Development/Plasticity/Repair

Semaphorin 3F Is Critical for Development of Limbic System Circuitry and Is Required in Neurons for Selective CNS Axon Guidance Events

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Little is known about the role of class 3 semaphorins in the development of CNS circuitry. Several class 3 semaphorins, including semaphorin 3F (Sema3F) bind to the receptor neuropilin-2 to confer chemorepulsive responses *in vitro*. To understand the role of Sema3F in the establishment of neural circuitry *in vivo*, we have generated *sema3F* null and *sema3F* conditional mutant mice. Inspection of the peripheral nervous system in *sema3F* null mice reveals that Sema3F is essential for the proper organization of specific cranial nerve projections. Analysis of the CNS in *sema3F* null mice reveals a crucial role for Sema3F in the rostral forebrain, midbrain, and hippocampus in establishing specific *Npn-2* (neuropilin-2)–expressing limbic tracts. Furthermore, we identify Sema3F and Npn-2 as the first guidance cue–receptor pair shown to be essential for controlling the development of amygdaloid circuitry. In addition, we provide genetic evidence in vertebrates for a neuronal requirement of a soluble axon guidance cue in CNS axon guidance. Our data reveal a requirement for neuronal Sema3F in the normal development of the anterior commissure in the ventral forebrain and infrapyramidal tract in the hippocampus. Thus, our results show that Sema3F is the principal ligand for Npn-2–mediated axon guidance events *in vivo* and is a critical determinant of limbic and peripheral nervous system circuitry.

Key words: **semaphorin; neuropilin; plexin; axon guidance; limbic system; stria terminalis; amygdala; synapsin; Cre**

Introduction

The exquisite complexity of the nervous system reflects the remarkable ability of neurons to form precise connections. A myriad of guidance cues are required during development to help axons navigate to their proper targets, and one such family of guidance cues is the semaphorins. The semaphorins include seven different classes of proteins that are defined by their mode of membrane attachment and the presence of various structural motifs C-terminal to the signature semaphorin (sema) domain (Semaphorin Nomenclature Committee, 1999). Class 3 semaphorins are vertebrate secreted proteins and include six members that have been shown in various contexts to act as neuronal chemorepellents or chemoattractants. Little is known about the role of secreted semaphorins in the establishment of CNS circuitry. Class 3 semaphorins signal through a holoreceptor complex consisting of a ligand-binding subunit and a signal-transducing component. The ligand-binding specificity of this holoreceptor complex is conferred by members of the small neuropilin (Npn) protein family that consists of the type 1 transmembrane proteins Npn-1 and Npn-2. Plexins are the signal-transducing component of the class 3 semaphorin holoreceptor complex and are type 1 transmembrane proteins with highly conserved cytoplasmic domains (He et al., 2002). Mice deficient for *sema3A*, *npn-1*, *npn-2*, and *plexin-A3* have proven invaluable for understanding the molecular basis of semaphorin-mediated axon guidance events *in vivo* (Behar et al., 1996; Kitsukawa et al., 1997; Taniguchi et al., 1997; Chen et al., 2000; Giger et al., 2000; Cheng et al., 2001).

Analysis of *npn-2* null mice reveals that this semaphorin coreceptor is critical for both axon guidance and cell migration (Chen et al., 2000; Giger et al., 2000; Marin et al., 2001; Cloutier et al., 2002). Certain class 3 semaphorins (Sema3F, Sema3B, and Sema3C) can bind and signal through Npn-2 *in vitro* (Adams et al., 1997; Chen et al., 1997; Giger et al., 1998; de Castro et al., 1999; Steup et al., 2000; Zou et al., 2000a). However, these class 3 semaphorins can also bind to Npn-1, an obligate coreceptor for Sema3A, and the possibility that they may also act as Sema3A competitive antagonists is supported by cell culture experiments (Takahashi et al., 1998). In addition to binding select class 3 semaphorins, Npn-2 also is an isoform-specific vascular endothelial growth factor (VEGF) receptor that binds $VEGF_{165}$, $VEGF_{145}$, and VEGF-C (Karkkainen et al., 2001; Neufeld et al., 2002). The observation that Npn-1 can function as a cell-surface adhesion molecule suggests that Npn-2 also might share this attribute with Npn-1 (Shimizu et al., 2000). Therefore, it is unclear from the spectrum of phenotypes observed in *npn-2* null mice

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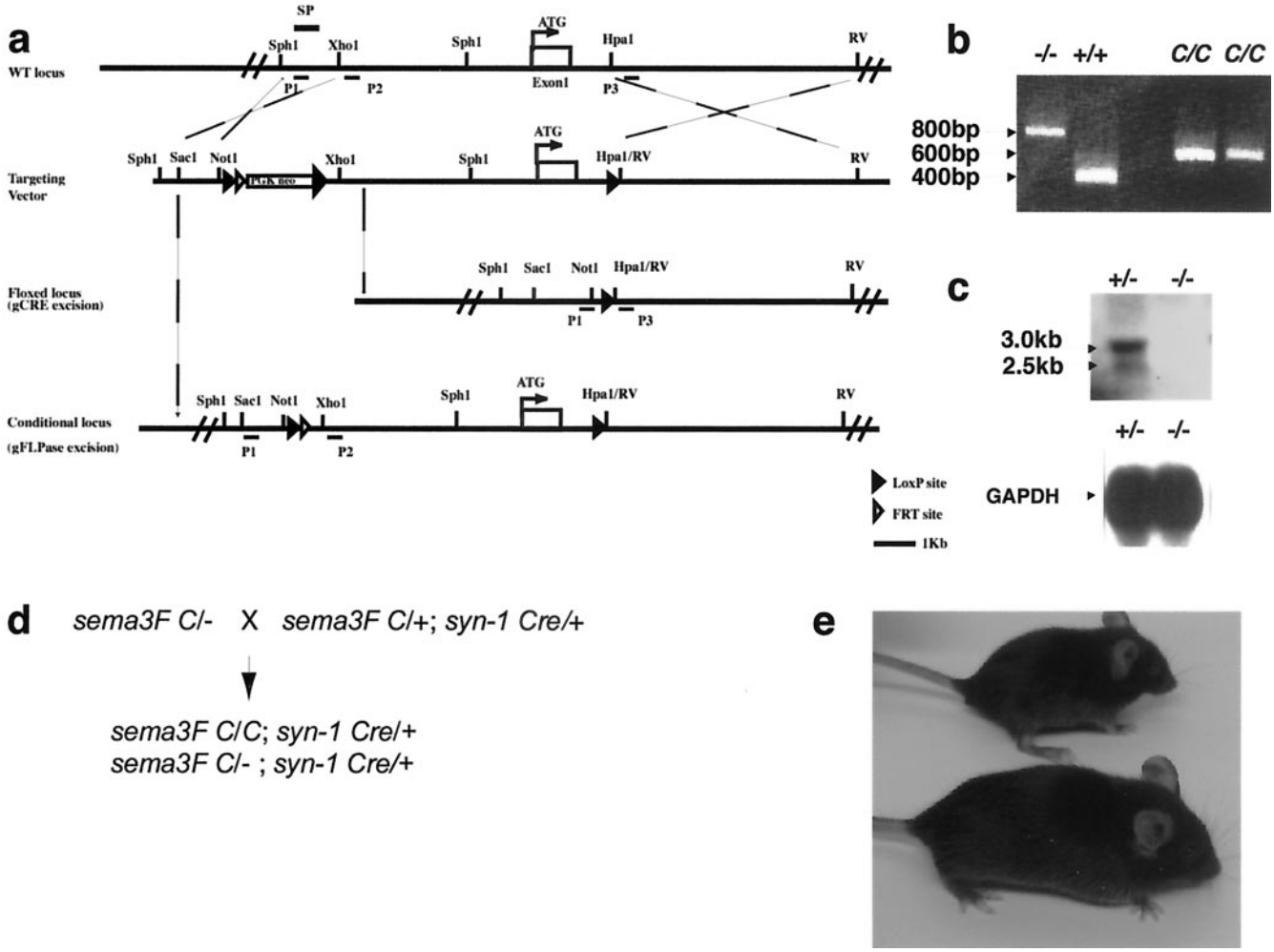

Figure 1. Generation of *sema3F* null and *sema3F* conditional mice. *a,* Schematic depiction of targeting vector, wild-type *sema3F* locus, *sema3F* null, and *sema3F* conditional alleles. Targeting of exon 1, which encodes the first 37 aa, including the entire signal sequence and 4 kb of presumptive promoter sequence, should functionally eliminate *sema3F* expression. The 5′ probe used (SP) for Southern blot genotyping and the PCR-primer pairs (P1, P2, P3) used to detect the *sema3F* wild-type, null, and conditional alleles are indicated. *b,* PCR genotyping analysis of P5 pups from two different crosses to show wild-type *sema3F* allele (400 bp), *sema3F* null allele (800 bp), and *sema3F* conditional allele (600 bp). *c,* Northern blot analysis of RNA isolated from E16 *sema3F* $^{+/-}$ and *sema3F* $^{-/-}$ embryos. Both *sema3F* transcripts are absent in *sema3F* $^{-/-}$ embryos. A GAPDH probe was used to show total RNA levels in *sema3F* $^{+/-}$ and *sema3F* $^{-/-}$ lanes (bottom). *d,* Breeding scheme used to generate mice that lack a neuronal source of *sema3F*. *e,* Photograph showing size difference between a 5-week-old *sema3F* $^{-/-}$ mouse (male, far back) and a *sema3F* $^{+/-}$ littermate (female, front). *Sema3F* $^{-/-}$ mice and control littermates are indistinguishable by ~3 months of age.

which Npn-2 ligands are required *in vivo*. The expression pattern of *sema3F* during embryonic development and its ability to repel *npn-2*–expressing neurons *in vitro* qualify Sema3F as a candidate Npn-2 ligand that signals through this receptor during axon guidance events *in vivo*.

In both axon guidance and regeneration, specific cell types are recruited to serve distinct guidance functions. For example, in the visual system glial cells have been proposed to express specific guidance cues to steer retinal ganglion cell axons, whereas in the thalamocortical system it is likely that pioneering neurons provide some of these cues (Hevner et al., 2001; Lemke, 2001). Class 3 semaphorins are expressed in a variety of cell types in the embryonic and adult nervous systems, raising the possibility that specific Npn-2–dependent functions rely on the production of these ligands by distinct cell types (Chen et al., 1997; Giger et al., 1998; Pasterkamp et al., 1999; Holtmaat et al., 2002).

In this study, we analyze *sema3F* null mice and also mice that lack *sema3F* specifically in neurons. We show that in the CNS, Sema3F is critical for limbic circuitry. *Sema3F* null mice exhibit profound axon guidance defects in distinct *npn-2*–expressing projections, including the anterior commissure and stria terminalis in the forebrain, the infrapyramidal tract in the hippocampus, and the fasciculus retroflexus in the midbrain. In the periphery, Sema3F is required for the normal development of specific cranial nerve projections. Moreover, Sema3F is required in neurons for some of its axon guidance functions *in vivo*, because mice lacking neuronal Sema3F show anterior commissure and infrapyramidal tract defects. Thus, Sema3F is the principal Npn-2 ligand required for the development of specific CNS and PNS projections *in vivo*.

Materials and Methods

Generation of sema3F *null and* sema3F *conditional mice.* To generate the targeting vector a 129SVJ lambda FixII library (Stratagene, La Jolla, CA) was screened using a sequence upstream of rat *sema3F* exon 1 as a probe, and a 20 kb genomic sequence λ clone (λ 1.1) was identified, isolated, restriction-mapped, and partially sequenced. Homologous recombination was performed in embryonic stem (ES) cells using a targeting vector designed to

introduce lox P sites 4 kb upstream and 1 kb downstream of exon 1 along with an Flp recombinase target-flanked phosphoglycerate kinase–neomycin cassette immediately juxtaposed to the 5′lox P site. Three targeted ES cell clones were identified by Southern blotting; one of two that were injected into blastocysts gave rise to germ-line-transmitting male chimeras. Heterozygous *sema3F* mutant and *sema3F* conditional mice were generated by crossing males that were heterozygous for the targeted allele with C57BL/6 female mice expressing either Cre (Schwenk et al., 1995) or Flpase recombinases (Susan Dymecki, Harvard University, Cambridge, MA) in their germ line, respectively. For Southern blot analysis, genomic DNA was digested by *Sph*I and hybridized with a radiolabeled 5′ probe that included the short arm of the targeting vector (Fig. 1 *a*). Using this probe, both wild-type (4.4 kb fragment) and targeted (2 kb fragment) alleles are detected. To distinguish the targeted, mutant, and conditional alleles, three primer PCRs were performed using primers 1, 2, and 3 (P1, P2, and P3). Primers P1 (5′-GAATGCCCGGGTAAACACCA-3′) and P2 (5′-TCGAAGCGTACCC-TGGCTCT-3′) detect both wild-type and conditional alleles, indicated by 400 and 600 bp, respectively, whereas primer set P1 and P3 (5′-AAGGAGCGCACAGAGGACCA-3′) amplifies an 800 bp fragment indicative of the null allele. Northern analysis was performed with a ^{32}P-dCTP-labeled 1107 bp rat PCR fragment spanning amino acids 146–516 of *sema3F,* which includes most of the sema domain, with total RNA isolated from E16 *sema3F* $^{+/-}$ and *sema3F* $^{-/-}$ embryos (Sambrook et al., 1989).

In situ *hybridization and AP-fusion protein binding to tissue sections.* The rat *sema3F* template for riboprobe synthesis (nucleotides 697–2995) spans most of the ORF as described previously, and the rat *Npn-2* template is a 2558 bp *Eco*RI fragment of the Npn-2 ectodomain beginning 102 bp downstream of the first translation initiation codon (Kolodkin et al., 1997; Giger et al., 1998). Timed-pregnant females [plug day is embryonic day 0.5 (E0.5)] were killed to obtain E10.5–E17.5 embryos. AP-Sema3F binding to tissue sections was performed as described previously (Feiner et al., 1997).

Immunohistochemical procedures. Mice were anesthetized and perfused transcardially with 120 ml of ice-cold perfusion solution (PBS containing 4% paraformaldehyde). Brains were dissected, postfixed overnight at 4°C in perfusion solution, and cryoprotected in PBS containing 30% sucrose. Cryoprotected brains were sectioned using a freezing microtome (40 μm) and subsequently processed as free-floating sections. Endogenous peroxidase activity was quenched by the incubation of tissue sections in methanol containing 0.03% H_2O_2 for 15 min, followed by several washes in PBS. Then, sections were blocked for 2 hr in PBS containing 3% BSA, 0.3% Triton X-100, and 1% normal goat serum. Primary antibodies used included anti-neurofilament 2H3 (1:20, supernatant from hybridoma cells, developmental; Hybridoma Bank, Iowa City, IA), anti-MAP-2 (1:1000; Sigma, St. Louis, MO), and anti-calbindin (1:5000; Swant, Bellinzona, Switzerland). Antibody incubations were performed overnight at 4°C in block solution. Sections were washed (six times for 15 min each) in PBS and incubated with secondary antibody for 1 hr at room temperature. Secondary antibodies included rat adsorbed biotinylated horse anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) and biotinylated goat anti-rabbit IgG (1:200; Vector). After incubation with secondary antibodies, sections were processed using the Vectastain ABC kit. Peroxidase-stained brain sections were dehydrated in a graded ethanol series, cleared in Xylene and embedded in Entellan New (Electron Microscopy Sciences, Fort Washington, PA). For whole-mount anti-neurofilament immunohistochemistry of E10.5 and E11.5 embryos, anti-neurofilament (2H3) supernatant (1:50) and sheep anti-mouse IgG HRP (1:200; Amersham Biosciences, Buckinghamshire, UK) were used as described previously (Kitsukawa et al., 1997).

Results

The ability of multiple secreted semaphorin ligands to bind and signal through a single receptor underscores the need to define *in vivo* the functions of these ligands. In this instance, there are several different potential ligands of Npn-2 whose roles in axon guidance are unknown. By genetic ablation of *sema3F* in the mouse we sought to define *in vivo* the role for Sema3F as a ligand for Npn-2 for PNS and CNS axon guidance events. To accomplish this we carried out a systematic and detailed analysis of *npn-2*–expressing fiber tracts in the CNS and PNS of mice deficient for *sema3F.*

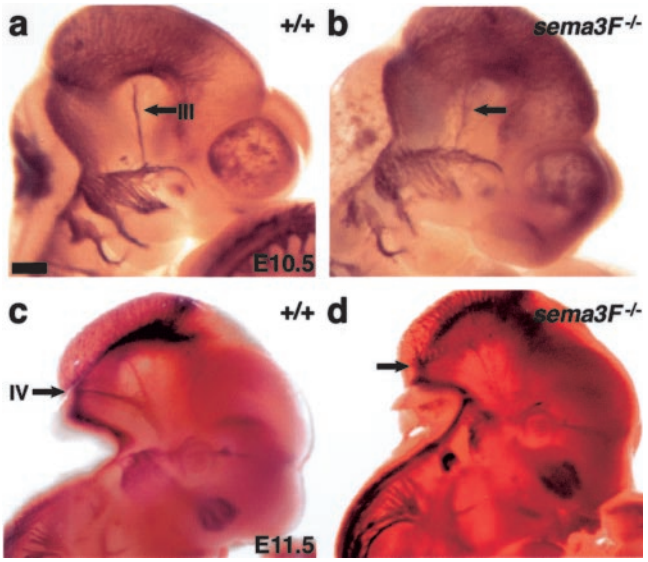

Figure 2. *Npn-2*-expressing cranial nerves are defective in *sema3F* null mice. *a–d,* Whole-mount immunostaining using neurofilament antibodies (2H3) of E10.5 and E11.5 wild-type embryos (*a, c,* respectively) and *sema3F* null embryos (*b, d,* respectively). The oculomotor nerve is severely defasciculated (arrow in *b*), and only a few trochlear axons exit the dorsal midline and project into the periphery in *sema3F* null mice (arrow in *d*). III, Oculomotor nerve; IV, trochlear nerve. For E10.5, $n = 5$ (+/+), 9 (+/−), and 7 (−/−). For E11.5, $n = 4$(+/+), 3(+/−), and 3 (−/−). Scale bar, 100 μm.

Generation of sema3F null and *sema3F* conditional mice

To define the role of *sema3F* in the patterning of neuronal circuitry, we generated *sema3F* null and *sema3F* conditional mutant mice. We targeted exon 1 of *sema3F,* which encodes the first 37 aa of *sema3F,* including the entire signal sequence. Through our targeting strategy we also deleted 4 kb of presumptive promoter sequences upstream of exon 1 that include the first splice donor site (Fig. 1*a*). Homozygous *sema3F* mutant mice are viable and fertile. They are smaller in size than wild-type littermates but achieve normal size by 3 months of age (Fig. 1*e*). Genotype analysis of mice at E17 and of mice after weaning revealed approximate mendelian ratios of mutant and wild-type *sema3F* alleles. The targeted, mutant, and conditional alleles are all distinguishable by PCR and Southern blotting (Fig. 1*b*) (data not shown).

We assessed the expression of *sema3F* in mice harboring the *sema3F* mutant allele. Northern blot analysis using total RNA extracted from E16 embryos revealed two transcripts of ~2.5 and 3.0 kb in *sema3F* heterozygous mice, both of which are absent in *sema3F* $^{-/-}$ embryos (Fig. 1*c*). Thus, *sema3F* $^{-/-}$ mice lack *sema3F* transcripts and are likely to harbor a null mutation at the *sema3F* locus.

Npn-2–expressing cranial motor neurons require peripheral Sema3F *in vivo*

Specific cranial nerve nuclei such as the oculomotor and trochlear nuclei express *npn-2* during early prenatal stages. In embryos lacking Npn-2, the oculomotor nerve is severely defasciculated and trochlear axons fail to project into the periphery. Whole-mount RNA *in situ* hybridization analysis at E11 in the mouse reveals prominent *sema3F* expression in the caudal midbrain and at the rostral hindbrain with a conspicuous corridor devoid of

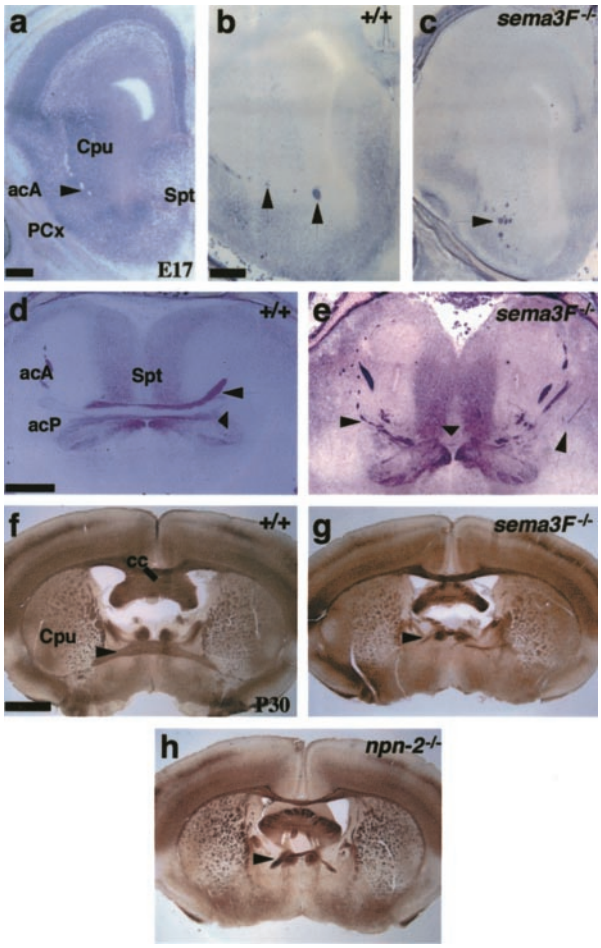

Figure 3. The anterior commissure is disrupted in *sema3F* null mice. *a*, E17 mouse coronal section showing *in situ* hybridization for *sema3F* in the ventral forebrain. *Sema3F* is expressed in the caudoputamen, piriform cortex, and lateral septum. *b–e*, Binding of AP-Sema3F to coronal (*b*, *c*) and horizontal (*d*, *e*) sections of E17 wild-type and *sema3F* null mouse brains. The anterior and posterior limbs of the anterior commissure are defasciculated (arrows in *c*, *e*) in *sema3F* null mice. A tiny fraction of axons decussate normally (arrowhead in *d*, *e*). *f–h*, 2H3-neurofilament immunohistochemistry on coronal sections of P30 wild-type (*f*), *sema3F* null (*g*), and *npn-2* null (*h*) mouse brains. The decussation of the anterior commissure is disorganized in *sema3F* and *npn-2* null mice. Small fascicles of anterior commissure axons traverse the midline in a chaotic and unregulated manner (arrow in *g*, *h*). acA, Anterior commissure, anterior limb; acP, anterior commissure, posterior limb; Cpu, caudoputamen; cc, corpus callosum; PCx, piriform cortex; Spt, septum. For E17 coronal and horizontal, $n = 1(+/+), 3(+/-)$ and $3(-/-)$; for P30, $n = 2(+/+), 3(+/-)$, and $5(-/-)$. Scale bars: *a*, 200 μm; *b*, *c*, 300 μm; *d*, *e*, 500 μm; *f–h*, 1 mm.

sema3F expression at the level of the midbrain–hindbrain junction (Giger et al., 2000). This corridor corresponds to the path taken by trochlear nerve axons once they exit the CNS. *Sema3F* transcripts are also seen flanking trochlear axons on either side of the aqueduct as these axons leave the ventrally embedded fourth nuclei. Sema3F can repel trochlear motor axons *in vitro* (Giger et al., 2000). Therefore, we assessed the contribution of Sema3F to Npn-2 signaling in the development of these specific cranial nerve projections by carrying out whole-mount immunostaining for neurofilament on E10.5 and E11.5 *sema3F* null embryos and wild-type littermates.

At E10.5, the oculomotor nerve in wild-type embryos normally projects ventrally as a compact fiber bundle from the mesencephalic flexure toward the ciliary ganglion and extrinsic ocular muscles (Fig. 2*a*). At this stage, trochlear neuron axons can be seen in transverse sections projecting circumferentially and dorsally around the aqueduct toward the midbrain–hindbrain junction (not shown). By E11.5, trochlear axons of wild-type embryos have exited the CNS, decussated at the dorsal midline, and course along a narrow path to establish synaptic contacts with the superior oblique muscle of the eye (Fig. 2*c*). In dramatic contrast, the trochlear nerve is largely absent in *sema3F* null embryos and only a few axons exit the hindbrain–midbrain junction (Fig. 2*d*). The oculomotor nerve is severely defasciculated in *sema3F* null embryos, but it maintains its peripheral trajectory (Fig. 2*b*). These findings are identical to those seen in *npn-2* null mice and demonstrate an indispensable role for Sema3F–Npn-2 signaling in the normal development of third and fourth cranial nerves (Chen et al., 2000; Giger et al., 2000).

The anterior commissure is defasciculated and fails to decussate normally in *sema3F* null mice

Sema3F is expressed in the ventral forebrain, in the developing hypothalamic–preoptic area, and in the striatum during the formation of the anterior commissure (Fig. 3*a*). The anterior commissure is comprised of an anterior limb, a horseshoe-shaped tract connecting the two olfactory bulbs (pars anterior, acA), and a posterior limb that forms a laterally directed tract carrying projections between the two temporal lobes (pars posterior, acP) (Jouandet and Hartenstein, 1983). Alkaline phosphatase–tagged Sema3F (AP-Sema3F) binds robustly to endogenous Npn-2 and with lower affinity to Npn-1 in brain sections of wild-type mice. Specific limbic projections, including the anterior commissure, stria terminalis, and fasciculus retroflexus express Npn-2 but not Npn-1, and AP-Sema3F no longer binds to these CNS projections in brain sections of *npn-2* null mice (Giger et al., 2000) (data not shown). Therefore, we used AP-Sema3F section binding to visualize the integrity of the anterior commissure in *sema3F* null mice. AP-Sema3F binding in horizontal and coronal sections of an E17 mouse brain reveals high levels of Npn-2 protein on axons that leave the anterior olfactory nuclei (AON) via the anterior limb of the anterior commissure (Fig. 3*b*). Cortical axons coursing through the posterior limb of the anterior commissure are also visualized by AP-Sema3F binding (Fig. 3*d*). The anterior limb axons form tightly fasciculated structures on either side of the midline, are restricted to the same plane of projection along the dorsal–ventral axis, and decussate in a highly organized manner (Fig. 3*b*,*d*). To evaluate the contribution of Sema3F to Npn-2 signaling in the development of this major commissural projection, we performed AP-Sema3F section binding on brains of E17 *sema3F* null mice. In striking contrast to wild-type mice, analysis of E17 *sema3F* null mouse brains revealed severely defasciculated anterior limb axons on either side of the midline. These axons appear to be directed both dorsally and ventrally to the normal horizontal plane of projection both during and after decussation (Fig. 3*c*,*e*). At 4 weeks postnatally, the anterior and posterior limbs of the anterior commissure can be visualized by neurofilament (2H3) immunostaining. Axons of the anterior commissure decussate in a highly organized manner in wild-type mice (Fig. 3*f*). In sharp contrast, neurofilament immunostaining of *sema3F* null littermates revealed few, if any, anterior commissure axons crossing the midline in the ventral forebrain in an organized manner. Instead, the majority of these axons chaotically traverse the midline as tightly bundled small fascicles (Fig. 3*g*). These results indicate that Sema3F in the ventral forebrain is essential for anterior commissure axons to form fascicles and to decussate normally at the CNS midline. It is remarkable that the anterior commissure defect in *sema3F* null mice precisely phenocopies what we observe in age-matched *npn-2* null mice (Fig. 3*h*). Taken

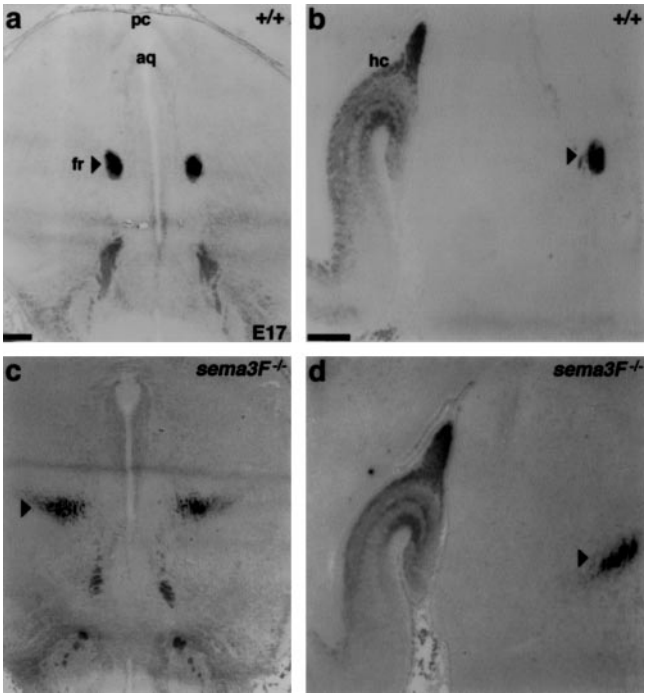

Figure 4. The fasciculus retroflexus is defasciculated in *sema3F* null mice. *a–d,* AP-Sema3F section binding on coronal (*a, c*) and horizontal (*b, d*) sections of brains of E17 wild-type and *sema3F* null mice. The fr is defasciculated in *sema3F* null mice (arrowhead in *c* and *d*). pc, Posterior commissure; aq, aqueduct; hc, hippocampus. For E17 coronal and horizontal, $n = 1(+/+), 3(+/-)$, and $3(-/-)$. Scale bars: *a, c,* 175 μm; *b, d,* 500 μm.

together, these results demonstrate that Sema3F–Npn-2 signaling plays a critical role in the channeling and fasciculation of AON and cortical axons through the anterior commissure.

Projections from the medial habenula to the interpeduncular nucleus are defasciculated in *sema3F* null mice

We next examined a specific limbic projection in the midbrain, the fasciculus retroflexus (fr), which has been shown previously to require Npn-2 for its normal development. This prominent projection from the epithalamus is the last link in a pathway that extends from the basal forebrain through the anterior hypothalamic nuclei to the ventral midbrain tegmentum. *Npn-2* is expressed at high levels in the medial habenula of the thalamus starting at E12, and Npn-2 protein is found along the entire length of fr axons as they project caudoventrally toward the interpeduncular nucleus (Giger et al., 1998) (data not shown). During the development of the diencephalon, *sema3F* is expressed in the rostral prosomere 1 adjacent to the developing fr. Moreover, Sema3F exerts a potent chemorepulsive effect on neurites of perinatal stage habenular explants *in vitro* (Funato et al., 2000). To test the hypothesis that Sema3F directs fr axons *in vivo*, we analyzed this projection by AP-Sema3F section binding at E17 in *sema3F* null mice and their wild-type littermates. In perinatal brains of wild-type mice, fr axons are tightly fasciculated and project caudoventrally and ipsilaterally on either side of the midline (Fig. 4*a*,*b*). However, in brain sections of *sema3F* null mice the fr is defasciculated and is wider, although growth of fr axons to the interpeduncular nucleus does not appear to be altered (Fig. 4*c*,*d*) (data not shown). These results are consistent with our observations on adult *sema3F* null mice using immunostaining for microtubule-associated protein-2 (MAP-2) and myelin basic protein (data not shown). These observations, taken together with the strikingly similar fr phenotype seen in *npn*-2 null mice, show that Sema3F is the Npn-2 ligand that serves to guide fasciculus retroflexus axons from the medial habenula to the interpeduncular nucleus.

Sema3F is required in the hippocampus for infrapyramidal tract development

Hippocampal mossy fibers extend from granule cells in the dentate gyrus and synapse on the apical dendrites of hippocampal CA3 pyramidal neurons. In addition to the main mossy fiber projection that courses along the stratum lucidum, a smaller group of granule cell axons travel below the pyramidal cell layer of CA3, traverse the pyramidal cell layer, and join the main mossy fiber projection. These axons constitute the infrapyramidal tract and, along with the main mossy fiber projection, can be visualized by calbindin immunostaining (Fig. 5*a*). Postnatally, *npn-2* is expressed in granule cells of the dentate gyrus and also in a subpopulation of cells in the hilus, including mossy cells. *Npn-2* is also expressed in pyramidal neurons in CA1 and CA3, and this pattern of expression persists into adulthood (Giger et al., 2000; Holtmaat et al., 2002) (data not shown). Between E15 and postnatal day 0 (P0), *sema3F* is expressed uniformly in the CA1 and CA3 fields and at higher levels in the subiculum (Chedotal et al., 1998) (data not shown). In the adult hippocampus, *sema3F* expression is seen in pyramidal neurons in CA1 and CA3 and, to a lesser extent, in granule cell neurons (Holtmaat et al., 2002; Barnes et al., 2003). *In vitro*, Sema3F strongly repels neurites from perinatal–dentate gyrus and CA3 explants (Chedotal et al., 1998; Chen et al., 2000; Pozas et al., 2001).

To investigate the role of Sema3F in the guidance and fasciculation of main mossy fiber axons and the infrapyramidal tract, we examined the development of these projections in 4-week-old *sema3F* null mice and wild-type littermates using calbindin immunostaining. In contrast to wild-type littermates, *sema3F* null mice show an aberrantly targeted infrapyramidal tract with axons of the infrapyramidal tract extending into the stratum oriens of CA3 (Fig. 5*b*). The main mossy fiber projection appears mostly intact in all mutants examined. Nissl staining of the hippocampus in *sema3F* null mice did not reveal any obvious difference in the number or distribution of granule cells in the dentate gyrus (data not shown). This infrapyramidal tract defect is identical to that reported in both *npn-2* null and *plexin-A3* null mice (Chen et al., 2000; Cheng et al., 2001). Taken together, these observations show that Sema3F signaling, through a holoreceptor complex that includes Npn-2 and Plexin-A3, is required for proper development of the infrapyramidal tract.

Identification of novel limbic requirements for Sema3F and Npn-2 in development of amygdaloid circuitry

The amygdala is a central component of the limbic system with major efferents to the rostral forebrain. Little is known about guidance cues or receptors that control the development of amygdaloid circuitry. The CNS defects that we observe in *sema3F* null mice argue for a critical role for Sema3F in the development of different limbic projections in the forebrain, midbrain, and hippocampus. Therefore, we wanted to know if Sema3F and Npn-2 also play a role in the establishment of amygdaloid circuitry.

The stria terminalis is a prominent limbic tract comprised of axons that course between the amygdala and the ventral forebrain. It arises principally in specific amygdalar nuclei and follows the inner curvature of the caudate nucleus to the rostral forebrain area (Fig. 6*c*). Stria terminalis fibers terminate in the septal area, in the medial preoptic area of the hypothalamus and

in the bed nucleus of the stria terminalis (Bst). Specific amygdalar nuclei project to different parts of the Bst. The central nucleus and certain amygdalar nuclei associated with the main olfactory system preferentially innervate various parts of the lateral and medial halves of the bed nuclear anterior division. However, the medial nucleus and the rest of the amygdalar nuclei associated with both the accessory and main olfactory systems target the posterior division and the medial half of the anterior division of the Bst (Dong et al., 2001). Thus far, the guidance cues required for pathfinding or targeting of the stria terminalis are unknown.

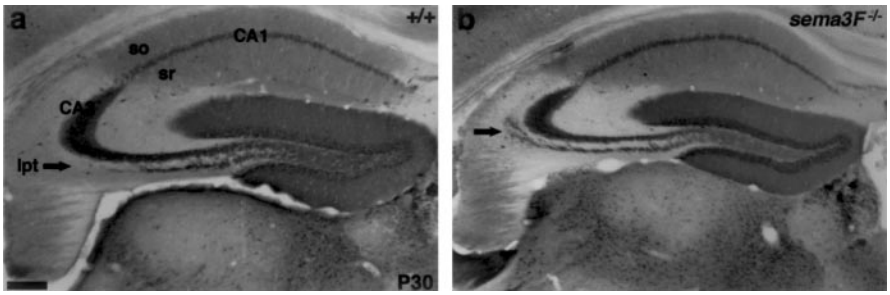

Figure 5. The infrapyramidal tract projects abnormally in *sema3F* null mice. *a, b,* Calbindin immunostaining on coronal sections of brains of P30 wild-type (*a*) and *sema3F* null mice (*b*). Infrapyramidal tract axons aberrantly extend into the stratum oriens of CA3 in *sema3F* null mice (arrow in *b*). lpt, Infrapyramidal tract; so, stratum oriens; sr, stratum radiatum. $n = 3$ (+/−) and 5 (−/−). Scale bars: *a, b,* 200 μm.

Npn-2 and *sema3F* are expressed during development of the stria terminalis in nuclei of amygdalar efferents and in target areas in the ventral forebrain, respectively. Analysis of *npn-2* transcripts at E17 revealed specific labeling of the medial aspect of the central amygdaloid nucleus, the medial amygdaloid, and the cortical amygdaloid nuclei (Fig. 6*b*). *Sema3F* transcripts, on the other hand, are found in the developing hypothalamus, caudoputamen, and Bst (Fig. 6*a*) (data not shown). Thus, *sema3F* expressed in the ventral forebrain might guide incoming *npn-2*–expressing amygdalar axons.

To assess the requirement for Sema3F and Npn-2 in stria terminalis pathfinding, we examined the fate of this tract in both *sema3F* and *npn-2* null mice using neurofilament and calbindin (data not shown) immunostaining in age-matched *sema3F* and *npn-2* null mice. In brains of P30 wild-type mice, amygdalar efferents enter the Bst as a tightly fasciculated and organized structure on either side of the midline (Fig. 6*d*). In contrast, immunostaining of *npn-2* null brains revealed disrupted targeting of these efferents (Fig. 6*e*). Inspection of *sema3F* null brains also revealed that stria terminalis targeting is disorganized and is indistinguishable from that seen in *npn-2* null mice (Fig. 6*f,* 7*i*) (data not shown).

To better visualize this defect in stria terminalis targeting, we performed AP-Sema3F section binding on brains of E17 *sema3F* null mice and their wild-type littermates. In both wild-type and *sema3F* null mice, axons leave the amygdala as an intact fasciculated bundle and weave their way around the caudate (Fig. 6*g,h,k,l*). In wild-type mice, axons of the stria terminalis enter the hypothalamus and the Bst as a compact fascicle on either side of the midline (Fig. 6*i,j*). However, in *sema3F* null mice, targeting of these axons is severely disrupted in the hypothalamus and also more rostrally in the Bst (Fig. 6*m,n*). The axons that enter both of these target fields are disorganized and severely defasciculated. These observations demonstrate a role for Sema3F in guiding amygdalar efferents to their destinations, the hypothalamus and Bst. Moreover, these results show that Sema3F–Npn-2 signaling is required for proper targeting of the stria terminalis to the hypothalamus and the Bst.

Neuron-specific requirements for Sema3F in development of the anterior commissure and infrapyramidal tract

Numerous neuronal migration and axon guidance events occur in the ventral forebrain at prenatal and perinatal stages, and guidance cues expressed in specific cell types instruct these processes. For example, analysis of the ontogeny of the anterior commissure in the mouse, rat, opossum, and hamster suggests a role for pioneer axons, GFAP-expressing cells at the midline, and ependymal cells at the rostral pole of the third ventricle in influencing the channeling and decussation of axons in the anterior commissure (Wahlsten, 1981; Pires-Neto and Lent, 1991; Santacana et al., 1992; Cummings et al., 1997; Pires-Neto et al., 1998). Unlike the anterior commissure, which develops perinatally, the hippocampal infrapyramidal tract forms postnatally. At postnatal stages, s*ema3F* is expressed, albeit at low to moderate levels, in specific hippocampal cell populations, including granule cells of the dentate gyrus and pyramidal neurons of both CA3 and CA1 (Holtmaat et al., 2002). The identities of specific cell types in which *sema3F* is required *in vivo* are not apparent simply from *sema3F* RNA *in situ* analysis. To distinguish the neuronal *sema3F* contribution to axon guidance in the ventral forebrain and hippocampus from that of other cell types, we used a *synapsin-1 Cre* (*syn-1 Cre*) transgenic mouse line to generate mice that lack *sema3F* specifically in neurons. In *syn-1 Cre* mice, Cre expression is controlled by the rat *synapsin-1* promoter, which drives transgene expression exclusively in almost all neuronal cells (Hoesche et al., 1993). Characterization and use of this line in other studies reveals expression of functional Cre recombinase as early as E12.5 in most differentiated neurons outside the ventricular zones of the brain and spinal cord (Ma et al., 1999; DeFalco et al., 2001; Zhu et al., 2001). Furthermore, no Cre expression has been observed in astroglia or other non-neuronal cell types in this line. To selectively ablate *sema3F* in neurons, we generated mice that were heterozygous for the *syn-1 Cre* allele and either homozygous for the *sema3F* conditional allele or heterozygous for both the *sema3F* null and *sema3F* conditional alleles (*syn-1 Cre/+; C/C* or *syn-1 Cre/+; C/−*, respectively) (Fig. 1*d*).

Analysis of the anterior commissure in P30 brains from mice heterozygous for the *syn-1 Cre* allele and either homozygous for the conditional *sema3F* allele (*syn-1 Cre/+; C/C*) or heterozygous for the *sema3F* conditional and *sema3F* null alleles (*syn-1 Cre/+; C/−*) using neurofilament (2H3) immunostaining revealed phenotypes reminiscent of those seen in *sema3F* null mice (3/3 *syn-1 Cre/+; C/−* and 5/5 *syn-1 Cre/+; C/C*). At rostral levels, in contrast to control littermates, the anterior limb of the anterior commissure was dramatically reduced and defasciculated (Fig. 7*a,d*). Although a small number of axons of the anterior commissure decussate properly, most axons cross the midline aberrantly as smaller tightly bundled fascicles (Fig 7. *b,e*). Interestingly, we did not observe any defects in stria terminalis targeting in *syn-1 Cre/+; C/−* and *syn-1 Cre/+; C/C* mice (3/3 *syn-1 Cre/+; C/−* and 5/5 *syn-1 Cre/+; C/C*) (Fig. 7*f*). Taken together, these results indicate that a neuronal source of *sema3F* is critical for normal fasciculation and decussation of the anterior commissure.

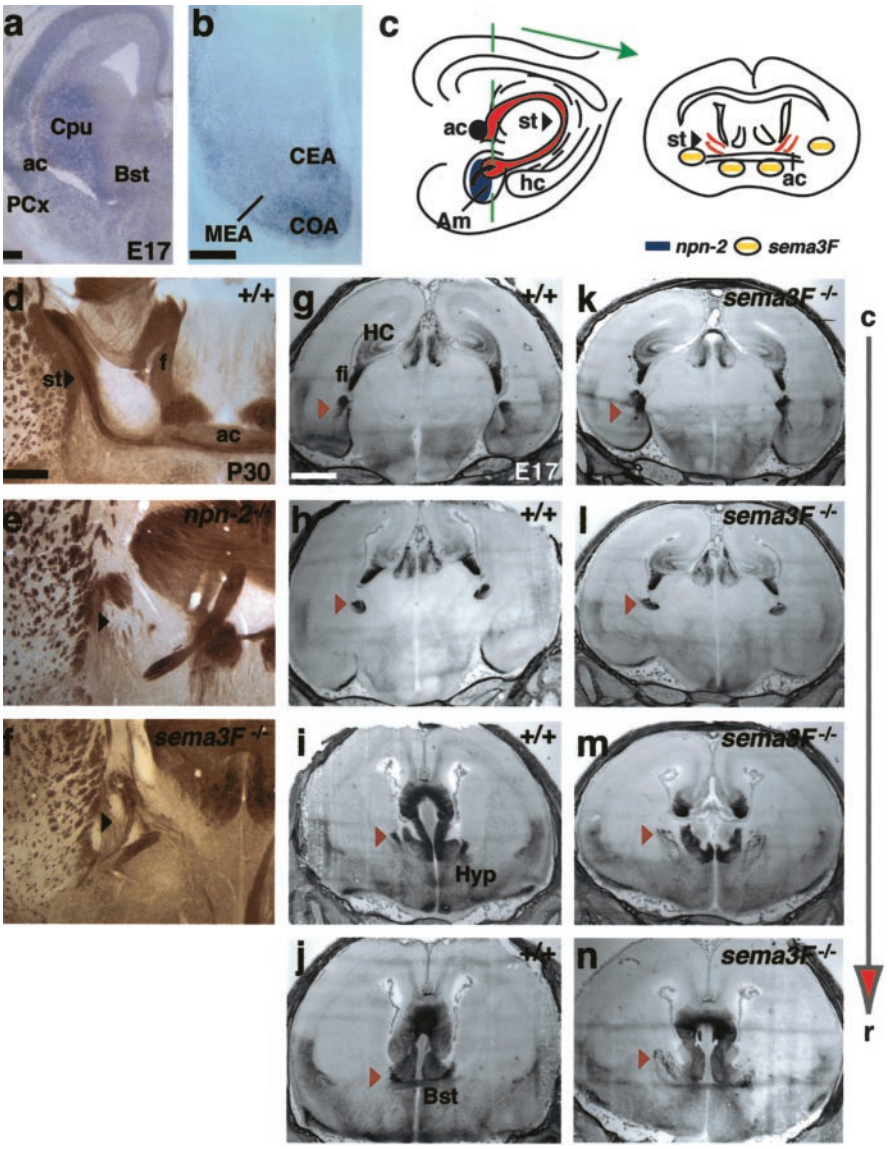

Figure 6. Abnormal targeting of stria terminalis in *sema3F* and *npn-2* null mice. *a,* E17 mouse coronal section showing *in situ* hybridization for *sema3F* in ventral forebrain. *Sema3F* is expressed in the caudoputamen, piriform cortex, and Bst. *b,* E17 mouse coronal section showing *in situ* hybridization for *npn-2* in the amygdala. *Npn-2* is expressed in central, medial, and cortical amygdaloid nuclei. *c,* Schematic diagram showing *sema3F* and *npn-2* expression in the context of the projection of the stria terminalis. The stria terminalis carries axons from the amygdala to the Bst (shown here) and the hypothalamus. *d–f,* Neurofilament (2H3) immunostaining of brains of P30 wild-type (*d*), *npn-2* null (*e*), and *sema3F* null mice (*f*). Stria terminalis axons entering the Bst are disorganized (black arrowhead in *e, f*). *g–n,* AP-Sema3F section binding on coronal sections of E17 wild-type brains (*g–j,* caudal to rostral) and *sema3F* mutant brains (*k–n,* caudal to rostral). The targeting of stria terminalis axons is disrupted, both in the hypothalamus (red arrowhead in *m*) and more rostrally in the Bst (red arrowhead in *n*). CEA, central amygdaloid nucleus; MEA, medial amygdaloid nucleus; COA, cortical nucleus amygdala; fi, fimbria; f, fornix; Hyp, hypothalamus. For E17, $n = 1(+/+)$, $3(+/-)$, and $3(-/-)$; for P30, $n = 2(+/+)$, $3(+/-)$, $5(-/-)$, and 3 ($npn\text{-}2^{-/-}$). Scale bar: *a,* 200 μm; *b,* 250 μm; *d–f,* 500 μm; *g–n,* 1 mm.

Calbindin immunostaining of P30 brains from *syn-1 Cre/+; C/−* and *syn-1 Cre/+; C/C* mice also revealed an infrapyramidal tract defect in a subset of these mice (two of three *syn-1 Cre/+; C/−* and one of five *syn-1 Cre/+; C/C*). Analogous to the *sema3F* null mouse, neuron-specific deletion of *sema3F* results in infrapyramidal tract axons extending far into the stratum oriens of CA3, beyond the level at which they normally turn dorsally into the stratum radiatum (Fig. 7*g,h*). Thus, neuronal *sema3F* is required for establishing specific granule cell–pyramidal neuron circuitry.

Discussion

The formation of functional neuronal circuits is contingent upon the completion of numerous highly stereotyped events, such as fasciculation, channeling, and targeting of growing axons. Class 3 semaphorins are expressed in the developing and adult nervous system in specific cell types and may have disparate functions such as chemorepulsion or chemoattraction on extending neurons. Mice lacking *npn-1* or *npn-2*, the coreceptors for class 3 semaphorins, exhibit severe defects in nervous system development. Thus, characterization of mice lacking the different class 3 semaphorins allows us to define precisely the requirements for semaphorin-neuropilin signaling *in vivo*. Our analysis of *sema3F* null reveals that Sema3F is the principal ligand for Npn-2 in axon guidance events and allows for a better understanding of the complete range of secreted semaphorin functions throughout neural development *in vivo*. Furthermore, using *sema3F* conditional mutant mice, we demonstrate a requirement for Sema3F in neurons to guide select *npn-2* expressing neurons *in vivo*, thereby underscoring a role for neuron–neuron signaling in axon pathfinding.

Sema3F is the principal ligand for Npn-2 in axon guidance *in vivo* and is a critical determinant of limbic circuitry

To define the role of Sema3F in axon guidance we have generated *sema3F* null mice; these mice show profound central and peripheral axon guidance defects in *npn-2*–expressing neurons. A unifying feature of the neural defects observed in *sema3F* and *npn-2* null mice is that many of the CNS circuits affected are components of the limbic system. We show here that Sema3F is required in the ventral forebrain to channel axons of the anterior commissure as they decussate and course toward their respective targets, the contralateral olfactory bulb and temporal lobe. In the hippocampus of *sema3F* null mice, we observe an infrapyramidal tract defect identical to that found in *npn-2* and *plexin-A3* null mice (Chen et al., 2000; Cheng et al., 2001). In the diencephalon, we show that Sema3F is required for the fasciculation of axons as they leave the medial habenula and project toward the interpeduncular nucleus. The fasciculus retroflexus defect in *sema3F* null mice is commensurate with a model of surround repulsion in which Sema3F is indeed the chemorepellent in rostral prosomere 1 in the developing diencephalon.

Interestingly, the peripheral *sema3F* expression and severe trochlear nerve defect in *sema3F* null mice is also consistent with a Sema3F-mediated surround repulsion mechanism for channeling *npn-2*–expressing trochlear axons as they exit the hindbrain–

midbrain junction. In addition to the trochlear nerve, the oculomotor nerve is severely defasciculated in *sema3F* null mice, a phenotype consistent with the pattern of *sema3F* expression in the developing midbrain (Giger et al., 2000). Taken together, our results show that Sema3F plays a critical role in both central and peripheral axon guidance.

We show here that neuronal defects found in *sema3F* null mice strictly phenocopy those observed in *npn-2* null mice (Chen et al., 2000; Giger et al., 2000). These observations indicate that Sema3F is necessary and sufficient for Npn-2–mediated functions in axon guidance; other class 3 semaphorins such as Sema3B and Sema3C do not compensate for the loss of Sema3F function in the different systems we have examined. The expression of Sema3B in the spinal cord and its ability to repel commissural axons point to a potential role for this Npn-2 ligand in axon pathfinding (Zou et al., 2000b). More recently, implication of Sema3B as a tumor-suppressor gene suggests that Sema3B might have an important role in tumor metastasis (Tomizawa et al., 2001; Tse et al., 2002). Whether or not these effects require Npn-2 is unclear at present. In contrast, *sema3C* null mice exhibit heart defects not observed in *npn-2* null mice, suggesting that this secreted semaphorin can signal through an Npn-2-independent mechanism (Feiner et al., 2001). Additional analysis of mice lacking these different secreted semaphorins will reveal the degree to which they contribute to Npn-2 signaling in non-neuronal systems.

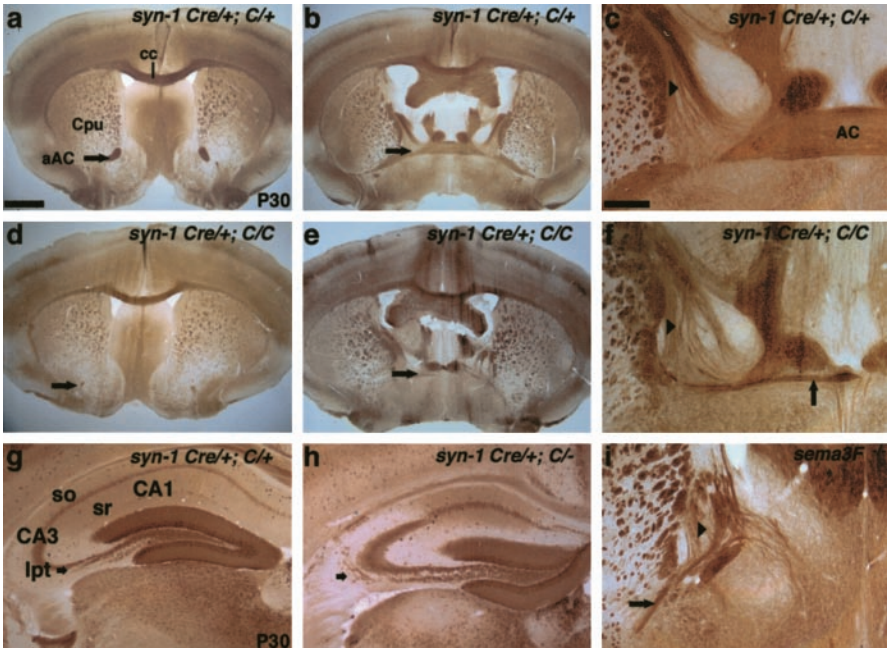

Figure 7. The anterior commissure and infrapyramidal tract are defective in mice lacking *sema3F* in neurons. *a–f, i,* 2H3-neurofilament immunohistochemistry on coronal sections of P30 control (*a–c*) and *syn-1 Cre/+; C/C* (*d–f*), and *sema3F*$^{-/-}$ (*i*) mice. The anterior commissure is defasciculated (arrow in *d*), and decussation is disrupted (arrow in *e, f, i*). The arrowheads in *c, f,* and *i* indicate the stria terminalis. The stria terminalis and anterior commissure develop independently of each other. *g, h,* Calbindin immunostaining on the brains of P30 control (*g*) and *syn-1Cre/+; C/−* (*h*) mice. Infrapyramidal tract axons aberrantly extend into the stratum oriens of CA3 in *syn-1Cre/+; C/−* mice (arrow in *h*). $n = 5$ (*syn-1 Cre/+; C/C*), 3 (*syn-1Cre/+; C/−*), and 3 (*syn-1 Cre/+; C/+*). Scale bars: *a, b, d,* 1 mm; *g, h,* 200 μm; *c, f, i,* 50 μm.

Identification of a guidance cue–receptor pair that controls development of amygdaloid circuitry

The amygdala is a principal component of the limbic system with a wide range of roles in human emotion. The identity of guidance cues that play a role in the establishment of amygdaloid circuitry is unknown. We show that *npn-2* and *sema3F* are expressed in the amygdala and the rostral forebrain perinatally, respectively, suggesting that Sema3F and Npn-2 may play a role in guiding projections from the amygdala to the rostral forebrain. Using *sema3F* and *npn-2* null mice, we demonstrate here that Sema3F–Npn-2 signaling is required for the targeting of the stria terminalis, a major output of the amygdala. Thus, Sema3F is required in the ventral forebrain by distinct *npn-2*–expressing fiber tracts such as the anterior commissure and the stria terminalis for fasciculation, decussation, and targeting. It will be interesting to assess the behavioral consequences of these defects in specific amygdala-related learning paradigms such as the fear-potentiated startle reflex and the light-enhanced startle effect (Davis and Shi, 1999).

Neuron-specific requirement for a soluble axon guidance cue in vertebrate CNS axon guidance

Precise spatial distributions of guidance cues are required to establish proper neuronal connectivity *in vivo*. However, little is known about the mechanisms by which such distributions are established or how they are maintained. Moreover, there is a paucity of *in vivo* data to corroborate models postulating axon–axon or axon–glia interactions for proper axon pathfinding. To begin to understand how Sema3F acts on different populations of neurons to facilitate axon-tract fasciculation and proper targeting, we assessed cell-type requirements for Sema3F functions. Using the *sema3F* conditional mutant and *syn-1 Cre* mice, we generated mice lacking Sema3F solely in neurons. In these mice we found that establishment of both the anterior commissure in the ventral forebrain and the infrapyramidal tract in the hippocampus requires neuronal Sema3F. The anterior commissure defect was found in all *syn-1 Cre/+; C/−* and *syn-1 Cre/+; C/C* mice examined and is reminiscent of that seen in *sema3F* null mice. This result suggests that residual *sema3F* expressed in glial cells cannot compensate for the loss of neuronal *sema3F* in the development of this major commissural projection. The observation that at least some anterior commissure axons do cross the midline, albeit in a haphazard manner, shows that other guidance cues must still be operative in this region. Indeed, anterior commissure defects are seen in *netrin-1* and *EphB2* mutant mice (Serafini et al., 1996; Cowan et al., 2000). Furthermore, the absence of a phenotype in the stria terminalis in *syn-1 Cre/+; C/C* or *syn-1 Cre/+; C/−* mice indicates that these two closely apposed limbic tracts develop independently from one another. Importantly, these data suggest that anterior commissure and stria terminalis axons rely on distinct sources of Sema3F. Thus, even though axons of these two limbic projections journey through a common terrain within the ventral forebrain, they differ in their spatial requirements for Sema3F.

The infrapyramidal tract defect observed in mice lacking neuronal *sema3F* is also similar to that seen in *sema3F* null mice. However, in contrast to the consistently observed defects in the anterior commissure, we find the penetrance of this defect in the hippocampus to be somewhat lower in mice that lack a neuronal source of Sema3F than in *sema3F* null mice. This may reflect

either a low efficiency of Cre recombination in the hippocampus of *sema3F* conditional mutant mice or additional requirements for Sema3F in non-neuronal cells in the hippocampus. Although the neuron-specific *sema3F* ablation experiments define the contribution of neuronal *sema3F*, they do not specify the neuronal source for *sema3F* in the ventral forebrain and in the hippocampus. These neuron-specific *sema3F* ablation data should motivate additional inquiry into determining the identity of these neuronal populations and subsequent analysis of the mode of action of Sema3F, whether it be autocrine, paracrine, or juxtacrine, on *npn-2*–expressing neurons.

Sema3F–Npn-2/Plexin-A3 signaling is required for normal development of the infrapyramidal tract

In our analysis of *npn-2*–expressing neurons in the hippocampus of *sema3F* null mice we show that *sema3F* is required for the normal targeting of the infrapyramidal tract. This same infrapyramidal tract defect is also observed in *npn-2* and *plexin-A3* null mice, suggesting that Sema3F interacts with an Npn-2/Plexin-A3 holoreceptor complex to elicit normal development of the infrapyramidal tract (Chen et al., 2000; Cheng et al., 2001). Based on *sema3F* mRNA distribution and the neuron-specific requirement for Sema3F in infrapyramidal tract development, it is plausible that Sema3F is required in a cell-autonomous manner, such that dentate granule cells projecting through the infrapyramidal tract secrete and respond to Sema3F. A more parsimonious model consistent with recent observations (Bagri et al., 2003) is that Sema3F released by CA3 neurons acts on axons of the infrapyramidal tract to shape its final architecture.

Plexin-A3 null mice share only a subset of the cranial nerve defects observed in *sema3F* and *npn-2* null mice (Cheng et al., 2001). Therefore, it is likely that Sema3F signals through a holoreceptor complex of Npn-2 and a different class A Plexin in the cranial nerve projections that are defective in *sema3F* and *npn-2* null mice but not in *plexin-A3* null mice. *In vitro*, Sema3F can collapse cells co-expressing Npn-2 and Plexin-A1 (Takahashi and Strittmatter, 2001). *Plexin-A1* is also expressed in many *npn-2*–expressing neuronal structures during embryonic development. These observations qualify Plexin-A1 as an excellent candidate Sema3F coreceptor in the normal development of systems such as the anterior commissure, fasciculus retroflexus, and specific cranial nerve projections (Murakami et al., 2001). Additional studies will reveal the precise combinations of Npn-2 and A-class Plexins required to confer Sema3F responsiveness to neurons *in vivo*.

Defining a role for class 3 semaphorins in the adult nervous system has remained elusive. In the adult hippocampus, *sema3F* is expressed in pyramidal neurons of CA1 and CA3 and also in granule cells of the dentate gyrus (Holtmaat et al., 2002; Barnes et al., 2003). Evidence of neurogenesis in the adult dentate gyrus and a role for aberrant granule cell circuitry in seizure generation underscore the need to define the etiology of the infrapyramidal tract defect in *sema3F* null mice (McNamara, 1994; Parent et al., 1997; van Praag et al., 2002). Experiments aimed in this direction will shed light on potential functions for Sema3F in the adult brain, which may extend beyond its role in axon guidance.

Although we have focused on the role of Sema3F in the nervous system, *sema3F* is expressed in a multitude of non-neuronal tissues during fetal development and in the adult, including the lung. Functional assays using cultured fetal lung tissue show that Sema3F can enhance branching morphogenesis, suggesting that Sema3F may play a role in lung development (Kagoshima and Ito, 2001). Interestingly, *sema3F* in humans is localized to the region 3p21.3 on chromosome 3, and in this region several lung cancer cell lines exhibit homozygous deletions indicative of the presence of a tumor-suppressor gene (Roche et al., 1996; Xiang et al., 1996; Lerman and Minna, 2000). Experiments performed to assess a role for Sema3F in tumor metastasis suggest that it can act in an autocrine manner to suppress tumor growth (Xiang et al., 2002). It will be interesting to see whether analysis of *sema3F* null mice unveils parallels between nervous system development and mechanisms of tumor progression.

In summary, we show here that the class 3 semaphorin Sema3F is the major Npn-2 ligand for axon guidance events *in vivo*. Furthermore, we show a neuronal requirement for *sema3F* in CNS development, underscoring the significance of neuron–neuron interactions in axon pathfinding. We also present *in vivo* evidence consistent with a requirement for a Npn-2–Plexin-A3 holoreceptor complex in mediating Sema3F responses. The integral role played by Sema3F–Npn-2 signaling in the patterning of neuronal circuitry demonstrated here may be applicable to the adult nervous system in neuronal processes such as regeneration and synaptic plasticity, and may also be important for non-neuronal events, including tumorigenesis.

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