Published in final edited form as:

Mol Cell Neurosci. 1999 April ; 13(4): 281–292. doi:10.1006/mcne.1999.0747.

Role of Semaphorin III in the Developing Rodent Trigeminal System

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Abstract

Semaphorins are a large family of secreted and transmembrane glycoproteins. Sema III, a member of the Class III semaphorins is a potent chemorepulsive signal for subsets of sensory axons and steers them away from tissue regions with high levels of expression. Previous studies in mutant mice lacking sema III gene showed various neural and nonneural abnormalities. In this study, we focused on the developing trigeminal pathway of sema III knockout mice. We show that the peripheral and central trigeminal projections are impaired during initial pathway formation when they develop into distinct nerves or tracts. These axons defasciculate and compromise the normal bundling of nerves and restricted alignment of the central tract. In contrast to trigeminal projections, thalamocortical projections to the barrel cortex appear normal. Furthermore, sema III receptor, neuropilin, is expressed during a short period of development when the tract is laid down, but not in the developing thalamocortical pathway. Peripherally, trigeminal axons express neuropilin for longer duration than their central counterparts. In spite of projection errors, whisker follicle innervation appears normal and whisker-related patterns form in the trigeminal nuclei and upstream thalamic and cortical centers. Our observations suggest that sema III plays a limited role during restriction of developing trigeminal axons to proper pathways and tracts. Other molecular and cellular mechanisms must act in concert with semaphorins in ensuring target recognition, topographic order of projections, and patterning of neural connections.

Introduction

Axonal pathfinding, target selection, and formation of synapses are major steps in wiring of the nervous system. In recent years, increasing attention is directed toward families of diffusible or membrane bound molecules that dramatically influence growing axons as they navigate toward their targets by chemoattraction or chemorepulsion (e.g., netrins, semaphorins, ephrins). Complementing *in vitro* and *in vivo* function deletion experiments suggest that changing expression patterns of these molecules (or their receptors) in developing neural and nonneural tissues play a major role in steering growing axons and streamlining axonal trajectories (Kennedy *et al.*, 1994; Cheng *et al.*, 1995; Drescher *et al.*, 1995; Messersmith *et al.*, 1995; Püschel *et al.*, 1995; Fujisawa *et al.*, 1997; see Tessier-Lavigne and Goodman, 1996 for review).

Semaphorins are a large family of secreted and transmembrane glycoproteins characterized by an extracellular sema domain of approximately 500 amino acids (Kolodkin *et al.*, 1992, 1993; Luo *et al.*, 1993; Messersmith *et al.*, 1995; Püschel *et al.*, 1995; Kolodkin and Ginty, 1997; Koppel *et al.*, 1997; Mark *et al.*, 1997; Zhou *et al.*, 1997). Classes I, IV, V, and VI semaphorins are transmembrane proteins, while classes II and III semaphorins are secreted proteins. They appear to play important roles in axon fasciculation, branching, neuronal migration, and tissue differentiation in both invertebrates and vertebrates (Mark *et al.*, 1997; Zhou *et al.*, 1997). A member of the Class III semaphorins, sema III (sema III/sema D {mouse}/H-sema III {human}/coll-1 {chicken}) is a potent chemorepulsive signal for subsets of sensory axons (Luo *et al.*, 1993; Messersmith *et al.*, 1995; Püschel *et al.*, 1995; Wright *et al.*, 1995; Giger *et al.*, 1996; Feiner *et al.*, 1997). *In vitro* assays show that sema III (collapsin-1) causes growth cone collapse in primary sensory axons (Luo *et al.*, 1993; Fan and Raper, 1995) and repels NGF-dependent nociceptive neurons (Messersmith *et al.*, 1995). Its expression is also spatiotemporally regulated in the CNS and a variety of nonneural tissues such as the precartilage primordium (Wright *et al.*, 1995).

Null mutation of the sema III gene in mice leads to abnormal projection patterns of primary sensory axons, hypertrophy of the right ventricle of the heart and dilation of the right atrium, abnormal bone and cartilage development, and defects in dendritic orientation of layer V neocortical pyramidal cells (Behar *et al.*, 1996; but see Catalano *et al.*, 1998 for the latter). Anomalous peripheral nerve trajectories in embryonic sema III knockout mice were also documented by Taniguchi *et al.* (1997). In this study, we examined peripheral and central trigeminal axon projections, somatosensory thalamocortical projections, and whisker-related central patterns in sema III knockout mice during perinatal development. We found that peripheral and central projections of the trigeminal ganglion (the first link between the sensory epithelium and the central nervous system) are severely defasciculated, disorganized, and exuberant in sema III knockout mice. In contrast, the last leg of the trigeminal pathway, thalamocortical projections from the ventrobasal complex of the dorsal thalamus, does not show any noticeable projection errors. Disarrayed organization of peripheral and central trigeminal ganglion projections correlates with a transient and well-defined expression of sema III receptor, neuropilin, during the initial stages of trigeminal tract formation. Despite the overall projection anomalies, peripheral trigeminal axons develop normal innervation of the whisker follicles, and their central counterparts form whisker-specific patterns, “barrelettes,” in the brain-stem. Patterned neural aggregates “barreloids” in the ventrobasal complex of the dorsal thalamus and “barrels” in the primary somatosensory cortex are also present in these mice, indicating that once the patterns are established in the brainstem they are readily transferred to upstream trigeminal centers.

Results

The generation of sema III knockout mice was described previously (Behar *et al.*, 1996). The mice used in this study were derived from a colony of breeding heterozygous mice at LSU Medical Center. The founder mice for this colony were provided by Drs. O. Behar and M. Fishman in February 1998. Most of the examined mice were embryonic day (E) 13–19 ($n = 17$ ko, 21 wt, 47 heterozygous). A few ($n = 8$ ko, 11 wt and 23 heterozygous) were postnatal pups. In a given litter of 7–8 embryos/postnatal pups, there were usually 1–2 knockout mice. Embryos and postnatal pups were genotyped using PCR amplification (Fig. 1). In general sema III knockout mice die within the first postnatal week. Postmortem analyses in these mice revealed severe heart defects as reported earlier (Behar *et al.*, 1996).

Our focus in this study was on the trigeminal pathway for the following reasons. First, previous studies on the sema III and neuropilin (sema III receptor) knockout mice, documented defasciculation of primary sensory axons in the periphery during embryonic

development (embryonic day E9.5–12.5; Kitsukawa *et al.*, 1997; Taniguchi *et al.*, 1997). Second, the trigeminal pathway of rodents is characterized by patterning of afferent axon terminals and postsynaptic target cells into an array of modules in the brainstem, thalamus, and somatosensory cortex (barrelettes, barreloids, barrels) that replicates the spatial organization of whiskers on the animal's snout (Woolsey, 1990). Thus, it provides an excellent model system to dissociate the role of specific axon guidance molecules in pathway development, topography of neural connections, and pattern formation.

Trigeminal and Somatosensory Thalamocortical Projections

We first examined peripheral and central projections of the trigeminal ganglion in mutant mice at a stage when these axons normally develop well-defined pathways. In mice, trigeminal axons venture out around E10 and reach the peripheral target fields such as the maxillary pad (where the whisker pad develops) by E11, and the central trigeminal tract is laid down fully by E13 (Stainier and Gilbert, 1990, 1991; A.D., E.U., and R.E., unpublished observations). Thus, by E13 the three major peripheral divisions of the trigeminal nerve (ophthalmic, maxillary, and mandibular), as well as the ascending and descending components of the central trigeminal tract can be visualized in their full extent. The major component of the maxillary nerve is the infraorbital (IO) branch which innervates the whisker pad. As illustrated in Fig. 2A normal trajectory of the developing trigeminal pathway can be visualized following lipophilic carbocyanine dye, DiI, implants into the ganglion in paraformaldehyde-fixed E13 embryos. In striking contrast to discrete projection pathways taken by the peripheral and central components of the trigeminal ganglion projections (Figs. 2A, 3A, and 3C), knockout littermate embryos displayed aberrant projection patterns (Figs. 2B, 3B, and 3D). All three divisions of the peripheral trigeminal nerve were defasciculated and it was not clear as to which bundles of axons belonged to each subdivision. The IO nerve, instead of being one tight bundle, was broken into a network of axon fascicles (compare Figs. 3A and 3B). Within the peripheral target fields, the axons fanned into overlapping fields. The disarrayed peripheral projections could also be observed by other axonal markers, such as neurofilament C, TrkA, and neuropilin immunostaining (see below). Defasciculation of peripheral axons was most conspicuous in E13 embryos but could still be visualized at later embryonic ages. The central root of the ganglion normally enters the brainstem in one thick, short trunk (Fig. 2A). In knockout embryos this central trunk was still short but broken into two, or three, bundles that entered the hindbrain at different points (Fig. 2B). In the mutant embryos, the ascending and descending components of the central trigeminal tract (Fig. 2B, arrows), as well as retrogradely labeled string of mesencephalic nucleus neurons could be easily discerned. However, the central trigeminal tract appeared wider and the axons were often disheveled (compare Figs. 3C and 3D).

To examine central trigeminal projections at a later developmental stage, we labeled the trigeminal ganglion with DiI in E17 embryos and sectioned the brainstem in the coronal plane. At this age different components of the brainstem trigeminal nuclei can be readily identified. In the mutant brainstem, central trigeminal axons explored beyond the medial boundaries of the trigeminal nuclei (Figs. 2C and 2D, arrowheads). These extensive projections cannot be attributed to possible differences in the amount of DiI injected into the trigeminal ganglion, because we did not see exuberant projections in wild-type embryos even after large amounts of DiI injections.

After documenting these projection defects along the first leg of the trigeminal pathway, we wanted to see if such projection errors were also present along the second (trigeminothalamic) and third (thalamocortical) segments, of the pathway from whiskers to the barrel cortex. Due to labeling difficulties and visualizing the lemniscal pathway in a few consecutive sections, in any given plane, we chose to examine only the thalamocortical

projections. DiI implants were placed in the ventrobasal nucleus in E17 embryos (a stage when thalamocortical axons have just arrived in the somatosensory cortex and collected under the cortical plate, Senft and Woolsey, 1991; Agmon *et al.*, 1993; Molnár *et al.*, 1998). This procedure did not reveal any noticeable targeting errors along the thalamocortical pathway (Figs. 2E and 2F).

Developmental Regulation of Sema III Receptor Neuropilin Expression along the Trigeminal Pathway

In a separate series of experiments we examined expression of neuropilin, sema III receptor, using a polyclonal antibody to neuropilin (Kawakami *et al.*, 1996). During the initial stages of trigeminal pathway development, the trigeminal ganglion, and its peripheral and central axons, were strongly neuropilin-positive in both the wild-type and sema III knockout embryos. Along the central trigeminal tract of wild-type mice, the expression of neuropilin is very strong during the initial stages of tract formation (E13), while the central axons are elongating without any noticeable collateralization (Fig. 4A). At later stages (after E15), when central trigeminal axons collateralize and form terminal arbors in the brainstem trigeminal nuclei, neuropilin expression is downregulated (Fig. 4C), and it is no longer present at E18 (Fig. 4E). We did not observe any neuropilin immunoreactivity along the developing thalamocortical pathway even at early stages of development (data not shown). This observation also correlates with normal projection patterns of thalamocortical axons in sema III knockout mice.

In the trigeminal tract of sema III knockout embryos neuropilin expression was also high between E13 and E15 (Figs. 4G–4I) and began downregulation after E15. In these cases, too, defasciculation and expansion of the central trigeminal tract could be detected (Figs. 4G–4I). We also processed alternate sections from brainstem wholemounts with trigeminal ganglion attached, for TrkA immunohistochemistry. The polyclonal TrkA antibody (gift of Dr. L. Reichardt) labels mostly NGF-dependent, sema III-sensitive primary sensory axons (Clary *et al.*, 1994; Messersmith *et al.*, 1995). In normal mouse embryos TrkA antibody heavily labels trigeminal ganglion cells and a discrete central trigeminal tract (Figs. 4B, 4D, and 4F). In sema III knockout mouse embryos at E15, TrkA-positive component of the trigeminal tract showed distinct defasciculation (Fig. 4J). However, comparison of TrkA and neuropilin immunoreactive components of the defasciculated trigeminal tract suggests that these projection abnormalities may not be unique to TrkA-positive axons in the trigeminal system. A detailed analyses of neuropilin expression by different axon populations (i.e., small and large caliber fibers or fibers that carry nociceptive, mechanoreceptive, and proprioceptive information) were beyond the scope of the present study. In contrast to central trigeminal projections, neuropilin expression is maintained in the peripheral trigeminal axons much longer. In the whisker pad, neuropilin expression was high between E13 and E15 and could be detected at E18 (Fig. 5).

Whisker Follicle Innervation and Pattern Formation along the Trigeminal Pathway

In sema III knockout mice, five rows of whisker follicles and an array of sinus hairs flanking these rows develop normally. Considering the severe projection errors in the peripheral and central target fields, we expected to find errors in innervation of the whisker follicles on the snout and defects in whisker-related pattern formation in central trigeminal pathways. We were surprised to see that individual follicles were innervated normally and whisker-specific patterns, barrelettes in the brainstem trigeminal nuclei, barreloids in the ventrobasal thalamus, and barrels in the somatosensory cortex all appeared normal in mutant animals (Figs. 5 and 6).

Disarrayed projections of the trigeminal ganglion in peripheral target fields could be visualized by a number of histological labeling techniques such as DiI, and immunohistochemistry using neurofilament C, TrkA, or neuropilin antibodies. In sema III knockout embryos, the infraorbital nerve projections were not organized. Distinct row nerves that travel from caudal to rostral between rows of follicles, characteristic of wild-type embryos, were disarrayed in the knockout embryos (compare Figs. 5A and 5B). Nevertheless, around single follicles, deep and superficial nerves penetrated the external root sheaths and branched out similar to that seen in wild-type embryos (compare Figs. 5C and 5D, 5E and 5F, 5G and 5H).

Centrally, in the brainstem trigeminal nuclei, cytochrome oxidase dense barrelette rows, each with distinct numbers of patches, were clearly visible in postnatal day 5 wild-type and knockout mice (Figs. 6A and 6B). Normally, the dorsal-most row of whiskers (row A) is represented ventrally and the ventral-most row of whiskers (row E) dorsally in the brainstem trigeminal nuclei. It is easy to discern rows A–E because of lesser number of barrelettes in row A (corresponding to lesser number of row A whisker follicles) than in row E. Furthermore, row E barrelettes are flanked by numerous smaller barrelettes corresponding to ventral sinus hairs on the snout. The organization of barrelettes in the brainstem trigeminal nuclei of sema III knockout mice exhibited all the features of normal barrelettes in normal mice as assessed by cytochrome oxidase histochemistry. This implies that topography of trigeminal projections in sema III knockout mice is not altered. Therefore, we did not perform anatomical topography analyses by implanting alternating or spatially restricted lipophilic tracers along the dorsoventral axis of the snout (see Erzurumlu and Jhaveri, 1992). Development of patterns in the brainstem trigeminal nuclei of sema III knockout mice most likely reflects normal innervation of whisker follicles and consequent activation of the properly connected nerves. Furthermore, exuberance of central trigeminal axons in the ventrally situated barrelette region of the principal sensory nucleus (PrV) was minimal in comparison to lush overshooting of trigeminal axons dorsomedially (Fig. 2). Cytochrome oxidase histochemistry also revealed whisker-related patterns, barreloids, in the dorsal thalamus (Figs. 5C and 5D) and barrels in the somatosensory cortex (Figs. 5E and 5F). Thus, once the patterns are established in the ventral PrV, they are readily transmitted to upper levels of the trigeminal neuraxis.

Discussion

Primary somatosensory axons are responsive to various members of families of axon guidance molecules as they lay down specific, orderly pathways and develop patterned connections with the central nervous system. Trigeminal ganglion axons, much like dorsal root ganglion (DRG) fibers, initially grow toward their peripheral and central targets in distinct bundles or fascicles and form the peripheral divisions of the trigeminal nerve in the head and the ascending and descending components of the trigeminal tract in the brainstem (Stainier and Gilbert, 1990, 1991; Erzurumlu and Jhaveri, 1992). During pathway formation, these axons grow in the elongation phase without any branching or arbor formation. Once the pathways are established, trigeminal axons elaborate specific arborization patterns peripherally and centrally. Axons which innervate the whiskers and perioral sinus hairs form discrete central arbors that replicate the patterned organization of follicles on the snout (Erzurumlu and Killackey, 1983; Erzurumlu and Jhaveri, 1992). In this report, we show that null mutation of the sema III gene severely disrupts the fasciculation of both peripheral and central trigeminal axon trajectories, but does not affect the thalamocortical pathway that carries trigeminal information to the neocortex. Differential spatial expression of sema III receptor, neuropilin along the first and last legs of the pathway between the whisker pad and the barrel cortex corroborates our observation that null mutation of the sema III gene leads to pathway errors of trigeminal ganglion cells but not in axonal trajectories of

thalamocortical neurons. Despite projection errors and defasciculation of trigeminal axons, innervation of the whisker follicles on the snout and whisker-specific pattern formation along the trigeminal pathway in sema III knockout mice are not affected. It might be that among the disarrayed projections to the periphery and the brain-stem, a population of axons that reach individual whisker follicles respond to local cues to develop normal innervation patterns. Consequently, their central counterparts can establish patterned arbors using other developmental mechanisms, such as correlated activity incoming from the whisker follicles. Thus, the present results, along with other reports on the peripheral trigeminal projections in sema III and neuropilin knockout mice (Kitsukawa *et al.*, 1997; Taniguchi *et al.*, 1997), indicate that sema III plays a crucial role in restricting trigeminal axons along specific routes.

In vitro function blocking studies show that neuropilin is required for chemorepulsive/collapsing actions of sema III (He and Tessier-Lavigne, 1997; Kolodkin *et al.*, 1997). These results are further corroborated by the observations that sensory neurons from neuropilin knockout mice do not respond to sema III as their wild-type counterparts do (Kitsukawa *et al.*, 1997). Furthermore, sema III and neuropilin knockout mice show similar phenotypic abnormalities in defasciculation of sensory axons and aberrant projection patterns (Kitsukawa *et al.*, 1997; Taniguchi *et al.*, 1997). Overexpression of neuropilin in chimeric mice also leads to abnormalities in peripheral neural projections and cardiovascular system development (Kitsukawa *et al.*, 1995). Our results show that neuropilin is expressed by trigeminal axons during a short period when they are forming the central tract and are growing in the elongation phase. Neuropilin expression by central trigeminal axons is downregulated as these axons begin emitting collaterals into the trigeminal nuclei and forming terminal arbors. Therefore it is likely that sema III–neuropilin-mediated interactions inhibit collateral formation and arborization until the trigeminal tract is laid down. In future studies, it will be interesting to find out if downregulation of neuropilin expression by the trigeminal axons initiates a signal for collateralization and arbor formation. At present, it is not known whether sema III is expressed in areas surrounding the trigeminal tract, thereby preventing these elongating axons from prematurely invading the presumptive brainstem targets. Curiously, peripheral trigeminal axons express neuropilin for longer periods than their central counterparts. In the periphery, various nonneural elements such as cartilage and bone tissues express sema III (Wright *et al.*, 1995). Perhaps the prolonged expression of neuropilin by the peripheral trigeminal axons is necessary for maintaining bundling of peripheral nerves in their proper trajectories as they are surrounded by developing cartilaginous tissues.

Previous reports on axonal projection patterns in sema III and neuropilin knockout mice did not focus on central trigeminal projections (Behar *et al.*, 1996; Kitsukawa *et al.*, 1997; Taniguchi *et al.*, 1997). However, both groups examined DRG projections to the spinal cord. DiI labeling of the thoracic DRGs in wild-type and homozygous embryos showed no significant difference in overall patterns of central projections to the spinal cord in one report (Taniguchi *et al.*, 1997), and abnormal projections of NGF-dependent CGRP-positive axons in the other (Behar *et al.*, 1996). These differences perhaps could be attributed to the ways by which the knockout phenotypes were generated. A more recent report documents axonal connections and neuronal projections in various regions of the sema III knockout mice brains (but not the trigeminal tract or DRG projections in the spinal cord), with no noticeable abnormalities (Catalano *et al.*, 1998). These authors reported that thalamocortical projections and barrel patterns in the somatosensory cortex appear normal in sema III knockout mice.

Numerous studies have underscored the role of target-derived chemoattractant molecules, such as NGF family of neurotrophins, in attracting developing sensory axons to their targets

and supporting cell survival following target invasion (Schechterson and Bothwell, 1992; Buchman and Davies, 1993; Snider, 1994; El Shamy and Ernfors, 1996). It is most likely that sensory axons orient toward the source of these target-derived signals en masse and use these long range cues in target recognition. As they grow toward their targets, differential expression of multiple semaphorins in surrounding tissues may navigate them into specific zones. Furthermore, sema III receptiveness of trigeminal axons might account for why they first grow in the elongation mode forming the trigeminal tract and do not form collaterals and arbors until after they downregulate neuropilin expression.

We do not think that semaphorins alone can dictate axon growth patterns of trigeminal ganglion cells. At a given time in development, many molecular signals must act in concert as growing axons undertake target-directed pathfinding, emit collateral branches, and form terminal arbors. Our recent *in vitro* studies show that when embryonic trigeminal ganglion and brainstem wholemounts are grown in serum free culture medium, central trigeminal axons maintain their growth in the elongation phase and remain restricted to the tract. When neurotrophins NGF or NT-3 are added to the culture medium, these axons readily form collateral arbors into the surrounding brainstem trigeminal nuclear regions, or as in the case of NGF, grow extensively into the medial and lateral regions of the brainstem (Ulupinar and Erzurumlu, 1998). These observations suggest that in the presence of excess concentrations of neurotrophins central trigeminal axons can overcome sema III–neuropilin-mediated restriction of their trajectories.

Clearly, no single axon guidance molecule and its specific receptor alone can account for the complex behavior of developing axons during target selection and innervation. While in the present report we show that sema III receptor is transiently expressed along the developing trigeminal tract during axon elongation phase and these axonal pathways are severely affected in sema III knockout mice, other families of axon guidance molecules (e.g., ephrins, netrins, cell adhesion, and extracellular matrix molecules) must also play a role in development of the trigeminal pathway. We postulate that different classes of developing sensory axons are equipped with different sets of receptors for a variety of growth promoting, chemoattractant and chemorepellent signals. Consequently, the growth cones summate all these environmental cues and elicit a weighted response.

Experimental Methods

Genotyping

Genotype analysis of the sema III mice was done by extracting DNA from tail samples using salt-out procedure. For PCR reaction, oligo sequences designed for Neo1 (GCTTGGGTGGAGAGGCTATTC), Neo2 (CAAG-GTGAGATGACAGGAGATC), sema 3F2 (CATTATCC-GGTGCCTGGCTCGATT), and sema 3R3 (TCCTTCCT-GTATTGTGCGGCCAGA) were used. The samples were heat denatured at 95°C for 5 min, amplified 30 cycles at 95°C for 30 s, then at 70°C for 1 min. PCR products were resolved on a 2% agarose gel. Neo oligo gives about 280-bp fragment and the sema oligo gives about 200-bp fragment (see Fig. 1).

Preparation of Embryonic Brainstem Wholemounts

Timed-pregnant mice were obtained from the sema III breeding colony established at Louisiana State University Medical Center Institutional Animal Care Unit. Embryos from 13- to 18-day pregnant mice (day of vaginal plug designated as embryonic day 0) were removed by cesarean section following intraperitoneal administration of sodium pentobarbital (50 mg/kg body weight). Embryos were collected in ice-cold Gey's balanced salt solution supplemented with dextrose (GBSS). First, tissue samples of each embryo were collected for DNA extraction and genotyping. Then brainstem whole-mounts with intact

trigeminal ganglia were dissected under a stereomicroscope using dark-field illumination. After dissecting the left and right whisker pads as explants, the remaining part of the body was used for postmortem evaluation. Brainstem wholemounts were placed on Millicell (Millipore) inserts with microporous (0.4 μm) membranes and excess GBSS was suctioned off. The Millicell inserts were placed in six-well plates, containing 1 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS) per well.

Immunohistochemistry

The fixed brainstem wholemounts from E13 to E15 embryos, horizontal sections through the brainstem of E18 embryos, and whisker pads from E13 to E18 embryos were cryoprotected with 30% sucrose and frozen sectioned at a thickness of 35 μm . Endogenous peroxidase activity was quenched by immersing sections in 10% methanol and 3% hydrogen peroxide in Tris-buffer for 15–20 min. For neuropilin immunohistochemistry sections were first treated with 5% skim milk in tris-buffered saline (TBS) for 30 min. Sections were incubated in rabbit anti-neuropilin antibody (1:200) in TBS overnight at 4°C. For TrkA immunohistochemistry, sections were first treated with 10% normal goat serum in TBS containing 0.3% Triton X-100 for 1 h at room temperature. Sections were incubated in rabbit anti-TrkA antibody (1:2000, gift of Dr. L. Reichardt) in TBS overnight at 4°C. The next day sections were washed in TBS and incubated in biotinylated goat anti-rabbit antibody (1:200, Sigma). All of the sections were treated with avidin-biotin-peroxidase complex (Vectastain Elite, Vector Laboratories) for 1 h at room temperature and placed in 0.025% DAB and 0.3% hydrogen peroxidase in TBS or PBS for 5–10 min. For neurofilament immunohistochemistry, sections were blocked in 4% normal goat serum with 0.3% Triton X-100 for 30 min at room temperature and incubated in rabbit anti-neurofilament C antibody (1:200, Chemicon) overnight at 4°C. The following day sections were incubated in FITC-conjugated goat anti-rabbit antibody (1:128, Sigma) for 2 h at room temperature. For all immunohistochemical staining procedures, control sections were processed as above except that the primary antibody was omitted.

Cytochrome Oxidase Histochemistry

Postnatal mice obtained from the sema III breeding colony were transcardially perfused with 4% paraformaldehyde in PBS. Fixed brains of postnatal day 3–5 mice were cryoprotected with 30% sucrose and frozen sectioned at a thickness of 50–60 μm . Sections were incubated in a solution of 90 ml phosphate buffer with 4 g sucrose, 10 mg DAB, and 40 mg cytochrome C at 37°C in a shaker incubator for several hours. The reaction was stopped by several rinses in PBS after the sections turned golden brown. Sections were mounted on subbed slides and cover slipped with an aqueous medium containing gelatin and glycerol.

DiI Labeling

Embryonic day (E) 13–18 mice were immersion fixed in 4% paraformaldehyde in PBS. To visualize peripheral and central axonal projection patterns along the trigeminal pathway crystals of the lipophilic tracer DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine per-chlorate, Molecular Probes) were inserted into the trigeminal ganglion or the ventral posterior medial nucleus of the thalamus. The labeled specimens were kept at 37°C for 2 weeks and then sectioned at 100- μm thickness using a vibratome. Labeled specimens were photographically documented using epifluorescence and a rhodamine filter set. Due to the thickness of sections, labeled axonal profiles could not be visualized in their entirety in a given section. To better visualize these axons, tracings were made from photographic slides projected onto a screen (see Fig. 3).

The protocols used in this study were approved by the Louisiana State University Medical Center Institutional Animal Care and Use Committee.

Acknowledgments

We are grateful to Dr. L. Reichardt for providing the TrkA antiserum. We also thank Mr. T. Ulupinar for his assistance with the figures. E. Ulupinar is supported by a predoctoral fellowship from the Higher Education Commission of Turkey and Osmangazi University. Research supported by NIH (NS32195 to R.E.).

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Fig. 1. Genotype analysis of *sema III* mice by PCR. The lower band (about 240-bp fragment) is specific for the wild-type *sema III* allele, and the upper band (about 280-bp fragment) is specific for the mutant allele.

Fig. 2. Peripheral and central projections of trigeminal ganglion in E13 wild-type (A) and sema III knockout (B) embryo from the same litter. Note the highly defasciculated and entangled peripheral projections in the knockout embryo. Central root of the trigeminal nerve is also broken into separate bundles (arrows), and the central tract is wider and exuberant in the knockout case. Photographic montages were made from two consecutive 100- μ m-thick sagittal sections through the embryo head. wp, developing whisker pad; mp, mandibular process; TG, trigeminal ganglion; atr, ascending trigeminal tract; dtr, descending trigeminal tract. Scale bar, 400 μ m in A and B. Central trigeminal projections in the PrV of E17 wild-type (C) and sema III knockout (D) littermate embryos (coronal sections through the brainstem). Note that there are exuberant projections that pass beyond the medial boundary of the nucleus in the knockout case (arrowheads). These overshooting projections are particularly pronounced in the dorsal portions of the brainstem. In PrV, whisker-related patterns, or barrelettes develop in the ventral $\frac{1}{3}$ of the nucleus. D, dorsal; M, medial. Scale bar, 350 μ m in C and D. DiI-labeled thalamocortical projections in the primary somatosensory cortex of an E17 wild-type (E) and knockout embryo (F). Unlike in the case of trigeminal ganglion projections, we could not detect any exuberant projections along this pathway. CP, cortical plate; WM, white matter. Scale bar, 200 μ m in E and F.

Fig. 3. Higher magnification views of aberrant trigeminal projections in sema III knockout mice. The photographic inset in the center of the figure shows DiI-labeled peripheral and central trigeminal projections in wild-type (A and C) and knockout (B and D) E13 embryos. Tracings of individual axons from such photographic images are shown next to each micrograph. Infraorbital nerve (ION) in wild-type embryos is initially a tightly fasciculated bundle and then fans out into presumptive whisker row nerves in the whisker pad (WP). In sema III knockout embryos, this nerve is defasciculated and forms a widespread arboreal projection in the snout. Similar defasciculation is also evident in the projections of the mandibular nerve (MN) as it invades the mandibular process (MP). The central trigeminal tract is a well-defined axonal pathway in wild-type E13 embryos, but it is expanded and defasciculated in the knockout case. Scale bars for the drawings are 200 μm in A and B and 100 μm in C and D.

Fig. 4. Developmental regulation of neuropilin expression in the trigeminal ganglion and the central trigeminal tract. (A–F) Neuropilin and TrkA immunoreactivity in the trigeminal ganglion and central tract of E13–E18 wild-type embryos. Between E13 and E15, the trigeminal tract (arrowheads) is laid down, and at this stage axons do not collateralize or invade the developing trigeminal nuclei. At these ages trigeminal axons are positive for both TrkA and neuropilin. After E15, when trigeminal axons begin collateralization into the trigeminal nuclei, neuropilin expression is downregulated along the central trigeminal tract. By E18, neuropilin expression virtually disappears from the central trigeminal tract (E), whereas TrkA immunoreactivity is still present (F). (G–I) Neuropilin expression in the central trigeminal tract of sema III knockout mice at E13 and E15. Defasciculation and mediolateral expansion of the central trigeminal tract can be seen at both ages. (J) High power photomicrograph of TrkA-positive central trigeminal tract from an E15 sema III knockout embryo showing defasciculation. Arrows show the entry zone of central trigeminal axons. All sections are in the horizontal plane and are oriented in the same way with rostral to the top and medial to the right. Scale bar, 150 μ m in A–F, 200 μ m in G–J.

Fig. 5. Whisker follicle innervation and distribution of whisker afferents in wild-type and semaphorin III knockout embryos. Neuropilin immunoreactive IO nerve axons in the whisker pad of E15 wild-type (A) and knockout (B) embryos. Note that IO axons separate into distinct whisker row nerves (asterisks) in the wild-type whisker pad (A), but this organization is not present in the knockout case (B). Neuropilin immunoreactivity in peripheral trigeminal axons is still present at E18 in both wild-type and knockout cases (C, D). In the whiskerpad, individual whisker follicles (asterisks) receive a ring of innervation (arrows) that is comparable in wild-type and knockout animals. Innervation of whisker follicles can be seen with neuropilin immunostaining (C, D), DiI labeling (E, F), and neurofilament immunohistochemistry (G, H). Photomicrographs A–D, G, and H are from sagittal sections through the whisker pads. In coronal sections through the whisker pads, innervation of the individual whisker follicles along the longitudinal axis can be seen (E, F). Folicle innervation in both cases appear similar (arrows). Scale bar, 500 μ m in A–D, 300 μ m in E–H.

Fig. 6. Whisker-related patterns along the central trigeminal pathway of postnatal day 5 wild-type and sema III knockout mice. Barrelettes in the subnucleus interpolaris of the brainstem trigeminal complex in wild-type and knockout pups are similar (A and B). Similarly, barreloids in the ventrobasal nucleus of the thalamus (C and D) and barrels in the somatosensory cortex (E and F) can be visualized with cytochrome oxidase histochemistry in both the wild-type and sema III knockout pups. Whisker-related rows a–e are indicated in A and B, and arrowheads point to whisker-specific patterns in other regions. All sections are in the coronal plane; in A–D, dorsal is up and medial is to the left. Scale bar, 350 μ m.