Slit2, a Branching–Arborization Factor for Sensory Axons in the Mammalian CNS

P. Hande Özdinler and Reha S. Erzurumlu

Department of Cell Biology and Anatomy, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112

Axons that carry information from the sensory periphery first elongate unbranched and form precisely ordered tracts within the CNS. Later, they begin collateralizing into their proper targets and form terminal arbors. Target-derived factors that govern sensory axon elongation and branching–arborization are not well understood. Here we report that Slit2 is a major player in branching–arborization of central trigeminal axons in the brainstem. Embryonic trigeminal axons initially develop unbranched as they form the trigeminal tract within the lateral brainstem; later, they emit collateral branches into the brainstem trigeminal nuclei and form terminal arbors therein. In whole-mount explant cultures of this pathway, embryonic day 15 (E15) rat central trigeminal axons retain their unbranched growth within the tract, whereas E17 trigeminal axons show branching and arborization in the brainstem trigeminal nuclei, much like that seen *in vivo*. Similar observations were made in E13 and E15 mouse embryos. We cocultured Slit2-expressing tissues or cells with the whole-mount explant cultures of the central trigeminal pathway derived from embryonic rats or mice. When central trigeminal axons are exposed to ectopic Slit2 during their elongation phase, they show robust and premature branching and arborization. Blocking available Slit2 reverses this effect on axon growth. Spatiotemporal expression of Slit2 and Robo receptor mRNAs within the brainstem trigeminal nuclei and the trigeminal ganglion during elongation and branching–arborization further corroborates our experimental results.

Key words: Slits; Robos; trigeminal ganglion; trigeminal system; choroid plexus; explant cocultures; axon branching; axon arborization

Slit proteins, which bind Robo receptors, were identified as repellent guidance cues that regulate midline crossing behavior of axons in *Drosophila* (Seeger et al., 1993; Kidd et al., 1998; Brose et al., 1999; Simpson et al., 2000) (for review, see Chisholm and Tessier-Lavigne, 1999; Guthrie, 1999; Harris and Holt, 1999; Van Vactor and Flanagan, 1999; Brose and Tessier-Lavigne, 2000). Subsequent experiments showed that the Slit family of proteins are conserved across species and play a major role in repulsive axon guidance, as well as in guiding migrating cells by repulsion (Brose et al., 1999; Hu, 1999; Kidd et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Wu et al., 1999, 2001; Erskine et al., 2000; Ringstedt et al., 2000; Kramer et al., 2001). A novel function for one of the members of the Slit family has been identified in axon branching. Wang et al. (1999) devised a biochemical assay to detect activities in brain extracts that could induce branching of dissociated dorsal root ganglion (DRG) cell axons. Purification assays led to the identification of a 140 kDa protein, which turned out to be the N-terminal portion of Slit2. *Slit2* gene encodes a large (190–200 kDa) secreted protein that is cleaved into two fragments, the 140 kDa N terminus and the smaller C terminus. It was found that the 140 kDa fragment promotes axon branching in dissociated DRG cells but not the full-length Slit2 protein (Wang et al., 1999). We tested the role of Slit2 in branching behavior of trigeminal axons in the brainstem and provide direct evidence that Slit2 induces axon branching–arborization in the CNS.

During embryonic development, central trigeminal axons enter the brainstem at embryonic day 12 (E12) in rats. They bifurcate to lay down the ascending and descending components of the trigeminal tract and grow unbranched in the elongation phase until E17. At E17, they emit radially oriented collaterals into the brainstem trigeminal complex (BSTC) (see Fig. 1*A–C*) in which they form terminal arbors that replicate the patterned array of whiskers on the snout (Erzurumlu and Killackey, 1983; Erzurumlu and Jhaveri, 1992; Waite et al., 2000). To investigate the role of Slit2 and Robo receptors (Robo1 and Robo2) in axonal arborization and branching at critical time points in development, we used whole-mount explant cultures of the trigeminal ganglion (TG) and brainstem derived from embryonic rats (see Fig. 1). Brainstem–TG intact whole-mount preparations do not require exogenous neurotrophin supply for survival and many aspects of trigeminal tract development mimic *in vivo* conditions (Ulupinar et al., 2000; Özdinler and Erzurumlu, 2001). This *in vitro* model is well suited for testing the role of a variety of molecules on axon growth behaviors. We show that ectopic Slit2 expression along the trigeminal tract during the axon elongation phase abruptly induces axon branching–arborization. Furthermore, expression of Slit2 mRNA in the BSTC during trigeminal axon branching–arborization and Robo receptor mRNAs in the TG suggest that Slit–Robo interactions might play a key role in the developmental regulation of central trigeminal axon branching and arborization.

MATERIALS AND METHODS

Preparation of whole-mount cultures. All of the protocols used were approved by the Louisiana State University Health Sciences Center

Received Aug. 16, 2001; revised March 13, 2002; accepted March 22, 2002.

This work was supported by the National Institutes of Health. We thank K. Brose and M. Tessier-Lavigne for generous supply of Robo1 and Robo2 cDNA, as well as the hSlit2, Robo1-Fc, Robo2-Fc expression vectors, D. Ornitz for mSlit1, mSlit2, and mSlit3 cDNA, P. Cserjesi for anti-c-myc antibody, A. K. Dubey for help with Western Blot, and P. Bark for help with the figures and manuscript preparation.

Correspondence should be addressed to Dr. Reha S. Erzurumlu, Department of Cell Biology and Anatomy, Louisiana State University Health Sciences Center, 1901 Perdido Street, New Orleans, LA 70112. E-mail: rerzur@lsuhsc.edu.

 0270-6474/02/224540-•$15.00/0

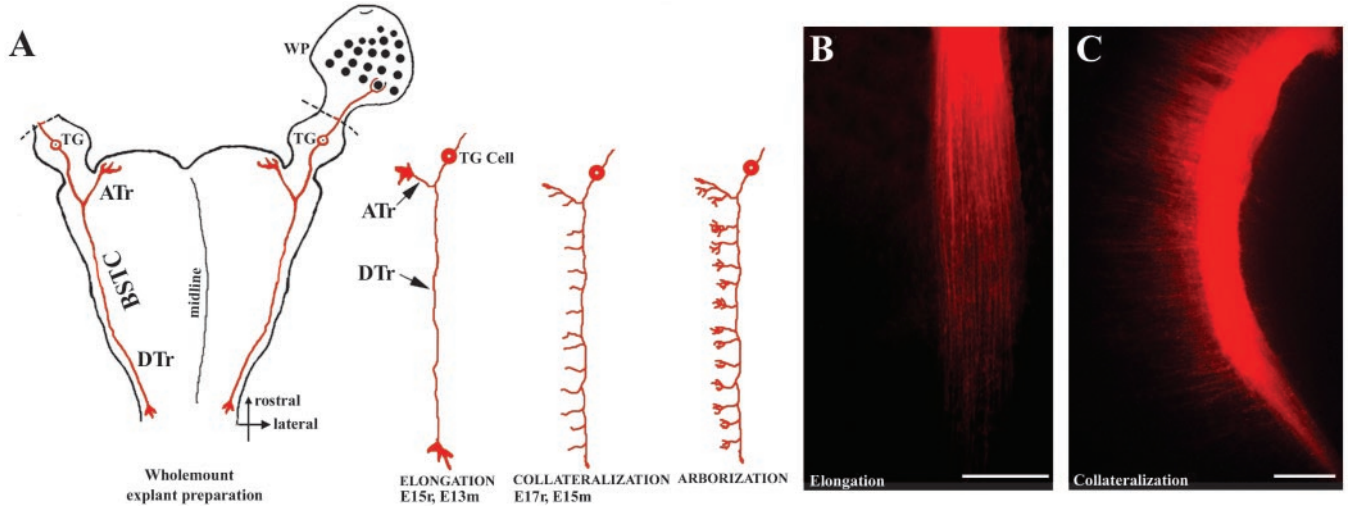


Figure 1. Brainstem–TG intact whole-mount pathway and axon outgrowth patterns of central trigeminal tract axons at different developmental stages. *A*, Schematic representation of brainstem–TG whole-mount preparation and the central trigeminal tract during elongation, collateralization, and arborization phases. In our cultures, the whisker pad (*WP*) portion of the pathway was left out (*dashed lines*). Previous work in our laboratory showed that absence of this peripheral target does not influence central trigeminal axon behavior *in vitro*. *B*, DiI-labeled trigeminal tract axons during the elongation phase (*E15r*). *C*, DiI-labeled trigeminal tract at the time of collateralization–arborization phase (*E17r*). The same type of axon growth is seen in mice at E13 (*E13m*) and E15 (*E15m*) preparations as well (data not shown). For all figures, *right* is lateral, and *top* is rostral. *ATr*, Ascending trigeminal tract; *DTr*, descending trigeminal tract. Scale bars, 100 μm.

Institutional Animal Care and Use Committee and conformed to the National Institutes of Health guidelines for use of animals. Day of sperm positivity (or presence of vaginal plug) was designated as E0. Embryos from timed pregnant mice and rats were removed by Cesarean section after killing the dam. Preparation of whole-mount cultures of the trigeminal pathway was detailed previously (Ulupinar et al., 2000; Özdinler and Erzurumlu, 2001). Choroid plexus (CP) was isolated from the lateral ventricles of adult rat or mouse brains and cut into smaller pieces. A single piece of CP was placed lateral to the central trigeminal tract in the TG brainstem intact whole-mount culture preparations ($n = 22$). As a negative control, a piece of skin tissue (Slit2-negative tissue) (Yuan et al., 1999) (our unpublished observations) isolated from E15 rats was used instead of CP ($n = 10$). Human embryonic kidney 293 (HEK293T) cells were lipofected (Lipofectamine; Invitrogen, Gaithersburg, MD) according to the protocol of the manufacturer. Robo1-Fc and Robo2-Fc cotransfected HEK293T cells (hereafter referred to as Robo-Fc-transfected cells) were trypsinized and embedded in collagen matrix [220 μl of collagen (3 mg/ml; Sigma, St. Louis, MO), 200 μl of DMEM–F-12, 50 μl of 10× MEM (Invitrogen), and 3 μl of 0.8 M $NaCO_3$, pH 7.4] the day after the lipofection. Collagen matrix containing either untransfected HEK293T cells or Robo-Fc-transfected cells were cut into pieces. CP and collagen matrix were placed lateral to the central trigeminal tract of the brainstem TG intact whole-mount preparations (see Fig. 3*B–D*). Cultures were incubated in DMEM–F-12 (Invitrogen) for 3 d in a humidified chamber (33°C) in the presence of 5% CO_2.

In a separate series of experiments, HEK293T cells, transfected with human Slit2 (hSlit2) expression vector, were plated into six-well plates. Brainstem–TG intact whole mounts were placed on Millicell membranes (Millipore, Bedford, MA), and the membranes were overlaid on the transfected HEK293T cells (see Fig. 4*A*). In some experiments, Robo-Fc conditioned medium (obtained from Robo-Fc-transfected HEK293T cells) was added to the cocultures with hSlit2-transfected HEK293T cells. As a negative control, untransfected HEK293T cells were used with the same experimental set up. All preparations were kept in culture for 3 d. Cocultures were then fixed with 4% paraformaldehyde (PFA) overnight and labeled by inserting small crystals of the fluorescent lipophilic tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR) into the TG. Most of the experiments reported below were performed in rat embryos and a few in mouse embryos. The development and differentiation of the rat and mouse trigeminal pathway are strikingly similar, with a slight shift in the timing of events. For trigeminal tract development, E13 in the mouse corresponds to E15 in the rat (trigeminal axon elongation) and E15 in the mouse to E17 in the rat (trigeminal axon branching–arborization) (Erzurumlu and Killackey, 1983; Erzurumlu and Jhaveri, 1992; Stainier and Gilbert, 1990, 1991; Waite et al., 2000). The results obtained from the rat and mouse embryos were indistinguishable. Our *n* for each experiment was much higher for the rat embryos; thus, we report here results from these experiments and their quantification.

Quantification of axon branching–arborization. In the first series of experiments, we observed localized axon branching only in the vicinity of the CP explants and none in the control or skin explant cases. The number of branch points of single, clearly visible axons along the lateral edge of the trigeminal tract facing the CP was counted at different focal planes. Results were classified according to the number of branch points observed. For each condition, axons were classified, based on the number of branches per axon, and counted (see Fig. 3*F*). For example, if three cases had an average of five branch points per axon, *3* is listed under the *column* corresponding to five branch points. The weighted average for each condition is presented as a bar graph in Figure 3*G*.

In the second series of experiments with hSlit2 secreting HEK293T cells, branching and arborization was profuse both within and along the lateral and medial edges of the tract. In control cases, there were no axons leaving the tract laterally or medially or branching. Rarely, budding branches were seen within the tract (see Fig. 5*B*). In these cocultures, a rectangular window (150 × 75 μm) in the ocular piece of the microscope was used as a unit area to sample branch points in the representative areas. For each condition, two similar locations (one rostral and the other caudal) were selected, and the number of branch points was counted. The average + SD number of branch points in the experimental and control cases was calculated with the help of the Excel program (Microsoft, Seattle, WA). One-tailed *t* test was used for statistical analysis (see Fig. 4*J*). Error bars in the graph represent one SD.

Immunohistochemistry. hSlit2 has a c-myc tag at the C terminus (Brose et al., 1999). HEK293T cells plated into six-well plates and transfected with hSlit2 were fixed with 2% PFA overnight. On the next day, the cells were washed with PBS and blocked for 30 min in blocking solution composed of 10% normal goat serum in 0.3% Triton X-100 dissolved in PBS. Primary antibody (anti-c-myc, 1:200; gift from Dr. P. Cserjesi, Louisiana State University Health Sciences Center, New Orleans, LA) in blocking solution was applied overnight at 4°C. Cells were washed with PBS and blocked for 30 min in blocking solution. Secondary antibody (FITC-conjugated goat anti-rabbit, 1:200 in blocking solution; Chemicon, Temecula, CA) was then applied for 2 hr at room temperature in the dark. Cells were washed with PBS and mounted on slides. For heparin-treated cells, 2 μg/ml heparin was added to the culture medium 2 hr before the removal of the conditioned medium and cell fixation. These cells were then processed for immunohistochemistry.

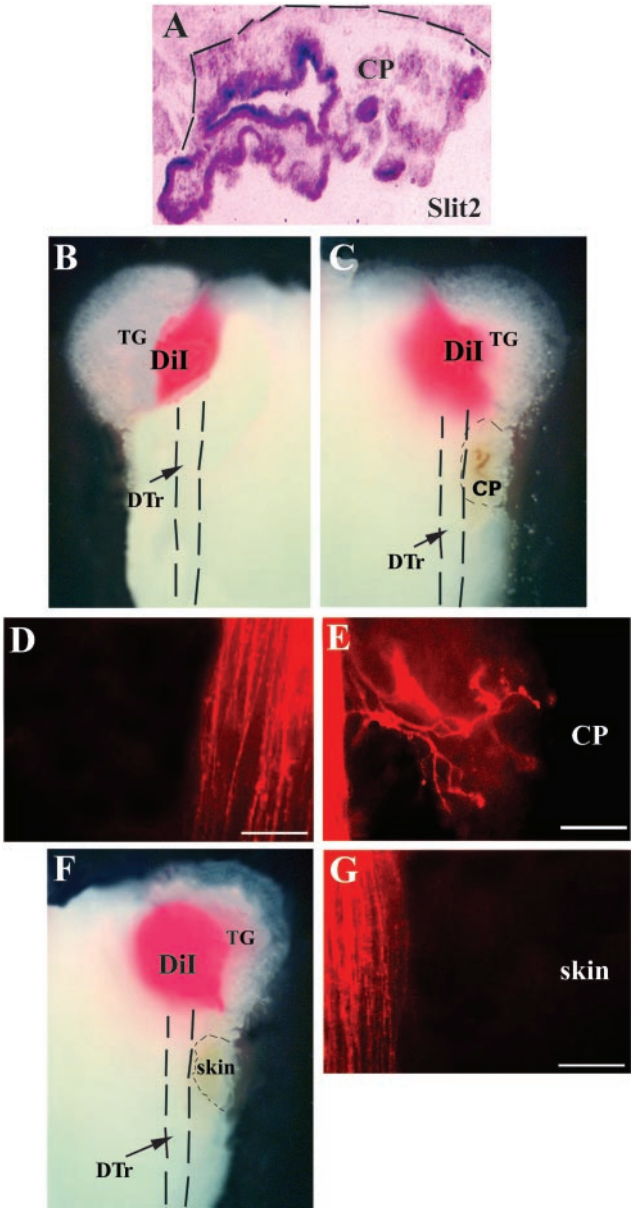
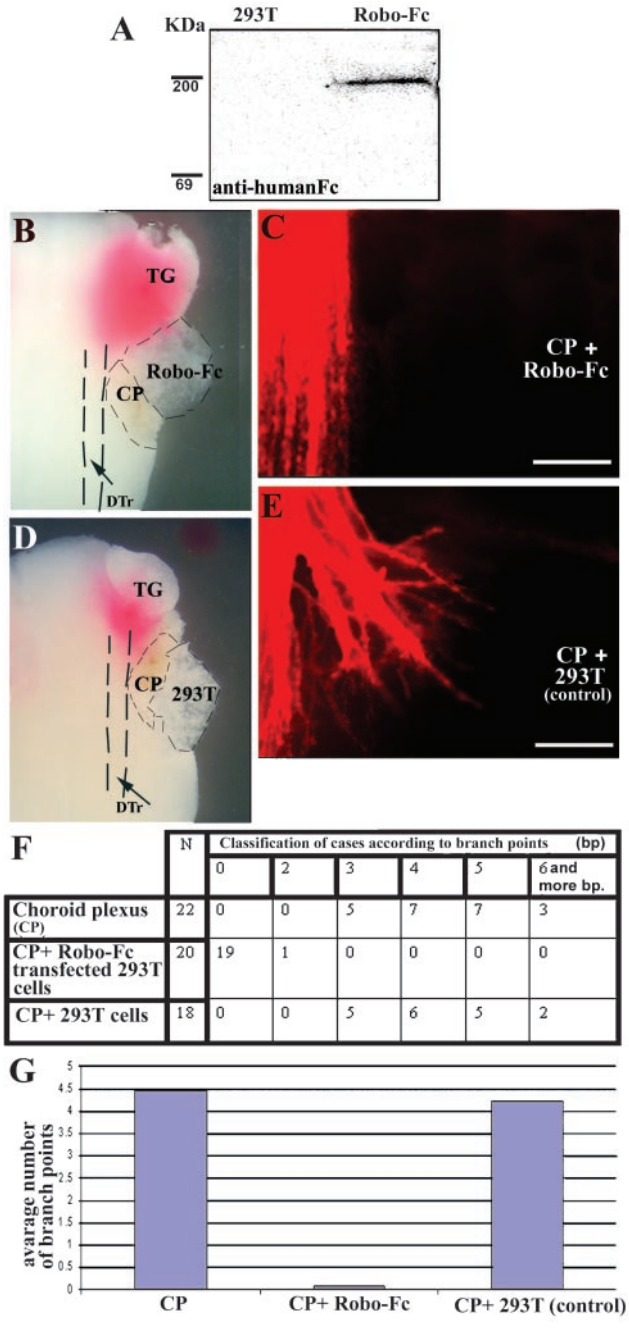


Figure 2. Effect of CP in branching and arborization of central trigeminal tract axons. *A*, Slit2 mRNA expression in adult mouse CP. *B*, E15 rat brainstem–TG whole-mount preparation (control, *left side*). *C*, Brainstem–TG whole mount cocultured with CP (experimental right side of the whole-mount preparation). *D*, High-power photomicrograph of DiI-labeled trigeminal tract axons in the control side as shown in *B*. *E*, High-power photomicrograph of DiI-labeled trigeminal tract axons next to CP explant in the experimental side as shown in *C*. *F*, Brainstem–TG whole mount cocultured with skin. *G*, DiI-labeled trigeminal tract axons next to skin explant. *DTr*, Descending trigeminal tract (indicated by the *dashed lines* in *B–D*). Note that, as a result of flattening of the whole-mount tissue in culture, the trigeminal tract has moved medially compared with its normal location *in vivo*. Scale bars, 50 μm.

Western blot. Twenty-four hours after lipofection, cells were incubated for 2 more days in the presence of Optimem (Invitrogen), and heparin treatment was done as described above. After 2 d, the conditioned medium was collected, separated on SDS-PAGE (8.8%) gel, and blotted on nylon membranes. After blocking for 30 min (5% milk in TBS), the membrane was incubated with anti-c-myc primary antibody (1:1000, in blocking solution) overnight at 4°C. On the next day, the membrane was washed with Tris buffer, and secondary antibody was applied (HRP-conjugated goat anti-rabbit IgG, 1:2000; Pierce, Rockford, IL) for 1 hr.



F

	N	Classification of cases according to branch points (bp)					
		0	2	3	4	5	6 and more bp.
Choroid plexus (CP)	22	0	0	5	7	7	3
CP+ Robo-Fc transfected 293T cells	20	19	1	0	0	0	0
CP+ 293T cells	18	0	0	5	6	5	2

Figure 3. Robo-Fc blocks CP-induced branching and arborization. *A*, Western blot of medium obtained from Robo-Fc-transfected cells and control untransfected cells. *B*, Brainstem–TG whole mount cocultured with CP and Robo-Fc-transfected HEK293T cells. *C*, DiI-labeled trigeminal tract axons next to CP and Robo-Fc-transfected HEK293T cells. *D*, Brainstem–TG whole mount cocultured with CP and untransfected HEK293T cells (control). *E*, DiI-labeled trigeminal tract axons next to CP and untransfected HEK293T cells. *F*, Classification according to branch points (see Materials and Methods). *G*, Bar graph showing average number of branch points for each condition. *DTr*, Descending trigeminal tract. Scale bars, 50 μm.

Color development was performed with Flour S-Max phosphoimager system. To detect Robo-Fc, conditioned medium was collected and blotted as described. The membrane was blocked for 30 min, and then HRP-conjugated anti-human Fc, the secondary antibody (1:2500; Jackson ImmunoResearch, West Grove, PA), was applied for 1 hr. Color was developed as above.

In situ *hybridization.* CP and brainstem–TG intact explants were removed in an RNase-free environment. They were fixed in 4% PFA and processed for *in situ* hybridization on tissue sections. Mouse Slit1 (mSlit1), mSlit2, and mSlit3 vector constructs (gift from D. Ornitz, Washington University, St. Louis, MO) and Robo1 and Robo2 containing vectors (gift from M. Tessier-Lavigne, Stanford University, Stanford, CA) were used for DIG-labeled probe preparation according to the protocol of the manufacturer (Boehringer Mannheim, Indianapolis, IN). *In situ* hybridization for Slit1, Slit2, and Slit3 probes were performed at 55°C overnight, and, for Robo probes, hybridization temperature was set to 60°C. After hybridization, unbound probe was removed by RNase treatment and with stringent washes. The bound probe was detected by alkaline phosphatase (AP)-conjugated anti-DIG Fab fragments, and color reaction was developed by BM purple AP substrate (Boehringer Mannheim).

RESULTS

Slit2-rich choroid plexus induces trigeminal axon branching

We first tested trigeminal axon growth patterns in cocultures with Slit2-rich tissue pieces. Previously, Hu (1999) reported that choroid plexus of embryonic, neonatal, and adult mice and rats expresses high levels of Slit2 and might play a role in repelling migrating forebrain neurons away from the ventricular zone. Thus, we used adult CP pieces to ectopically provide elongating central trigeminal axons with a focalized, rich source of Slit2. We verified Slit2 expression in CP by *in situ* hybridization (Fig. 2*A*). We then prepared whole-mount explant cultures of the TG and brainstem from E13–E15 rat embryos. We placed a small piece of CP (taken from the lateral ventricle of an adult rat) next to the descending trigeminal tract. In these experiments, CP explant was placed unilaterally, and the contralateral trigeminal tract was used as a control ($n = 22$) (Fig. 2*B*,*C*). After 3 d *in vitro*, labeling of the TG axons with the lipophilic tracer DiI revealed the central trigeminal tract on both sides of the whole-mount cultures. On the control side, the trigeminal tract axons were unbranched and restricted to the tract (Fig. 2*D*). In striking contrast, there was distinct axon growth and arborization outside the tract, within the vicinity of the CP on the experimental side (Fig. 2*E*). In a separate series of experiments, we used embryonic dorsal skin explants, a tissue with no known Slit expression (Yuan et al., 1999) (our unpublished observations). In such cocultures ($n = 10$), there was no axon branching from the central trigeminal tract (Fig. 2*F*,*G*). The experimental sides were indistinguishable from the control sides. It is also noteworthy that a previous study showed that embryonic brainstem explants (at times when Slit2 mRNA is not expressed, E15 rat) do not promote arborization of TG axons, whereas older brainstem tissue (at times when Slit2 mRNA is expressed at high levels; E17–E19 rat) does induce branching and arborization of TG axons (Erzurumlu and Jhaveri, 1995).

Verification of choroid plexus induced arborization is attributable to Slit2 effect

Our *in situ* hybridization results verified the previous documentation by Hu (1999) that CP is a rich source of Slit2 (Fig. 2*A*). However, this tissue could also express other molecules that might participate in the branching and arborization effect we obtained. To confirm that Slit2 secretion from the CP is indeed the major cause of the effects obtained, we transiently cotransfected HEK293T cells with both Robo1-Fc and Robo2-Fc expression constructs (Robo-Fc). In this study, we did not attempt to investigate effects of Robo1 or Robo2 alone, with the reasoning that both Robos bind Slit2 (Brose et al., 1999; Simpson et al., 2000a). Robo1-Fc and Robo2-Fc have a Slit-binding domain and are fused to the constant region (Fc) of the human Ig molecule. They bind to Slit but lack a functional cytoplasmic domain (Brose et al., 1999). Robo-Fc (Robo1-Fc plus Robo2-Fc) binds extracellular Slit2, and thus it can be used as a competitive inhibitor of ligand activity. To test whether the CP-induced arborization is mediated via Slit2, we repeated the coculture experiments with CP explants and brainstem–TG whole mounts with Robo-Fc expressing HEK293T cells placed next to the CP explants. We verified Robo secretion from transfected HEK293T cells by performing Western blot, using conditioned media obtained from HEK293T cells transfected with Robo-Fc. We detected Robo-Fc in the conditioned medium but not in medium obtained from untransfected (control) HEK293T cell cultures (Fig. 3*A*). In the presence of Robo-Fc, CP-induced branching and arborization was completely blocked ($n = 20$) (Fig. 3*B*,*C*). On the other hand, untransfected HEK293T cells (controls) did not block CP-induced arborization of central trigeminal axons ($n = 18$) (Fig. 3*D*,*E*). Results are classified according to the branch points per axon for each case (Fig. 3*F*) (for details, see Materials and Methods) and summarized with a bar graph (Fig. 3*G*).

Effect of hSlit2 on central trigeminal tract axons

To further confirm the role of Slit2 in trigeminal axon branching and arborization, we transiently transfected HEK293T cells with a hSlit2 expression vector. We then embedded them in collagen and cocultured with brainstem–TG intact whole-mount preparations. In these experiments, we did not observe axon branching at the site of the hSlit2-transfected cells (data not shown). We reasoned that this might be attributable to accessibility of hSlit2. It has been reported previously that efficient Slit2 isolation from transfected cells requires heparin treatment because the majority of secreted Slit2 is trapped on the cell membrane (Brose et al., 1999). We felt that heparin addition might introduce a confounding variable. Thus, we adopted a different approach. We first cultured hSlit2-transfected HEK293T cells in six-well culture plates, and then we placed Millicell membranes with brainstem–TG intact whole mounts over the transfected HEK293T cells (Fig. 4*A*) and cocultured the two preparations.

hSlit2 has a c-myc tag at the C terminus (Brose et al., 1999). To verify that transfected cells express Slit2, we performed c-myc immunocytochemistry and Western blot analysis of the conditioned medium to verify that they secrete Slit2 (Fig. 4*B–F*). The vast majority of the Slit2 protein was present on HEK293T cells (Fig. 4*B*,*C*), and the Slit2 protein level in the conditioned medium was low (Fig. 4*B*,*C*,*F*), suggesting that Slit2 was mainly trapped in the cell membranes and not secreted into the medium. To increase the levels of Slit2 in the medium and to detect the cleavage product, we treated hSlit2-transfected HEK293T cells with heparin at the end of the culture period and processed for immunohistochemistry and Western blot as described above. During treatment, we observed a drastic reduction in Slit2 levels on HEK293T cells, indicating secretion to the medium (Fig. 4*D*,*E*). Western blot analysis confirmed high levels of Slit2 in the heparin-extracted conditioned medium, as well as the presence of the 55–60 kDa cleavage product, the C-terminal fragment of Slit2 (Fig. 4*F*). We do not have direct evidence for the presence of the N-terminal fragment of Slit2 in the medium. However, detection of the 55–60 kDa C-terminal fragment in the medium indicates the presence of the N-terminal fragment of Slit2, because it would be available only after proteolytic cleavage. As a negative control, we collected conditioned medium from untransfected HEK293T cells (Brose et al., 1999; Ba-Charvet et al., 2001).

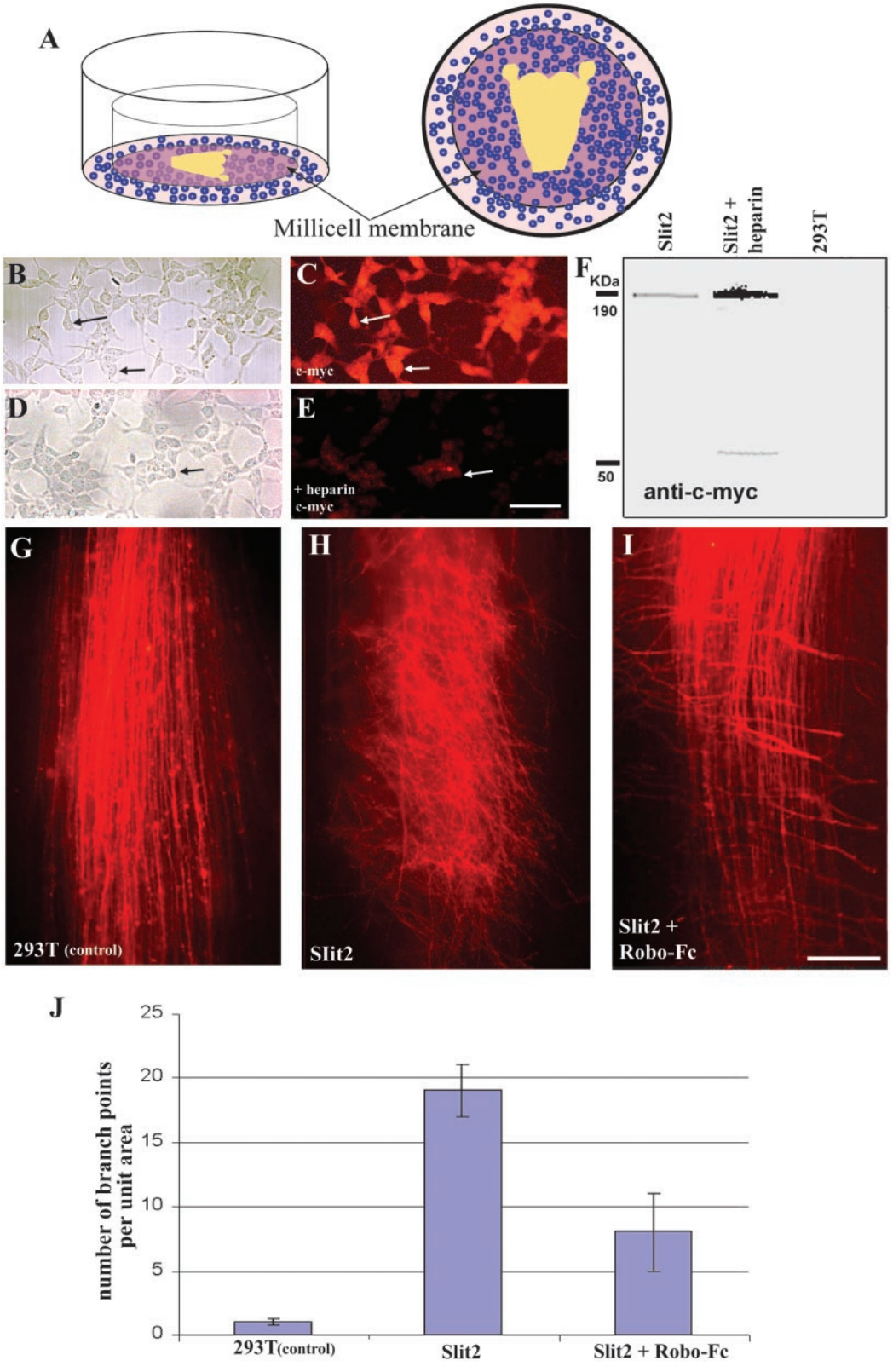


Figure 4. Effect of Slit2 on central trigeminal tract axons. *A*, Diagram of the culture setup. Explants are schematized within the center of the well; *blue circles* depict HEK293Tcells, which are just below and around the membrane with the whole-mount preparation. *B*, HEK293T cells transfected with hSlit2. *C*, c-Myc immunohistochemistry of cells shown in *B* (*arrows* in both micrographs point to the same cells). *D*, HEK293T cells transfected with hSlit2. *E*, c-Myc immunohistochemistry of cells shown in *D* after heparin treatment (*arrow* points to the same cell group in both micrographs). *F*, Western blot of the medium from hSlit-2-transfected cells and untransfected (control) cells. *G*, Low-magnification view of DiI-labeled trigeminal tract axons in a brainstem–TG intact whole-mount preparation grown in the presence of untransfected HEK293T cells (control). *H*, DiI-labeled trigeminal tract axons in a brainstem–TG intact whole-mount preparation grown in the presence of HEK293T cells transfected with hSlit2. *I*, (*Figure legend continues*)

In the culture setup described above, the cells are attached to the well, and the Millicell membrane with the explants is gently overlaid on the transfected HEK293T cells, with the trigeminal tract side (ventral surface) of the explant resting on the membrane. Thus, brainstem–TG intact whole-mount preparations are subject to both secreted Slit2 and Slit2 present on the cell membranes. The culture setup is illustrated in Figure 4*A*. As a negative control, we cocultured brainstem–TG whole mounts with untransfected HEK293T cells. After 3 d in culture, we labeled the TG with DiI and visualized the central trigeminal axons. In all cultures containing the Slit2-expressing cells ($n = 20$), we observed profuse branching and arborization within the central trigeminal tract (Figs. 4*H*, 5*C*,*D*). We quantified the branching by sampling two unit areas from each case (for details, see Materials and Methods). The increase in the number of branch points per unit area was highly significant ($p < 0.0005$) in the presence of Slit2-transfected HEK293T cells. In contrast, none of the control cultures with untransfected HEK293T cells ($n = 20$) showed any branching along the trigeminal tract (Figs. 4*G*,*J*, 5*A*,*B*). For additional controls, we repeated the same experiment with hSlit2-secreting HEK293T cells but also added culture medium collected from Robo-Fc-secreting HEK293T cells. In such cultures, branching effect was still observed, but there was a statistically significant ($p < 0.001$) reduction in the number of branch points per unit area compared with thecocultures of brainstem–TG whole mounts with hSlit2-secreting cells (Figs. 4*I*,*J*, 5*E*,*F*). In cocultures supplemented with Robo-Fc-conditioned medium, there was sporadic branching and arborization of the trigeminal tract axons. Often, axons within the tract were seen to fasciculate, leave the tract, and form arbors with lesser number of branches compared with cultures grown with hSlit2-secreting cells (compare Figs. 4*H*, 5*C*,*D* with 4*I*, 5*E*,*F*). In Figure 5, *E* and *F*, we only show branching axons from these cases. Quantitative analysis of branching under these conditions is presented in Figure 4*J*.

Slits and Robos are developmentally regulated during the elongation and branching–arborization phases of central trigeminal axons

The effects we obtained with Slit2-secreting CP explants and with hSlit2-secreting HEK293T cells were dramatic, suggesting that Slit2 might be a key player in inducing branching of the central trigeminal axons *in vivo*. Currently, there are no reliable antibodies available to detect the expression of Slit proteins within the nervous system. Thus, we performed *in situ* hybridization studies to visualize Slit and Robo mRNA expression during the elongation and branching–arborization phases of the central trigeminal tract in mouse and rat embryos. The Slit probes that we used are specific for mice and Robo probes are more specific for rat tissues. We used both rat and mouse trigeminal pathway derived from equivalent ages. As noted in Materials and Methods, development of the mouse and rat trigeminal pathways are very similar, and our culture results from both species were indistinguishable.

Slit expression in the developing mouse has been documented previously (Yuan et al., 1999). We examined Slit (Slit1, Slit2, and Slit3) and Robo (Robo1 and Robo2) mRNA expression during trigeminal axon elongation and arborization in E13–E15 mouse (*m*) and E15–E17 rat (*r*), respectively (Figs. 6, 7). Our results revealed a spatiotemporal regulation of Slit and Robo mRNA expression in the developing brainstem and TG. All Slit mRNAs are highly expressed in the brainstem midline (floor plate) at both stages (Fig. 6, *asterisks*). This is in accordance with previously published results (Yuan et al., 1999; Wang et al., 1999). Slit mRNAs are not detectable in the BSTC during axon elongation, and only Slit2 mRNA expression becomes distinct during the axon branching–arborization phase (Fig. 6*D*). All Slit mRNAs are also present in the TG with varying levels (Fig. 6). Whereas Slit1 and Slit3 mRNA levels are very high, Slit2 mRNA level is low in the ganglion.

Along with Slit mRNAs, TG cells express high levels of Robo1 and Robo2 mRNAs during axon elongation and arborization phases (Fig. 7). Coexpression of Slit and Robo mRNAs has been noted previously for the dorsal root ganglia and TG (Yuan et al., 1999; Wang et al., 1999). We also observed Robo mRNA expression in the BSTC along sensory and motor nuclei at later stages and along the midline (floor plate) at earlier stages. Robo mRNA expression within the BSTC corresponds to the development of axonal projections from these sensory nuclei to a variety of target regions in the brainstem and thalamus.

DISCUSSION

Most long-distance projection neurons follow distinct trajectories as they start their journey toward their targets. During this phase of development, axons grow unbranched at a rapid rate and lay down their characteristic pathways. Once they reach their targets, another characteristic phase in axon development is seen, that of interstitial branching to other targets or branching and arborization within the primary targets (Nakamura and O'Leary, 1989; Bhide and Frost, 1991; Jhaveri et al., 1991; Kuang and Kalil, 1994; Kalil et al., 2000). Molecular signals that direct these different phases of axon growth are primarily unknown. Some of these signals may be intrinsically programmed in the neuron, sending its axon to distant targets (Moya et al., 1988; Confaloni et al., 1997; Bhide and Frost, 1999), or derived from target tissues (Sato et al., 1994; Bastmeyer and O'Leary, 1996; Richards et al., 1997; Bastmeyer et al., 1998; Kalil et al., 2000). In recent years, several candidate molecules and their roles in axon elongation and branching have been tested. Most notably, the NGF family of neurotrophins attracts developing sensory axons in the periphery (O'Connor and Tessier-Lavigne, 1999; Tucker et al., 2001) and exerts distinct and differential effects on axonal morphology. For example, in dissociated sensory neuron cell cultures and in whole-mount explant cultures of the trigeminal pathway, high doses of NGF and neurotrophin-3 induce dramatic axon elongation and branching, respectively (Gallo et al., 1997; Gallo and Letourneau, 1998; Lentz et al., 1999; Ulupinar et al., 2000). We showed recently that the Rho family of GTPases mediate neurotrophin effects on axonal growth (Özdinler and Erzurumlu, 2001). Whether neurotrophins regulate axonal branching and arborization *in vivo* has not been resolved. Various mouse models with

←

DiI-labeled trigeminal tract axons in a brainstem–TG intact whole-mount preparation grown in the presence of HEK293T cells transfected with hSlit2 and Robo-Fc conditioned medium. *J*, Bar graph representation of the number of branch points per unit area for each case (see Materials and Methods). In the presence of Slit2, the number of branch points per unit area shows a highly significant increase ($p < 0.0005$). Addition of Robo-Fc conditioned medium causes a statistically significant reduction ($p < 0.001$) in the number of branch points per unit area. Error bars represent one SD. Scale bars: *B–E*, 30 μm; *G–I*, 100 μm.

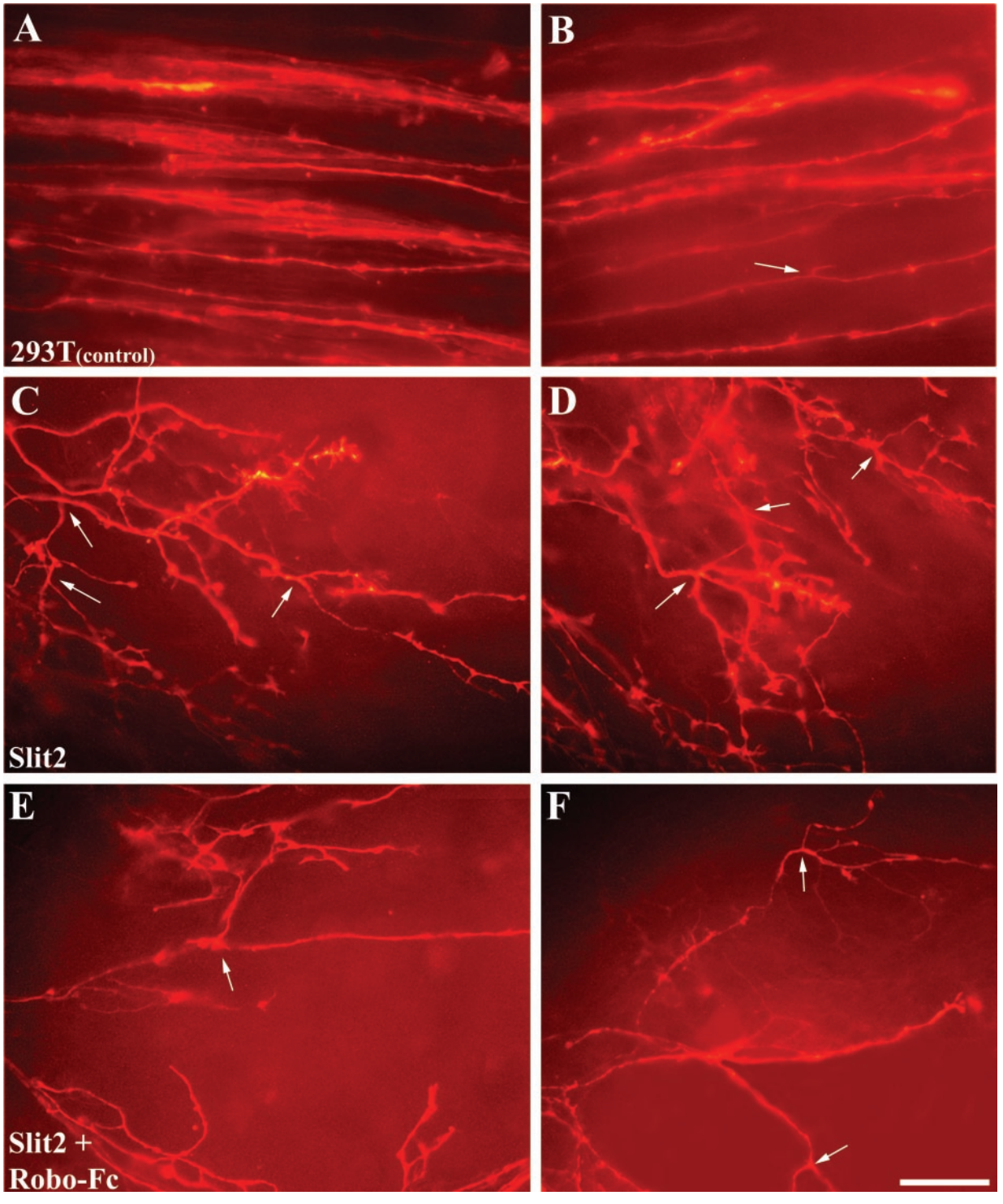


Figure 5. Role of Slit2 in branching and arborization of central trigeminal tract axons. *A*, *B*, High-power view of DiI-labeled central trigeminal tract axons grown in cocultures with untransfected HEK293T cells (control). *Arrow* in *B* points to a small budding branch-like process from one single axon. *C*, *D*, DiI-labeled central trigeminal tract axons grown in cocultures with hSlit2-transfected HEK293T cells. Note the density of branching, each branch tipped with large growth cones. *Arrows* indicate branch points. *E*, *F*, DiI-labeled central trigeminal tract axons grown with hSlit2-transfected HEK293T cells in the presence of Robo-Fc conditioned medium. *Arrows* indicate branch points. These two photomicrographs were taken in regions of branching, which was not uniform all along the trigeminal tract. Note that branching–arborization in these cases is significantly reduced. Scale bar, 20 μm.

targeted deletion of Trk receptors or neurotrophins do not show specific branching–arborization defects in the CNS (Patel et al., 2000). Although it is still not clear to what extent NGF family of neurotrophins are involved in regulating branching and arborization behavior of axons in the CNS, other target-derived molecules could also play a major role in this process.

In the present study, we found that a member of the Slit family of proteins, Slit2, induces premature branching–arborization of

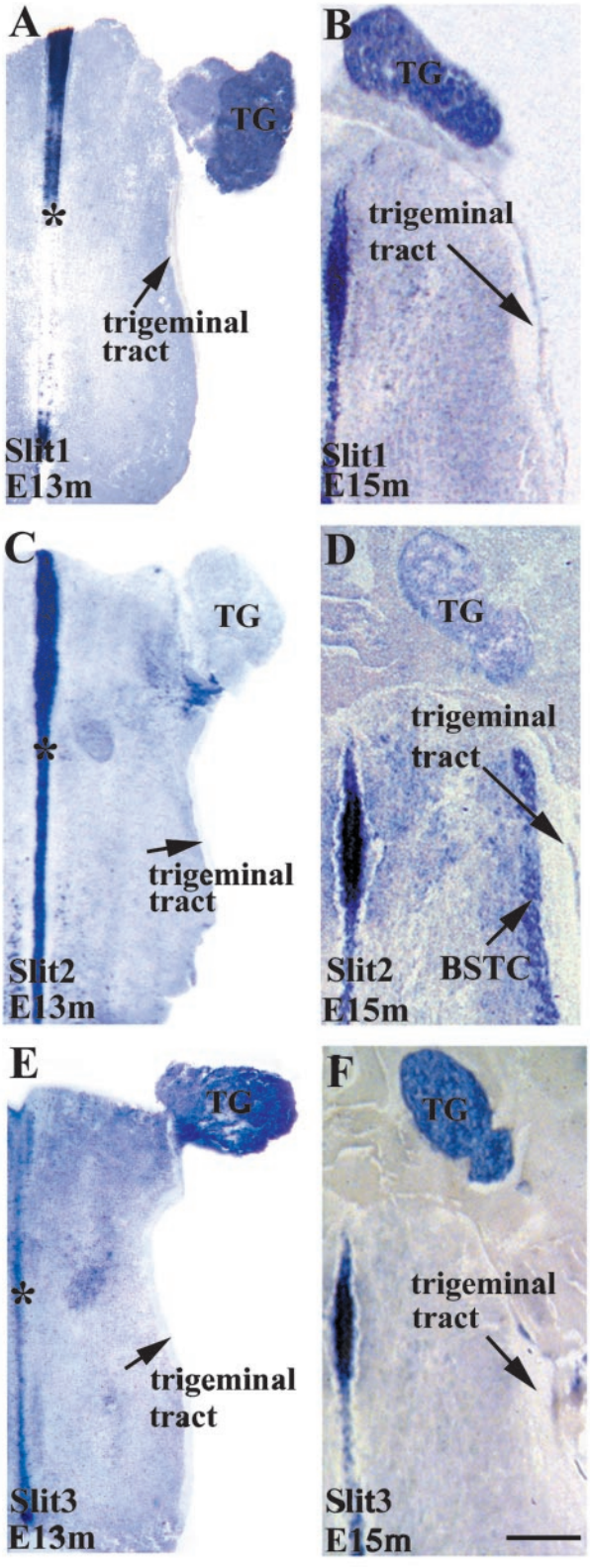


Figure 6. Slit mRNA expression patterns during elongation and arborization phases of trigeminal tract development in sections through brainstem–TG whole-mount preparations. *A*, Slit1 expression in mouse at E13 (*E13m*). *B*, Slit1 expression in mouse at E15 (*E15m*). *C*, Slit2 expression in mouse at E13. *D*, Slit2 expression in mouse at E15. *E*, Slit3 expression in mouse at E13. *F*, Slit3 expression in mouse at E15. Note that all Slit mRNAs are very low or absent in the BSTC compared with high levels in the midline (floor plate). Slit2 mRNA is absent at E13 but becomes very high within the BSTC on E15, when central TG axons are normally branching and arborizing within this region. Slit1 and Slit3 mRNA expression is also high within the TG. *Asterisks* mark the brainstem midline (floor plate). Scale bar, 500 μm.

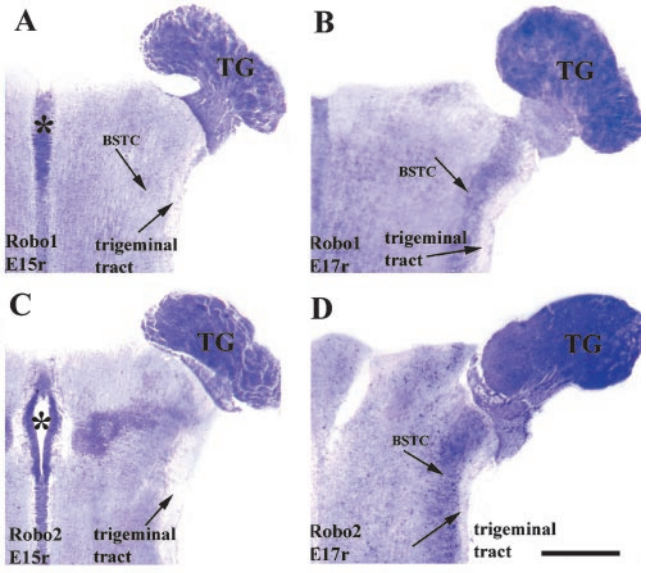


Figure 7. Robo mRNA expression during the elongation and arborization phases of trigeminal tract development in sections through brainstem–TG whole mounts. *A*, Robo1 expression in rat at E15 (*E15r*). *B*, Robo1 expression in rat at E17 (*E17r*). *C*, Robo2 expression in rat at E15. *D*, Robo2 expression in rat at E17. Note that the TG expresses high levels of both Robo mRNAs. In addition, there is a notable increase in the levels of Robo mRNA expression at E17. Moderate levels of Robo1 and Robo2 mRNA expression in the rat BSTC at E17 might be related to guidance and lateralization of axons from these nuclei destined for other brainstem and thalamic nuclei. *Asterisks* mark the brainstem midline (floor plate). Scale bar, 500 μm.

trigeminal axons in the brainstem. Increasing numbers of reports have underscored the role of Slit family of proteins and their receptors in axon guidance and cell migration in invertebrates and vertebrates (Brose et al., 1999; Hu, 1999; Kidd et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Wu et al., 1999; Erskine et al., 2000; Rajagopalan et al., 2000; Ringstedt et al., 2000; Simpson et al., 2000; Ba-Charvet et al., 2001; Chen et al., 2001; Kramer et al., 2001). A recent extensive study on Slit and Robo mRNA expression in the developing and adult rat brain has suggested the importance of Slits not only in axonal pathfinding and neural migration but also in synaptic plasticity (Marillat et al. 2002). In dissociated cortical neuron cultures, a role for Slit1 in dendritic growth and branching has been noted (Whitford et al., 2002). However, so far only one report documented axon branching effects of Slit2 in dissociated rat DRG cells cultured in the presence of exogenously added neurotrophins (Wang et al., 1999). In such cultures of bipolar sensory neurons, it is not possible to discern whether branching effect of Slit2 (or any other molecule) is on the peripheral or central processes or both. These authors also added NGF to their DRG cell cultures to prevent cell loss and most likely selected for only NGF-dependent small, nociceptive neurons. As mentioned previously, under certain culture conditions, neurotrophins can attract axons and promote axonal branching and arborization (Gallo et al., 1997; Gallo and Letourneau, 1998; Lentz et al., 1999; Ulupinar et al., 2000; Özdinler and

←

Erzurumlu, 2001). It is important to note that dissociated cells do not always reflect *in vivo* conditions. Several studies indicate that dissociated neurons can dramatically change their gene expression and requirements for survival (Moshnyakov et al., 1996; Friedel et al., 1997; Genc and Erzurumlu, 2000). The present study corroborates the previous findings of Wang et al. (1999) on branch-inducing effects of Slit2 on primary sensory neurons. This study also documents for the first time the branching–arborization effects of Slit2 in an intact, CNS pathway grown *in vitro* without any neurotrophins.

In brainstem–TG intact whole-mount preparations, the trigeminal pathway retains its characteristics of the day it is isolated from the embryo. The maturation and differentiation of the trigeminal pathway is considerably slowed down compared with *in vivo* development that proceeds at a rapid pace. For example, E15 rat explants do not show central trigeminal tract branching and arborization up to 5 d in culture. While keeping this caveat in mind, the brainstem–TG intact whole-mount preparations we used have several advantages. These whole-mount explants can be prepared such that the entire peripheral and central projection fields of the TG can be isolated intact at almost any developmental stage. In whole-mount cultures, many tissue-specific characteristics of the TG and the brainstem are retained. Different cell types within the TG survive, express Trk and p75 receptors, calcium binding proteins, and axonal growth and integrity, and electrophysiological characteristics closely match *in vivo* counterparts of the age the explant is isolated (Ulupinar and Erzurumlu, 1998; Ulupinar et al., 2000) (F.-S. Lo and R. S. Erzurumlu, unpublished observations). A major advantage of this culture model is the ease with which it is possible to assay the role of a variety of axon guidance or growth-promoting molecules under conditions that are far closer to *in vivo* conditions than in dissociated cell or sensory ganglion explant cultures. Another advantage of using an intact whole-mount preparation is the possibility of doing coculture experiments. Sometimes the molecules of interest could be unavailable as a soluble product that can be directly added to the culture medium. In such cases, tissues known to express and secrete that particular molecule could be cocultured with or transplanted into the intact whole-mount preparation. Effect of local application of this source could be easily assayed on axon outgrowth of central trigeminal tract axons.

Studies described in this report show that Slit2 is a branch-inducing factor for central trigeminal tract axons in an intact *in vitro* model. *In situ* hybridization studies further corroborate our experimental findings. Developing TG cells express both Robo1 and Robo2, and, of the three members of the Slit family examined, Slit2 mRNA becomes abundant in the BSTC right at the time of central trigeminal tract branching–arborization. At earlier times during the elongation phase of the central trigeminal tract axons, Slit2 mRNA is conspicuously absent in this region but is expressed at high levels along the brainstem midline structures together with other members of the Slit family.

Coexpression of Slit and Robo mRNAs in the TG is difficult to explain, because these cells do not receive any input on their soma. Curiously, the peripheral targets of most TG cells, the whisker follicles, also coexpress Slit and Robo mRNAs (Yuan et al., 1999; Özdinler and Erzurumlu, 2000). Slit2 and Slit3 expression is localized to the outer root sheath and surrounding the bulb of the follicles, whereas Robo1 expression is predominantly in the bulb and hair root. Thus, differential expression of Slit and Robos in the hair follicle complex and expression of both Slit and Robo mRNAs in the TG might be important in guiding specific innervation patterns in the periphery. Additional studies are needed to test this possibility.

Coexpression of Slit2 and Robo receptors in the BSTC during central trigeminal tract branching–arborization suggests that, while BSTC neurons are signaling incoming TG axons to branch, their own axonal projections to distant targets might be using similar cues for guidance. There is no direct evidence to support this scenario. However, previous studies in *Drosophila* (Rajagopalan et al., 2000; Simpson et al., 2000a) and recent studies on Slit1 and Slit2 knock-out mice (Bagri et al., 2002; Plump et al., 2002) indicate that Slits guide many axonal projection systems by repulsion. Studies in *Drosophila* suggest that differential and overlapping expression of Slits and Robos might play a role in not only midline crossing of some axons but also varying degrees of lateralization of different fiber pathways (Simpson et al., 2000b). Recent observations on Slit2 and Slit1 and Slit2 double knock-out mice have revealed serious axonal projection defects along the optic chiasm and many forebrain pathways (Bagri et al., 2002; Plump et al., 2002). It would be highly interesting to examine projections within the brainstem and any alterations in axonal branching and arborization timing and patterns for the central trigeminal tract axons in these mice.

REFERENCES

Ba-Charvet KT, Brose K, Ma L, Wang KH, Marillat V, Sotelo C, Tessier-Lavigne M, Chedotal A (2001) Diversity and specificity of actions of Slit2 proteolytic fragments in axon guidance. J Neurosci 21:4281–4289.

Bagri A, Marin O, Plump AS, Mak J, Pleasure SJ, Rubenstein JLR, Tessier-Lavigne M (2002) Slit proteins prevent midline crossing and determine the dorsoventral position of major axonal pathways in the mammalian forebrain. Neuron 33:233–248.

Bastmeyer M, O'Leary DDM (1996) Dynamics of target recognition by interstitial axon branching along developing cortical axons. J Neurosci 16:1450–1459.

Bastmeyer M, Daston MM, Possel H, O'Leary DDM (1998) Collateral branch formation related to cellular structures in the axon tract during corticopontine target recognition. J Comp Neurol 392:1–18.

Bhide PG, Frost DO (1991) Stages of growth of hamster retinofugal axons: implications for developing axonal pathways with multiple targets. J Neurosci 11:485–504.

Bhide PG, Frost DO (1999) Intrinsic determinants of retinal axon collateralization and arborization patterns. J Comp Neurol 411:119–129.

Brose K, Tessier-Lavigne M (2000) Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. Curr Opin Neurobiol 10:95–102.

Brose K, Bland KS, Wang KH, Arnott D, Henzel W, Goodman CS, Tessier-Lavigne M, Kidd T (1999) Slit proteins bind Robo receptors and have an evolutionary conserved role in repulsive axon guidance. Cell 96:795–806.

Chen JH, Wen L, Dupuis S, Wu JY, Rao Y (2001) The N-terminal leucine-rich regions in Slit are sufficient to repel olfactory bulb axons and subventricular zone neurons. J Neurosci 21:1548–1556.

Chisholm A, Tessier-Lavigne M (1999) Conservation and divergence of axon guidance mechanisms. Curr Opin Neurobiol 9:603–615.

Confaloni A, Lyckman AW, Moya KL (1997) Developmental shift of synaptic vesicle protein 2 from axons to terminals in the primary visual projection of the hamster. Neuroscience 77:1225–1236.

Erskine L, Williams SE, Brose K, Kidd T, Rachel RA, Goodman CS, Tessier-Lavigne M, Mason CA (2000) Retinal ganglion cell axon guidance in the mouse optic chiasm: expression and function of robos and slits. J Neurosci 20:4975–4982.

Erzurumlu RS, Killackey HP (1983) Development of order in the rat trigeminal system. J Comp Neurol 213:365–380.

Erzurumlu RS, Jhaveri S (1992) Trigeminal cell processes are spatially ordered prior to the differentiation of the vibrissa pad. J Neurosci 12:3946–3955.

Erzurumlu RS, Jhaveri S (1995) Target influences on the morphology of trigeminal axons. Exp Neurol 135:1–16.

Friedel RH, Schnurch H, Stubbusch J, Barde YA (1997) Identification of genes differentially expressed by nerve growth factor- and neurotrophin-3-dependent sensory neurons. Proc Natl Acad Sci USA 94:12670–12675.

Gallo G, Letourneau PC (1998) Localized sources of neurotrophins initiate axon collateral sprouting. J Neurosci 18:5403–5414.

Gallo G, Lefcort FB, Letourneau PC (1997) The trkA receptor mediates

growth cone turning toward a localized source of nerve growth factor. J Neurosci 17:5445–5454.
Genc B, Erzurumlu RS (2000) TrkA and TrkC co-expression in dissociated trigeminal ganglion cell cultures and axonal responses to NGF, NT-3. Soc Neurosci Abstr 26:319.
Guthrie S (1999) Axon guidance: starting and stopping with slit. Curr Biol 9:R432–R435.
Harris WA, Holt CE (1999) Slit, the midline repellent. Nature 398:462–463.
Hu H (1999) Chemorepulsion of neuronal migration by Slit2 in the developing mammalian forebrain. Neuron 23:703–711.
Jhaveri S, Edwards MA, Schneider GE (1991) Initial stages of retinofugal axon development in the hamster: evidence for two distinct modes of growth. Exp Brain Res 87:371–382.
Kalil K, Szebenyi G, Dent EW (2000) Common mechanisms underlying growth cone guidance and axon branching. J Neurobiol 44:145–158.
Kidd T, Brose K, Mitchell KJ, Fetter RD, Tessier-Lavigne M, Goodman CS, Tear G (1998) Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell 92:205–215.
Kidd T, Bland KS, Goodman CS (1999) Slit is the midline repellent for the robo receptor in *Drosophila*. Cell 96:785–794.
Kramer SG, Kidd T, Simpson JH, Goodman CS (2001) Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration. Science 292:737–740.
Kuang RZ, Kalil K (1994) Development of specificity in corticospinal connections by axon collaterals branching selectively into appropriate spinal targets. J Comp Neurol 344:270–282.
Lentz SI, Knudson CM, Korsmeyer SJ, Snider WD (1999) Neurotrophins support the development of diverse sensory axon morphologies. J Neurosci 19:1038–1048.
Li HS, Chen JH, Wu W, Fagaly T, Zhou L, Yuan W, Dupuis S, Jiang ZH, Nash W, Gick C, Ornitz DM, Wu JY, Rao Y (1999) Vertebrate Slit, a secreted ligand for the transmembrane protein Roundabout, is a repellent for olfactory bulb axons. Cell 96:807–818.
Marillat V, Cases O, Nguyen Ba-Charvet KT, Tessier-Lavigne M, Sotelo C, Chedotal A (2002) Spatiotemporal expression patterns of slit and robo genes in the rat brain. J Comp Neurol 442:130–155.
Moshnyakov M, Arumae U, Saarma M (1996) mRNAS for one, two or three members of trk receptor family are expressed in single rat trigeminal ganglion neurons. Mol Brain Res 43:141–148.
Moya KL, Benowitz LI, Jhaveri S, Schneider GE (1988) Changes in rapidly transported proteins in developing hamster retinofugal axons. J Neurosci 8:4445–4454.
Nakamura H, O'Leary DDM (1989) Inaccuracies in initial growth and arborization of chick retinotectal axons followed by course corrections and axon remodeling to develop topographic order. J Neurosci 9:3776–3795.
Nguyen Ba-Charvet KT, Brose K, Marillat V, Kidd T, Goodman CS, Tessier-Lavigne M, Sotelo C, Chedotal A (1999) Slit2 mediated chemorepulsion and collapse of developing forebrain axons. Neuron 22:463–473.
O'Connor R, Tessier-Lavigne M (1999) Identification of maxillary factor, a maxillary process-derived chemoattractant for developing trigeminal sensory axons. Neuron 24:165–178.
Özdinler PH, Erzurumlu RS (2000) Slit gene expression in the developing trigeminal pathway. Soc Neurosci Abstr 26:1357.
Özdinler PH, Erzurumlu RS (2001) Regulation of neurotrophin-induced axonal responses via Rho GTPases. J Comp Neurol 438:377–387.
Patel TD, Jackman A, Rice FL, Kucera J, Snider WD (2000) Development of sensory neurons in the absence of NGF/TrkA signaling in vivo. Neuron 25:345–357.
Plump AS, Erskine L, Sabatier C, Brose K, Epstein CJ, Goodman CS, Mason CA, Tessier-Lavigne M (2002) Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. Neuron 33:219–232.
Rajagopalan S, Nicolas E, Vivancos V, Berger J, Dickson BJ (2000) Crossing the midline: roles and regulation of Robo receptors. Neuron 28:767–777.
Richards LJ, Koester SE, Tuttle R, O'Leary DDM (1997) Directed growth of early cortical axons is influenced by a chemoattractant released from an intermediate target. J Neurosci 17:2445–2458.
Ringstedt T, Braisted JE, Brose K, Kidd T, Goodman C, Tessier-Lavigne M, O'Leary DDM (2000) Slit inhibition of retinal axon growth and its role in retinal axon pathfinding and innervation patterns in the diencephalon. J Neurosci 20:4983–4991.
Sato M, Lopez-Mascaraque L, Heffner CD, O'Leary DDM (1994) Action of a diffusible target-derived chemoattractant on cortical axon branch induction and directed growth. Neuron 13:791–803.
Seeger M, Tear G, Ferres-Marco D, Goodman CS (1993) Mutations affecting growth cone guidance in *Drosophila*: genes necessary for guidance toward or away from the midline. Neuron 10:409–426.
Simpson JH, Bland KS, Fetter RD, Goodman CS (2000a) Short-range and long-range guidance by Slit and its Robo receptors: a combinatorial code of Robo receptors controls lateral position. Cell 103:1019–1032.
Simpson JH, Kidd T, Bland KS, Goodman CS (2000b) Short-range and long-range guidance by slit and its Robo receptors. Robo and Robo2 play distinct roles in midline guidance. Neuron 28:753–766.
Stainier DY, Gilbert W (1990) Pioneer neurons in the mouse trigeminal sensory system. Proc Natl Acad Sci USA 87:923–927.
Stainier DY, Gilbert W (1991) Neuronal differentiation and maturation in the mouse trigeminal sensory system, in vivo and in vitro. J Comp Neurol 311:300–312.
Tucker KL, Meyer M, Barde YA (2001) Neurotrophins are required for nerve growth during development. Nat Neurosci 4:29–37.
Ulupinar E, Erzurumlu RS (1998) Peripheral target-specific influences on embryonic neurite growth vigor and patterns. J Comp Neurol 399:427–439.
Ulupinar E, Jacquin MF, Erzurumlu RS (2000) Differential effects of NGF, NT-3 on embryonic trigeminal axon growth patterns. J Comp Neurol 425:202–218.
Van Vactor D, Flanagan JG (1999) The midline and the end: Slit brings guidance and branching together in axon pathway selection. Neuron 22:649–652.
Waite PM, Ho SM, Henderson A (2000) Afferent ingrowth and onset of activity in the rat trigeminal nucleus. Eur J Neurosci 12:2781–2792.
Wang KH, Brose K, Arnott D, Kidd T, Goodman CS, Henzel W, Tessier-Lavigne M (1999) Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. Cell 96:771–784.
Whitford KL, Marillat V, Stein E, Goodman CS, Tessier-Lavigne M, Chedotal A, Ghosh A (2002) Regulation of cortical dendrite development by slit-robo interactions. Neuron 33:47–61.
Wu JY, Feng L, Park HT, Havlioglu N, Wen L, Tang H, Bacon KB, Jiang ZH, Zhang XC, Rao Y (2001) The neuronal repellent Slit inhibits leukocyte chemotaxis induced by chemotactic factors. Nature 410:948–952.
Wu W, Wong K, Chen J, Jiang Z, Dupuis S, Wu JY, Rao Y (1999) Directional guidance by neuronal migration in the olfactory system by the protein Slit. Nature 400:331–336.
Yuan W, Zhou L, Chen J, Wu JY, Rao Y, Ornitz DM (1999) The mouse Slit family: secreted ligands for ROBO expressed in patterns that suggest a role in morphogenesis and axon guidance. Dev Biol 212:290–306.