buds, first branchial arch, mandibular process, nasal foramen, eye, tongue, tissues surrounding the eye, auricle, and heart. However, in the CNS, there is a notable difference between *lacZ* expression in our mice and the distribution of *semaphorin III/D* mRNA. Previous studies showed significant levels of *semaphorin III/D* mRNA expression in the CNS (Wright et al., 1995; Giger et al., 1996). However, in this study, little discernible signal was detected in the CNS of E10–E16 embryos by *lacZ* expression. There are several possible explanations for this difference. First, it is possible that

the gene sequences which impart CNS-specific expression reside in the first exon, which is deleted in the present null mutant mice. However, *lacZ* expression was not seen in 121 mutant mice that still contained the first exon, suggesting that this is not the case. Second, it is possible that X-Gal solution might not have penetrated enough to reach the CNS. In order to test this possibility, we performed X-Gal staining on sections. However, little discernible signal was detected in the CNS, which may exclude this possibility. Third, though a considerable amount of *semaphorin III/D* mRNA was detected in the CNS by in situ hybridization analysis, it is possible that the amount of the transcripts was too small to detect them by X-Gal staining. Collapsin-1 is highly potent, acting at a concentration of 10 pM to induce the collapse of growth cones in in vitro culture (Luo et al., 1993). Thus, a small amount of the molecule might be enough to pattern sensory innervation in the CNS. Fourth, it is possible that *lacZ* expression is not completely faithful. However, further studies are necessary to clarify this difference.

Abnormal Neural Projection in *Semaphorin III/D* Null Mutants

In whole-mount staining, *semaphorin III/D* homozygous embryos showed severe abnormal peripheral axon projections. Abnormalities were only seen in selected nerves; among the cranial nerves, the trigeminal, facial, vagus, accessory, and glossopharyngeal nerves showed disrupted trajectories. The oculomotor nerve appears normal. Previous studies have shown that collapsin-1/semaphorin III specifically induces collapse of certain types of growth cones (Luo et al., 1993; Messersmith et al., 1995). Recently, Varela-Echavarría et al. (1997) showed that semaphorin D did not collapse the rat oculomotor nerve in a coculture study. Our present results confirmed these studies in vivo.

Semaphorin III/D has been hypothesized to act as a repulsive guidance cue for axons. Our demonstration of abnormal projection in *semaphorin III/D* homozygous mice verified this idea. Interestingly, although in homozygous mutants axon projections are more diffuse, and axons often wander on the way to their target, the direction of axon growth is roughly correct and several axons obviously reach their target field. The other interesting observation is that mutant mice are viable, and their usual behavior appears normal and coordinated. This indicates that spinal innervation may not be functionally perturbed in these mice. It has been proposed that the process of axon guidance can be divided into several phases. The first stage is to determine the initial direction of axon outgrowth. This is followed by axonal extension to the target in the second stage. This stage is achieved by cues acting at a long range (e.g., chemoattraction from the target or chemorepulsion from the opposite direction) or a short range (e.g., membrane- or extracellular matrix- [ECM-] bound repellents or attractants). In the third stage, axons recognize the target area and form an axonal arbor. At the final stage, synapses form at the target cells (this stage would be followed by the activity-dependent remodeling). Our results can distinguish clearly the role of semaphorin III/D in these different stages of axon guidance. The observation that the direction of axon growth is roughly correct indicates that these processes are achieved by other guidance cues (probably target-secreted chemoattractants). Mutant mice seem to behave normally, and thess results indicate that semaphorin III/D does not play a role in the target recognition process. However, the severe abnormalities seen in mutant mice show that semaphorin III/D is an essential component in the navigation process.

In grasshopper embryos, blocking of semaphorin I function by antibody caused various types of abnormalities in the trajectories of the Ti1 axons (Kolodkin et al., 1992); the Ti1 growth cones often extended across the semaphorin I boundary, and axons often were defasciculated or branched ectopically. The similarity in the phenotypes of grasshopper and mice with loss of function of semaphorin suggests that semaphorin III/D is a repulsive guidance cue in vivo.

Nature of the Guidance Role Played by Semaphorin III/D

By studying abnormal nerve projections in mutant mice, we can understand the role of semaphorin III/D in axon guidance.

Semaphorin III/D Inhibits Axon Extension to the Cartilage

Previous work has shown that nerves prefer not to grow over cartilaginous regions. Semaphorin III/D–collapsin-1 expression is detected in the cartilage during embryogenesis (Wright et al., 1995; Shepherd et al., 1996; Figure 5I; unpublished data) and could play a role in this avoidance. Also, in our knocked-in *lacZ* mutant mice, we saw that spinal nerves in forelimb buds avoided the cartilage expressing *semaphorin III/D* (Figures 5G and 5I). The fact that null mutants showed axon growth into these regions supports a role for semaphorin III/D in this shaping of spinal nerves in the forelimb buds.

Semaphorin III/D Regulates the Innervation Pattern around the Eye

In E12.5 homozygous embryos, nerve fibers entered in the eye, tunica fibrosa bulbi and lens vesicle. These nerve fibers could not be identified. However, they are among the three kinds of neurons—the trigeminal, ciliary, and superior cervical (sympathetic) neurons—which project to the near regions of the eye, and collapsin-1/semaphorin III/D acts on them (this study; H. Kobayashi and J. A. Raper, personal communication). Thus, semaphorin III/D may act in normal circumstances to exclude one or more of these types of endings from the immediate eye region.

Normal Projection of Dorsal Root Afferents in *Semaphorin III/D* Null Mutants

The present DiI-labeling of dorsal root afferents showed that *semaphorin III/D* homozygous embryos exhibited nearly normal projection of the nerves within the spinal cord, while semaphorin III/D has been suggested to act in the patterning of DRG afferent projection (Messersmith et al., 1995). This result may be attributed to the functional redundancy of semaphorin III/D. In fact, several members of the *collapsin*/*semaphorin* gene family

are differentially expressed in the developing spinal cord (Luo et al., 1995; Püschel et al., 1995; Püschel et al., 1996); however, the ultimate functions of these members as repulsive guidance cues are not yet clear. While we do not observe any gross defects in E15.5 and E16.5 homozygous embryos, much more work is required to determine whether there are any defects in the timing or patterning of initial projections into the spinal cord or in the projections of individual subclasses of axons.

Other *Semaphorin III* Homozygous Mice

Recently, another group generated *semaphorin III* homozygous mutants (Behar et al., 1996). The majority of these homozygotes die within a few days after birth, and they show some abnormalities in the projection of sensory axons into the spinal cord as well as abnormal orientation of neuronal processes in the cortex. Also, these homozygous mice show bone and cartilaginous structure abnormalities, hypertrophy of the right ventricle, and dilation of the right atrium. Although we have not examined bone and cartilaginous structure or the orientation of neuronal processes in the cortex in our mutant mice, the difference seen in the viability of mutant mice is striking. Further, our mutant mice show no cardiac defect (n = 14). It must be noted that the genetic backgrounds of the mice used to generate mutants differ between the two studies. The genetic background of our mice is C57BL/6, and the background of their mice is 129. Thus, the differences in mortality and cardiac defects can be attributed to the difference in the genetic backgrounds of homozygous mice. Recently, it has been pointed out that the mouse genetic background is important for the correct interpretation of phenotypic (especially behavioral) abnormalities observed in homologous recombinant mice (e.g., Gerlai, 1996). When compared to C57BL/6 mice, the 129 strain has a lesser gene compensating ability, and therefore one observes greater deficits in the *semaphorin III/D* null mice. The semaphorin III/D disruption in the CD-1 genetic background is even more severe, as the homozygotes die after birth (showing profound cardiac defects; unpublished data), a situation similarly found in the 129 *semaphorin III/D* null mice.

Conclusion

In this study, we generated *semaphorin III/D* mutant mice, and we showed that (1) *lacZ* inserted into the *semaphorin III/D* genomic locus is expressed at low levels in the CNS but strongly in the periphery during embryogenesis, (2) semaphorin III/D is a selective repulsive guidance cue in vivo during embryogenesis, (3) semaphorin III/D functions in certain processes of axon guidance, (4) semaphorin III/D inhibits axon extension to the cartilage and eye, (5) deficiency of semaphorin III/D does not affect the projection of dorsal root afferents, and (6) the phenotypes of *semaphorin III/D* homozygous mice differ according to their genetic background.

Further studies are needed to verify the in vivo functions of semaphorin III/D during development of the nervous system. Nevertheless, our results suggest that semaphorin III/D functions as an important repellent in axon guidance.

Experimental Procedures

Construction of the Targeting Vector, Electroporation, and Selection by pCre-Pac Plasmid

We obtained *semaphorin III/D* cDNA containing ~0.7 kb of 5′-untranslated region from the C57BL/6 adult brain cDNA library, with chick *collapsin-1* cDNA used as a probe. Genomic *semaphorin III/D* DNAs containing the initiation codon in the first exon were cloned from the TT-2 genomic DNA library. The genomic DNA library was screened with the 0.4 kb EcoRI-EcoRV fragment of cDNA (accession number D85028) containing the initiation codon. The 12.4 and 16.0 kb genomic DNA fragments were obtained, and SalI fragments were subcloned in pBluescript II SK(-) (Stratagene). The 12.4 kb DNA fragment was used for construction of targeting vector. The first exon of *semaphorin III/D* includes ~0.7 kb of 5′-untranslated region and encodes 38 amino-terminal amino acids. We generated a ploxP-3 plasmid consisting of three *loxP* sites. The XbaI-EcoRI (converted into a SmaI site) fragment containing a *loxP* site from pGH-1 (Gu et al., 1993) and the EcoRI-SalI (converted into a SmaI site) fragment containing a *loxP* site from pGEM-30 (Gu et al., 1993) were inserted into XbaI-SalI sites of pBluescript II SK(−) to get ploxP-2. The SmaI-blunted XhoI fragment containing a *loxP* site of ploxP-2 was inserted into the SmaI site of ploxP-2 to get ploxP-3 (consists of *loxP*, SmaI, *loxP*, SalI, and *loxP* sites). A ~0.7 kb BamHI-HindIII fragment containing the first exon and the *lacZ-neo* cassette (consists of *lacZ*, SV40 early pA signal, PGK promoter, *neo*, and PGK pA signal) was inserted into the SmaI and SalI sites of ploxP-3, respectively. The targeting vector was completed by ligation of 5′ and 3′ genomic flanking DNAs of the first exon of a 12.4 kb DNA fragment and DT-A containing PGK pA signal. Targeting vector (5 nM) linearized by Not I was electroporated into 1×10^7 TT-2 cells by use of a gene pulser (250 V, 960 μF; BioRad). Homologous recombinants were selected by G418 (200 μg/ml) and Southern blot hybridization. We selected clone 121 for selection by pCre-Pac plasmid. The 1.7 kb SalI fragment of pPGKPuro (Watanabe et al., 1995) was inserted into XhoI-SalI sites of pMC-Cre (Gu et al., 1993) to get pCre-Pac. Circular pCre-Pac plasmid (20 μg) was electroporated into 0.5×10^7 clone-121 cells. Selection with puromycin (1 μg/ml; Sigma) was performed transiently. These cells were subcultured on new feeder cells with normal ES cell culture medium 72 hr after electroporation. Each type of the Cre-mediated recombinants was confirmed by PCR and Southern blot hybridization.

Production of Chimeric Mice

Ten targeted TT-2 cells were microinjected into an eight-cell stage CD-1 mouse embryo (Yagi et al., 1993a). The microinjected embryos were cultured in M16 medium overnight to blastocysts. Seven blastocysts were transplanted into each uterus of a recipient CD-1 mouse. Chimeric mice were identified by eye and coat color. Chimeric mice were mated to strain C57BL/6 females.

Southern Blot Hybridization

DNAs (10 μg) were digested with EcoRV and HindIII and then separated on 1% agarose gels and blotted on nitrocellulose filters (S&S). Hybridization was performed with probes of internal (probe A) and external (probe B) parts of the targeting vector (shown in Figure 1A), in 6× SSC, 5× Denhardt solution, 0.2% SDS, and 100 μg/ml herring sperm DNA at 65°C overnight. The filters were washed with 2× SSC-0.2% SDS three times at 65°C.

Northern Blot Hybridization

Total RNAs were extracted from adult brains by the guanidiniumthiocyanate method (Chomczynski and Sacchi, 1987). Total RNAs (20 μg) were separated on 1% formaldehyde gels and blotted on nitrocellulose filters (S&S). Hybridization was performed in 50% formamide, 6× SSC, 5× Denhardt solution, 0.2% SDS, and 100 μg/ml herring sperm DNA at 42°C overnight. About 0.6 kb untranslated BamHI-PstI fragment of the first exon of *semaphorin III/D* cDNA was

used as a probe. The filters were washed with 2× SSC-0.2% SDS three times and with 0.2× SSC-0.2% SDS once at 60°C.

Polymerase Chain Reaction

The genotype analyses of *semaphorin III/D* mutants was done by PCR using Taq DNA polymerase (TaKaRa, Japan). For this, the following primers were used (shown in Figure 1B): primer P1 derived from the 5′ intron of the *semaphorin III/D* exon, ACAACGCTTGCCTC GGGAGGTAAA; primer P2 from the *lacZ* gene sequence, TGGGATA GGTTACGTTGGTGTAGA; and primer P3 from the 3′ intron of the *semaphorin III/D* exon, ATGGTTCTGATAGGTGAGGCATGG. PCR was carried out at 96°C for 5 min, 75°C for 5 min, 60°C for 3 min, and 75°C for 5 min, and then at 96°C for 30 s, 60°C for 30 s, and 72°C for 2 min for 35 cycles.

X-Gal Staining of Embryos

Embryos were fixed in 2.5% paraformaldehyde-0.2% glutaraldehyde in phosphate-buffered saline (PBS) at 4°C for 2–5 hr. After they were washed with PBS three times, embryos were stained with X-Gal solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal] in PBS). Incubation time was 4 hr overnight at 37°C. After the embryos were washed with PBS three times, the reaction was stopped with 4% paraformaldehyde solution. Then, the stained embryos were dehydrated by methanol for 2 days and cleared with benzyl benzonate and benzyl alcohol (2:1).

Immunohistochemical Analyses

For whole-mount staining with antineurofilament antibody (2H3), embryos were fixed in 4% paraformaldehyde at 4°C overnight. After they were washed with 0.9% NaCl, embryos were dehydrated and fixed with 30%, 50%, 80%, and 100% methanol for a few hours each. Then, embryos were treated with 10% H_2O_2 in 80% methanol-20% dimethyl sulfoxide (DMSO) for a few hours, washed with TST (10 mM Tris [pH 7.8], 0.9% NaCl, and 0.1% Triton X-100) and incubated with 2H3 antibody (purchase from Developmental Studies Hybridoma Bank, Iowa University; monoclonal antibody supernatant; diluted 1:200) for 2 days at room temperature. After they were washed with TST, embryos were incubated with horseradish peroxidase- (HRP-) coupled biotinylated mouse IgG antibody (BioRad) overnight at room temperature, washed with TST, and then developed with DAB (p-dimethylaminoazobenzene). The reaction was stopped with TST.

For the immunohistochemical staining of sections with antineurofilament antibody, embryos were immersed in 95% ethanol and 5% acetic acid at 4°C overnight. They were then dehydrated, embedded in paraffin, and sectioned at 10 μm. The sections were stained by using polyclonal anti-neurofilament (160 kDa) antibody (gift from Dr. Kuwano at Niigata University) as the primary antibody and HRP-labeled Fab fragment of anti-rabbit IgG (MBL, Japan) as the secondary antibody. The HRP reaction was performed in diaminobenzidine-H_2O_2 solution.

Double Staining with Anti-Neurofilament Antibody and X-Gal Staining

After they were stained with X-Gal solution, embryos were fixed again in 4% paraformaldehyde at 4°C overnight and then were stained with 2H3 antibody as described above.

DiI Labeling

Projection of dorsal root afferents in the spinal cord was studied by labeling the nerves with the lipophilic fluorescent dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; Molecular Probes). After fixation of embryos with 4% paraformaldehyde, crystals of DiI were placed on the DRGs of the thoracic nerves between the fifth and tenth segments. The specimens were incubated at 37°C. Following the appropriate time interval (~5 days for E15.5 and 8 days for E16.5 embryos), the spinal cords were embedded in 3.5% agar and sectioned on a vibratome at 125 μm.

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