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Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling

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In vertebrate embryos, neural crest cells migrate only through the anterior half of each somite while avoiding the posterior half. We demonstrate that neural crest cells express the receptor neuropilin 2 (*Npn2*), while its repulsive ligand semaphorin 3F (*Sema3f*) is restricted to the posterior-half somite. In *Npn2* and *Sema3f* mutant mice, neural crest cells lose their segmental migration pattern and instead migrate as a uniform sheet, although somite polarity itself remains unchanged. Furthermore, Npn2 is cell autonomously required for neural crest cells to avoid Sema3f in vitro. These data show that Npn2/Sema3f signaling guides neural crest migration through the somite. Interestingly, neural crest cells still condense into segmentally arranged dorsal root ganglia in *Npn2* nulls, suggesting that segmental neural crest migration and segmentation of the peripheral nervous system are separable processes.

KEY WORDS: Trunk neural crest migration, Sclerotome, Neuropilin 2, Semaphorin 3F, Mouse, Chick

INTRODUCTION

The neural crest is a multipotent population of migratory cells that gives rise to a wide variety of different lineages in vertebrates. During development, neural crest cells arise in the central nervous system, but subsequently migrate away and follow defined stereotypic pathways. In the trunk, for example, neural crest cells invade only the anterior but not the posterior portion of each somitic sclerotome (Bronner-Fraser, 1986; Rickman et al., 1985; Serbedzija et al., 1990). This selective migration results in the formation of segmentally arranged streams of migrating neural crest cells. This pattern appears to be imposed by the somites, with the anterior sclerotome being permissive and posterior sclerotome repulsive for neural crest migration (Bronner-Fraser and Stern, 1991; Kalcheim and Teillet, 1989). Accordingly, surgical or genetic modification of anteroposterior somite polarity results in loss of segmental neural crest migration (Kalcheim and Teillet, 1989) and formation of fused neural crest-derived dorsal root ganglia (Bussen et al., 2004; Kalcheim and Teillet, 1989; Leitges et al., 2000; Mansouri et al., 2000). Thus, the segmental pattern of neural crest migration is believed to be responsible for the metameric organization of the ganglia of the peripheral nervous system (Kuan et al., 2004; LeDouarin and Kalcheim, 1999). The segmented arrangement of these ganglia relative to the somites, which will form the vertebrae, is crucial for proper wiring of the ganglia and peripheral nerves to targets in the periphery.

The identity of the molecular cues that direct neural crest migration exclusively through the anterior sclerotome is still open to debate. Although previous reports suggested that Eph/ephrin signaling might pattern trunk neural crest migration (Krull et al., 1997; Wang and Anderson, 1997), the *Eph* and ephrin mutant mice that have been examined fail to exhibit trunk neural crest migration defects (Adams et al., 2001; Davy et al., 2004; Orioli et al., 1996; Wang et al., 1998). Likewise, neuropilin 1 and its ligand semaphorin 3A have been suggested to play a role (Eickholt et al., 1999), but are not expressed at the right time (reviewed by Kuan et al., 2004) and are not required in the mouse for appropriate trunk neural crest migration (Kawasaki et al., 2002). It is not clear whether the inability to identify a trunk neural crest mutant phenotype is due to redundancy or whether the true regulatory molecules have not been found.

We isolated chick neuropilin 2a1 (*Npn2a1*) in a screen for genes upregulated as a consequence of neural crest induction (Gammill and Bronner-Fraser, 2002). Npn2 is a receptor for class 3 secreted semaphorins (Sema) 3C and 3F as well as vascular endothelial growth factor (Bagri and Tessier-Lavigne, 2002; Neufeld et al., 2002). Npn2 is required for appropriate axon guidance and fasciculation in the central and peripheral nervous system (Chen et al., 2000; Cloutier et al., 2002; Giger et al., 2000). However, the importance of Npn2 and its ligands during trunk neural crest development has not been examined.

Here, we explore the role of Npn2 signaling during neural crest migration. We demonstrate that the Npn2 receptor on neural crest cells detects a Sema3f repellant cue in the posterior sclerotome that guides neural crest migration through the somites. Surprisingly, individualized dorsal root ganglia still form, albeit less well separated than normal, suggesting that the pattern of neural crest migration alone does not dictate the arrangement of the peripheral ganglia, and that multiple signaling pathways are required to create a segmented peripheral nervous system.

MATERIALS AND METHODS

Embryos

Fertile chicken eggs were incubated at 37°C to the desired stage (Hamburger and Hamilton, 1992). Embryos were isolated in Ringers Saline and fixed overnight at 4°C in 4% paraformaldehyde. Mouse embryos were surgically isolated, with day 0.5 being the day of the plug, into ice cold phosphate-buffered saline (PBS) and fixed for 2 hours at room temperature or overnight at 4°C in 4% paraformaldehyde. Embryos were genotyped by polymerase chain reaction (PCR) (Giger et al., 2000).

In situ hybridization

Chick in situ hybridization was performed as described previously (Gammill and Bronner-Fraser, 2002). Mouse in situ hybridization was performed as described previously (Wilkinson, 1992), except that hybridization was

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performed in 50% formamide, 1.3× SSC (pH 5), 5 mM EDTA, 50 μg/ml yeast RNA, 0.2% Tween 20, 0.5% CHAPS and 50 μg/ml heparin at 70°C. Embryos were washed twice in hybridization mix at 70°C, three times in wash solution I at 65°C, and antibody pre-treatment was performed in 100 mM maleic acid, 150 mM NaCl, 0.1% Tween (pH 7.5) with 2% Blocking Reagent (Boehringer Mannheim). Templates for digoxigenin-labeled antisense riboprobes were as follows: chick *Npn2* (Gammill and Bronner-Fraser, 2002), mouse *Npn2* (Giger et al., 2000), *Sox10* (Kuhlbrodt et al., 1998), *Sema3f* (Giger et al., 2000), ephrinB2 (Wang and Anderson, 1997), *Tbx18* (Kraus et al., 2001) and *Uncx4.1* (Mansouri et al., 1997). Stained embryos were infiltrated with 5% sucrose, 15% sucrose and 7.5% gelatin in 15% sucrose, frozen in liquid nitrogen, sectioned at 20 μM by cryostat (Microm) and mounted in permafluor (Thermo Electron Corporation).

Immunohistochemistry

Neural crest cells with were stained with 1:50 anti-HNK-1 (American Type Culture; Tucker et al., 1984) followed by 1:400 anti-mouse-IgM-Rhodamine Red X (Jackson Immuno Research) or 1:2000 anti-p75 (Weskamp and Reichardt, 1991) followed by 1:400 anti-rabbit-Rhodamine Red X (Jackson Immuno Research). Sema3f spots were visualized using an anti-mouse IgG Alexa 488 secondary at 1:1000 (Molecular Probes). Unstained embryos were infiltrated with 5% sucrose, 15% sucrose and 7.5% gelatin in 15% sucrose, frozen in liquid nitrogen, sectioned at 15 μM by cryostat (Microm) and degelatinized for 20 minutes at 42°C in PBS. Dorsal root ganglia were stained with 1:500 anti-TUJ1 (neuron specific class III β-tubulin; Babco) followed by 1:500 anti-mouse-Biotin (Jackson Immuno Research), and developed using the ABC-horseradish peroxidase kit (Vector Laboratories) and 0.1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) with 0.009% hydrogen peroxide according to the manufacturer's instructions.

Conditioned medium

293T cells were transfected with 24 μg of AP-Sema3f (Giger et al., 2000) or AP empty vector using 45 μl Lipofectamine 2000 (Invitrogen) per 10 cm dish according to the manufacturer's instructions. Media (DMEM + 0.1% BSA + Pen Strep) was collected after 3 days and concentrated using a Centriplus YM-100 filter device (Millipore). Alkaline phosphatase activity was determined using AP Assay Reagent A (GenHunter Corporation) and the molarity of the collected protein calculated according to the manufacturer's instructions.

Sema3f spot preparation

Aminopropyltriethoxysilane (2%; APTES; Sigma) was prepared in 95% ethanol and allowed to hydrolyze for 5 minutes in a fume hood. Thermanox (25 mm; Nunc) cover slips in a wafer basket (Fluoroware) were incubated in the 2% APTES for 10 minutes, and washed three times for 5 minutes with 95% ethanol. Coverslips were cured for 15 minutes at 100°C in a vacuum oven, immobilized onto 35 mm tissue culture plates with four spots of silicone vacuum grease, and UV sterilized in a tissue culture hood for 15 minutes. AP-Sema3f (75 nM) was preincubated at room temperature for 1 hour with 50 μg/ml mouse anti-human placental alkaline phosphatase (Chemicon), 0.2 μl drops were spotted manually in a grid on the coverslip, and the location of the spots was marked on the underside of the dish. After 1 hour at 37°C, the coverslips were washed three times with 4 ml of 1× Hank's buffered saline solution (HBSS; Invitrogen). After all remaining traces of HBSS had been aspirated, 150-200 μl of 125 μg/ml fibronectin (BD Biosciences) was laid over the spots and incubated for 1.5 to 2 hours at 37°C. After aspirating the fibronectin, the coverslips were washed once with 4 ml of HBSS and stored overnight at 4°C in 2 ml DMEM-F12 (Invitrogen) + 1 mg/ml BSA.

Mouse neural tube culture

E9.5 embryos (14-24 somites) were isolated into ice cold HBSS. The region of the trunk containing the last 10 somites was dissected, trimming the membranes lateral to the somites and removing the gut tube. Trunk pieces were incubated for 8 minutes at 37°C in room temperature 3 μg/ml dispase made fresh in HBSS and 0.2 μm filter sterilized. After rinsing several times with DMEM-F12 + 10% fetal bovine serum (Hyclone), neural tubes were isolated by trituration with a fire-polished pasteur pipette and plated on the spotted region of the coverslips in 1 ml of pre-warmed neural crest complete medium, prepared as described (Stemple and Anderson, 1992) except that DMEM-F12 was used and retinoic acid was omitted. The tubes were allowed to stick to the coverslip for one hour at 37°C, then 2 ml of additional complete medium was added slowly down the side of the dish, and explants were cultured for an additional 28-48 hours at 37°C.

RESULTS

Neural crest migrates through anterior and posterior sclerotome in *Npn2* mutants

We identified chick *Npn2* as an early response to neural crest induction (Gammill and Bronner-Fraser, 2002). *Npn2* is expressed in premigratory neural crest cells in the dorsal neural tube as well as on migratory neural crest in both the chick (Fig. 1A,B) (Gammill and Bronner-Fraser, 2002) and the mouse (Fig. 1C-F). In the trunk, *Npn2* was clearly expressed in a segmental pattern in both species. Longitudinal sections through chick embryos revealed that *Npn2* was expressed by neural crest cells, identified by HNK-1 immunoreactivity, as they migrate through the anterior half of each somitic sclerotome (Fig. 1B). Similarly, in transverse sections of mouse embryos, *Npn2* expression colocalized with the neural crest marker p75 (Fig. 1D-F).

The distribution of *Npn2* on neural crest cells made this receptor a potential candidate for influencing neural crest formation and migration. To address the requirement for Npn2 during neural crest development, we assessed the loss-of-function phenotype by

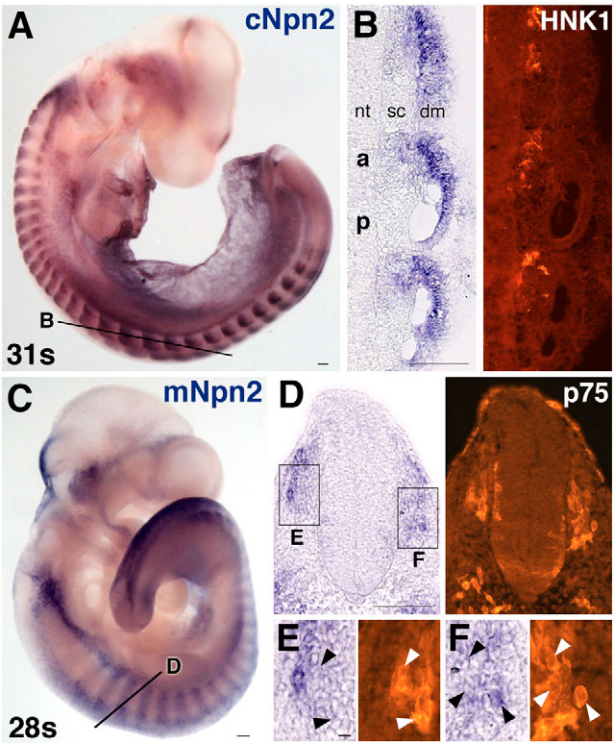

Fig. 1. *Npn2* is expressed in migrating neural crest cells. *Npn2* expression was revealed by whole-mount in situ hybridization of 31 somite stage chick (**A**) and E10.0 (28 somite) mouse (**C**) embryos. Neural crest cells were immunostained with anti-HNK-1 in longitudinal sections (**B**) and anti-p75 in transverse sections (**D-F**) through the embryos shown in A and C. Plane of section is indicated on the whole-mount view. (E,F) Higher magnification view of the regions boxed in D. Arrowheads indicate migrating neural crest cells. Identical results were obtained at E9.5. a, anterior; p, posterior; dm, dermomyotome; sc, sclerotome; nt, neural tube. Scale bars: 0.1 mm in A-D; 0.01 mm in E,F.

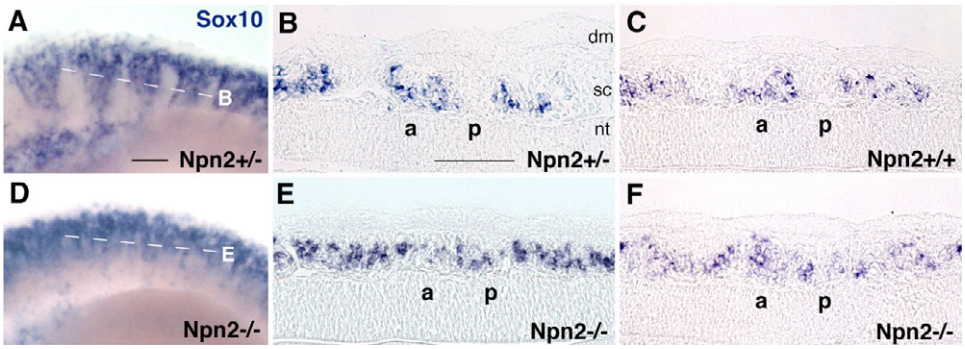

Fig. 2. Npn2 is required to pattern segmental trunk neural crest migration. Trunk neural crest normally migrates in streams (**A**) restricted to the anterior-half sclerotome (**B**,**C**). In the absence of *Npn2*, segmental migration is lost (**D**) and neural crest cells migrate throughout both anterior- and posterior-half sclerotomes (**E**,**F**). Neural crest was visualized at E9.5 by in situ hybridization for *Sox10*. In A,D, anterior is towards the left, dorsal is upwards. (B,C,E,F) Longitudinal sections; B and E are sections through the embryos shown in A and D at the levels indicated. a, anterior; p, posterior; dm, dermomyotome; sc, sclerotome; nt, neural tube. Scale bars: 0.1 mm.

characterizing neural crest marker gene expression in *Npn2*-null mutant mice (Giger et al., 2000). In situ hybridization with probes for the neural crest markers *Sox10*, *Foxd3* and *Pax3* at E8-8.5 (4 to 12 somites) showed no obvious effects on the specification or generation of neural crest cells in wild-type, heterozygous or *Npn2* mutant mice (data not shown).

Profound defects were observed, however, on the pattern of neural crest migration in *Npn2*-null mice. In the trunk of wild-type (not shown) or *Npn2* heterozygous (Fig. 2A) embryos, neural crest migration appeared as segmentally iterated blocks of *Sox10* staining cells. In sections through these embryos, *Sox10*-positive neural crest cells were found only in the anterior-half sclerotome at levels adjacent to the intermediate region of the neural tube (Fig. 2B,C) (Serbedzija et al., 1990). In *Npn2* mutant mice, however, neural crest cells migrated in a uniform sheet rather than in streams (Fig. 2D), and *Sox10*-positive cells were present throughout both anterior and posterior portions of the sclerotome (Fig. 2E,F). More ventrally, approaching the dorsal aorta where the neural crest-derived sympathetic ganglia will form, some segmental neural crest migration was still apparent in *Npn2*-null mice (Fig. 2D). Interestingly, motor axons, which normally are restricted to the anterior sclerotome, also project into both anterior and posterior sclerotome in *Npn2* mutants, although ventral roots still form (data not shown). Together, these data suggest that Npn2 signaling is required for neural crest guidance events through dorsal and intermediate levels of the somite.

Npn2 and *Sema3f* exhibit complementary expression patterns

Npn2 can bind to three different ligands: Sema3C, Sema3f and vascular endothelial growth factor (Chen et al., 1997; Gluzman-Poltorak et al., 2000). To determine which ligand was mediating the patterning functions revealed by the *Npn2* mutant, we next assessed the expression patterns of these molecules by in situ hybridization and immunohistochemistry. *Sema3c* is first expressed in the somites around E10, and then only in the dermomyotomal compartment (Adams et al., 1996). Immunostaining for vascular endothelial growth factor was also detected at low levels in the dermomyotome but not in the sclerotome at E9.5 (data not shown). By contrast, *Sema3f* was expressed in a pattern complementary to that of *Npn2* (Fig. 3A,B),

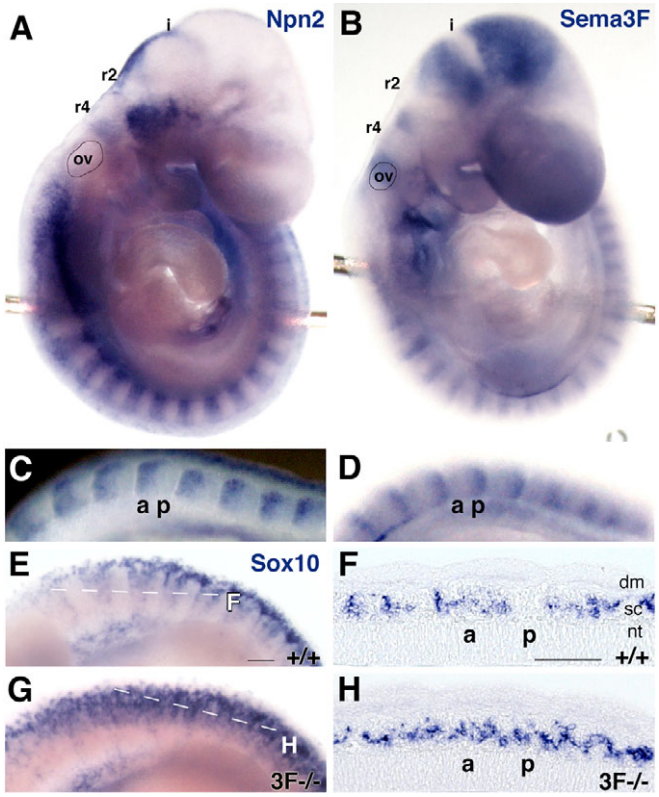

Fig. 3. *Sema3f* expression is complementary to *Npn2* and required to pattern neural crest migration. *Npn2* (**A**) and *Sema3f* (**B**) are expressed in reciprocal patterns at E9.5, most notably in the hindbrain, branchial arches and trunk, where *Npn2* is expressed in the anterior-half somite (**C**) and *Sema3f* in the posterior-half somite (**D**). At E9.5, the segmental appearance of migrating trunk neural crest (**E**) that results from migration exclusively through the anterior-half sclerotome (**F**) is disrupted in *Sema3f* mutants (**G**) because neural crest cells migrate throughout the sclerotome (**H**). (A-D) In situ hybridization for *Npn2* (A,C) or *Sema3f* (B,D). (E-H) Neural crest was visualized by in situ hybridization for *Sox10*. (E,G) Anterior is towards the left. (F,H) Longitudinal sections of embryos shown in E and G. i, isthmus; r2, rhombomere 2; r4, rhombomere 4; ov, otic vesicle; a, anterior; p, posterior; dm, dermomyotome; sc, sclerotome; nt, neural tube. Scale bars: 0.1 mm.

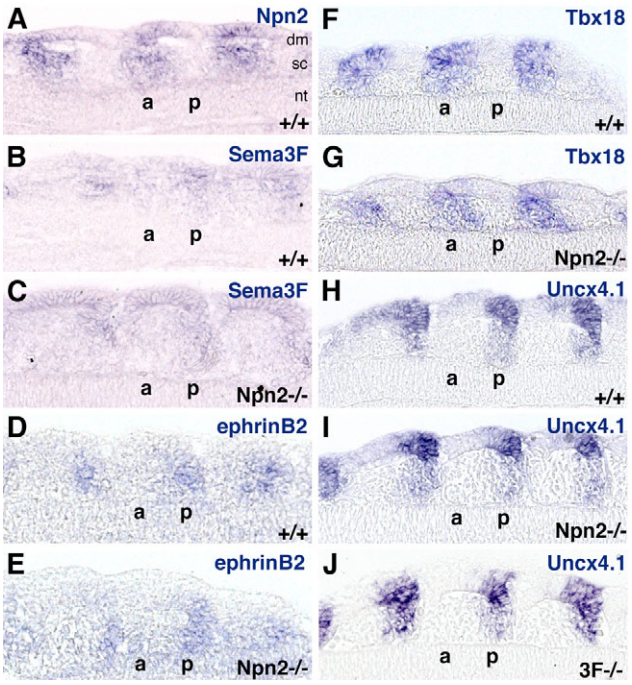

Fig. 4. Somite polarity is normal in *Npn2* and *Sema3f* mutant mice. In E9.5 embryos, *Npn2* is expressed in anterior sclerotome (**A**), and *Sema3f* in posterior sclerotome (**B**). (**C**) In *Npn2* mutants, *Sema3f* is still posteriorly restricted. EphrinB2 is expressed in the posterior sclerotome (**D**), *Tbx18* is expressed in the anterior sclerotome (**F**) and *Uncx4.1* is expressed in the posterior sclerotome (**H**). Expression of these three genes remains unchanged in *Npn2* mutants (**E**,**G**,**I**). (J) *Uncx4.1* also remains restricted to the posterior sclerotome of *Sema3f* mutants. Gene expression was visualized by in situ hybridization and embryos sectioned longitudinally. a, anterior; p, posterior; dm, dermomyotome; sc, sclerotome; nt, neural tube.

with *Npn2* expression in the anterior somite (Fig. 3C) mirrored by *Sema3f* expression in the posterior somite (Fig. 3D). In sections, *Npn2* was clearly expressed in the anterior half of each sclerotome (Fig. 4A), and *Sema3fF* in the posterior half (Fig. 4B). This expression pattern made *Sema3f* an ideal candidate for signaling through the *Npn2* receptor during trunk neural crest migration. The complementary distribution of *Npn2* and *Sema3f* expression has also been observed at later stages of development (Giger et al., 2000; Giger et al., 1998).

Sema3f mutants phenocopy *Npn2*-null mice

We next assessed the requirement for *Sema3f* during neural crest development by examining *Sox10* expression in *Sema3f* mutant mice (Sahay et al., 2003). Strikingly, the *Sema3f*-null neural crest migration phenotype was identical to that observed in *Npn2* mutants. Instead of segmentally arranged streams of neural crest cells in the trunk (Fig. 3E), *Sema3f* nulls contained uniform sheets of migrating neural crest (Fig. 3G). In sections, alternating *Sox10*-positive and -negative regions were observed in the anterior- and posterior-half sclerotome, respectively, of wild-type mice (Fig. 3F), whereas uniformly distributed *Sox10*-labeled cells were seen throughout the sclerotome of *Sema3f* mutants (Fig. 3H). Together, these results demonstrate that signaling between the receptor Npn2 and its ligand Sema3f is required to restrict neural crest migration to the anterior somite.

Somite patterning is normal in *Npn2* and *Sema3f* mutants

Two alternate mechanisms could explain the trunk neural crest migration defects observed in the *Npn2* (Fig. 2) and *Sema3f* (Fig. 3) mutant mice. The phenotype could reflect a requirement for signaling between the Sema3f repulsive ligand in the posterior somite and the Npn2 receptor on neural crest cells to guide neural crest migration. Alternatively, Npn2/Sema3f signaling between the anterior and posterior somite could be important for maintenance of anterior and/or posterior sclerotomal identity. Disrupting this signaling could affect somite polarity and thus the environment through which neural crest migrates, secondarily impacting the pattern of neural crest migration. For example, anteroposterior somite polarity is abolished in *Delta1* mutant mice (deAngelis et al., 1997), and as a consequence, neural crest cells migrate aberrantly through the posterior sclerotome of these animals (DeBellard et al., 2002).

To differentiate between these two possibilities, markers of anterior and posterior sclerotome were examined in wild-type, *Npn2* mutant and *Sema3f* mutant mice to determine whether anterior and posterior somite identity was retained in the mutants. *Sema3f* was expressed in the posterior sclerotome of both wild-type (Fig. 4B) and *Npn2* mutant mice (Fig. 4C). Ephrin B2, a ligand that repels migrating neural crest cells in vitro (Wang and Anderson, 1997), was also equivalently restricted to the posterior sclerotome of wild-type (Fig. 4D) and *Npn2* mutant embryos (Fig. 4E). Thus, two posteriorly expressed guidance molecules are appropriately localized in *Npn2* mutants.

Somite polarity is established during segmentation of the somitic mesoderm, with anterior and posterior somite identity maintained and promoted by two different transcription factors, Tbx18 (Bussen et al., 2004) and Uncx4.1 (Leitges et al., 2000; Mansouri et al., 2000). *Tbx18* was restricted to the anterior sclerotome of wild-type (Fig. 4F) and *Npn2* mutant embryos (Fig. 4G). *Uncx4.1* was also properly expressed in the posterior sclerotome of wild-type (Fig. 4H), *Npn2* mutant (Fig. 4I) and *Sema3f* mutant mice (Fig. 4J). These results demonstrate that anterior and posterior sclerotomal character is maintained in *Npn2* and *Sema3f* mutants, suggesting that the requirement for Npn2/Sema3f signaling is likely to reside in the neural crest.

Npn2 is required in the neural crest for Sema3f-mediated repulsion

To test the requirement for Npn2 in the neural crest directly, we explanted wild-type and *Npn2* mutant neural tubes, and cultured them on fibronectin-coated substrates containing spots of Sema3f. Wild-type neural crest cells avoided Sema3f (Fig. 5A), with the majority of cells remaining at the spot border and only individual, rare cells migrating onto the Sema3f substrate, consistent with their behavior in vivo (Kasemeier-Kulesa et al., 2004). By contrast, *Npn2* mutant neural crest cells migrated equally well on fibronectin with or without Sema3f protein (Fig. 5B,C). This demonstrates that the Npn2 receptor on neural crest cells detects a Sema3f repulsive cue in the environment, and supports a cell-autonomous requirement for Npn2 on the neural crest during trunk neural crest migration.

Segmentally arranged dorsal root ganglia form in *Npn2* mutant mice

The segmental migration of neural crest through the somite is thought to prefigure the segmented organization of the neural crest-derived ganglia of the peripheral nervous system. This pattern ensures that the ganglia and the vertebrae, which differentiate from

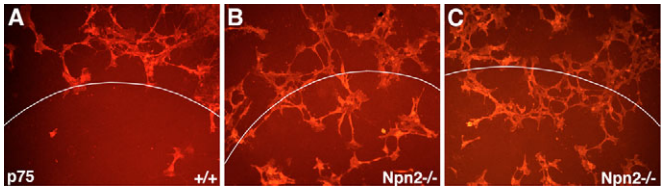

Fig. 5. Npn2 is cell autonomously required for neural crest cells to avoid Sema3f in vitro. Neural tubes from 14 to 24 somite mouse embryos were cultured on Thermanox coverslips coated with fibronectin and spotted with AP-Sema-3F conditioned medium conjugated with anti-placental alkaline phosphatase. Neural crest cells normally avoid immobilized Sema3f (**A**), while neural crest cells lacking the Npn2 receptor migrate equally well on fibronectin and Sema3f substrates (**B**,**C**; *n*=5 spots in three separate experiments). Neural crest cells were labeled with anti-p75, while spots (outlined in white) were visualized using anti-mouse IgG-Alexa 488.

the somites, will form in register with one another. For example, in each somite, neural crest cells in the anterior sclerotome coalesce to form the dorsal root ganglia. As a result, in *Npn2* mutants, one might expect a continuous mass of dorsal root ganglia to form instead of individualized ganglia. This is the case when neural crest migrates non-segmentally through somites that are genetically (deAngelis et al., 1997; DeBellard et al., 2002) or surgically manipulated (Kalcheim and Teillet, 1989) to contain only anterior character. In wild-type embryos at E10.5 and E11.5, the streams of *Sox10*-expressing neural crest cells in the trunk condensed into ganglia in an anterior to posterior progression (Fig. 6A,C). Strikingly, neural crest cells in *Npn2* mutant mice also coalesced into recognizable ganglia. At E10.5, neural crest cells were still distributed throughout the somites, but became excluded from the somite boundaries (Fig. 6B). By E11.5, individualized ganglia appeared to have sorted out from the sheet of migrating neural crest in the somite (Fig. 6D, arrowheads mark the same axial levels in all panels). In longitudinal sections at E11.5, TUJ1 immunoreactivity confirmed the apparent segmentation, with space between each *Npn2* mutant dorsal root ganglion (Fig. 6F). Although morphologically similar to those in wild-type embryos (Fig. 6E), the mutant ganglia were not as well separated, suggesting that the process of gangliogenesis occurred but was somewhat compromised. The sympathetic ganglia, which form in a segmental pattern ventral to the somites, are normal in *Npn2* mutants (Giger et al., 2000).

DISCUSSION

We have examined the importance of the receptor Npn2 and its repulsive ligand Sema3f during neural crest development in the trunk. We demonstrate that *Npn2* is expressed in migrating neural crest cells, and that Npn2/Sema3f signaling is required for segmental neural crest migration but not for somite patterning. Surprisingly, we found that segmental migration was not essential for the formation of individualized dorsal root ganglia.

Npn2/Sema3f signaling patterns neural crest migration in the trunk

In *Npn2* and *Sema3f* mutant mice, trunk neural crest cells migrate through both the anterior and posterior sclerotome, rather than exclusively through the anterior-half sclerotome as in wild-type mice. This demonstrates that signaling between the receptor Npn2 and its ligand Sema3f is required to restrict neural crest migration to the anterior somite.

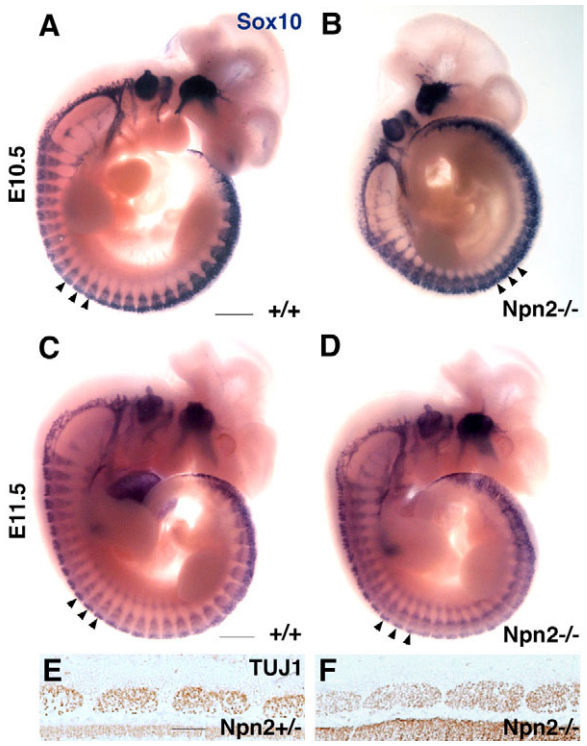

Fig. 6. Non-segmentally migrating neural crest cells give rise to segmental dorsal root ganglia in *Npn2* mutant mice. In E10.5 (**A**) and E11.5 (**C**) wild-type embryos, in situ hybridization for *Sox10* reveals segmental streams of migrating neural crest cells in the posterior trunk, and condensed dorsal root ganglia anteriorly. (**B**,**D**) In *Npn2* mutant embryos, in the absence of segmental neural crest migration posteriorly, individualized dorsal root ganglia segregate anteriorly. Black arrowheads indicate condensing dorsal root ganglia at the same axial level in all panels. Scale bars: 0.5 mm. (**E**,**F**) In sections of *Npn2* heterozygous (E) and mutant (F) E11.5 embryos stained with anti-TUJ1, dorsal root ganglia had a similar appearance but were less well separated in the mutant. Scale bar: 0.1 mm.

Murine *Npn2* is expressed by neural crest cells (Fig. 1D-F) as well as cells of the anterior sclerotome (Fig. 4A). Therefore, the migration defects in *Npn2* mutants could reflect a requirement for Npn2 in one or both cell types. One possibility is that Npn2 receptors on neural crest cells detect a Sema3f repulsive cue in posterior sclerotome, leading to anterior-only migration. Alternatively, Npn2 receptors on anterior sclerotomal cells may interact with the Sema3f ligand secreted by cells of the adjacent posterior sclerotome to affect the environment through which neural crest cells migrate, thus influencing neural crest migration in a non-cell-autonomous manner. The latter mechanism seems unlikely for several reasons. First and foremost, in contrast to wild-type cells, *Npn2* mutant neural crest cells do not avoid Sema3f in culture. This indicates a cell autonomous requirement for Npn2 in the neural crest. Second, as anteroposterior polarity of the somites is indistinguishable in wild-type, *Npn2* and *Sema3f* mutant embryos (Fig. 4), Npn2/Sema3f signaling does not appear to be required in this process. In support of this conclusion, neural crest cells are present all along the anteroposterior axis in the *Npn2* (Fig. 2E,F) and *Sema3f* mutants (Fig. 3H), even at somite borders. This contrasts with the *Delta1* mutant mouse, in which somite polarity is lost (deAngelis et al., 1997), where neural crest migration has a pseudo-segmental

appearance, with migrating neural crest cells present throughout the sclerotome but avoiding somite boundaries (DeBellard et al., 2002). This comparison suggests that, unlike *Delta1*, the *Npn2* defect is not in the somites themselves. Third, as *Npn2* is expressed on anterior sclerotomal cells, if Npn2/Sema3f were involved in somite patterning, loss of signaling through this receptor would most probably posteriorize the sclerotome. A posteriorized somite impedes neural crest migration, leading to reduced numbers of migratory neural crest cells and their derivatives (Bussen et al., 2004; Kalcheim and Teillet, 1989). However, this is not the case in *Npn2*-null mice. For these reasons, we favor the idea that Npn2 is required in the neural crest.

What is the purpose, then, of *Npn2* expression in both the neural crest and the sclerotome through which it migrates? Interestingly, like *Npn2*, *Npn1* (Eickholt et al., 1999) and *Ephb3* receptors (Krull et al., 1997), both of which have been postulated to play a role in patterning trunk neural crest migration, are also distributed on both neural crest and anterior sclerotomal cells. One intriguing possibility is that these receptors do not play a signaling role in the somite, but rather serve as a sink to bind up any repulsive ligand diffusing from the posterior sclerotome, thus ensuring a sharp boundary such that the anterior sclerotome is devoid of the repulsive cue.

This is the first report of a single receptor/ligand pair that is absolutely required to pattern trunk neural crest migration. The molecular basis for segmental neural crest migration has preoccupied this field since the phenomenon was first observed. Many different cell adhesion molecules, extracellular matrix molecules and receptor/ligand pairs have been identified that are expressed in anterior or posterior sclerotome or in the neural crest, and in some cases they have been shown to be sufficient to direct neural crest migration (Kuan et al., 2004). But in no case has a requirement for any molecule been previously demonstrated in the embryo. Other signals, such as Eph receptor/ephrin ligand interactions, might fine tune neural crest migration, or in the case of Npn1/Sema3A, be involved in later steps in the process. However, Npn2/Sema3f signaling is clearly the key determinant patterning anterior-only migration through the sclerotome.

Segmental neural crest migration may not be required for segmental dorsal root ganglion formation

The requirement for Npn2/Sema3f signaling in neural crest migration has uncovered an additional, previously unrecognized process that results in dorsal root ganglion segmentation irrespective of the neural crest migration pattern. Despite the fact that neural crest cells migrate through the anterior and posterior sclerotome of *Npn2* mutant mice, segmentally arranged dorsal root ganglia still form. This result suggests that segmental neural crest migration and subsequent sequestration of ganglia are separable events. One explanation is that there may be independent signals restricting neural crest migration and the pattern of ganglion aggregation. For example, either a cell-adhesive, 'sorting' signal or a repulsive cue in the posterior sclerotome could promote aggregation within the anterior sclerotome, irrespective of the starting location of the neural crest cells within the sclerotome. In favor of this possibility, fused dorsal root ganglia form when anteroposterior somite polarity is abolished by either surgical or genetic manipulation (Bussen et al., 2004; Kalcheim and Teillet, 1989; Leitges et al., 2000; Mansouri et al., 2000). This indicates that anteroposteriorly patterned signals in the somite are required for segmental formation of dorsal root ganglia. Candidates for such signals include F-spondin (Debby-Brafman et al., 1999) and Npn1 (Kitsukawa et al., 1997).

A second possibility is that dorsal root ganglia form in the absence of segmental neural crest migration simply because neurons tend to aggregate (M. Bronner-Fraser, unpublished). In support of this stochastic mechanism, when normal somites are surgically replaced with multiple anterior or posterior somite halves, a giant mass of ganglia forms that exhibits a pseudo-segmental appearance, with alternating thick and thin regions at random intervals within the giant ganglion (Kalcheim and Teillet, 1989). That they are fused, however, argues that a combination of these two mechanisms is normally at play.

Finally, it is also possible that the physical structure of the somite itself can impose segmentation during gangliogenesis. In addition to regionally restricted molecular cues, such as *Sema3f* expression posteriorly, there are embryological boundaries and differences within the sclerotome. These include, most notably, the intersomitic space, as well as von Ebner's fissure between anterior and posterior sclerotome, and the various subdomains within the sclerotome (reviewed by Christ et al., 2004). Anterior sclerotome is less cell dense than posterior sclerotome (Christ et al., 2004), is mitogenic for dorsal root ganglia (Goldstein et al., 1990) and will undergo apoptosis in the absence of neural crest cells (see Christ et al., 2004). All of these segmentally restricted differences could have a morphological impact during gangliogenesis. In the case of the *Npn2* mutants, the uniform sheet of migrating neural crest cells appears to segment into individual dorsal root ganglia at the somite border (Fig. 6). Interestingly, when chick embryos are surgically modified to contain only anterior sclerotome, in other words have no somite boundary, neural crest migrates non-segmentally and dorsal root ganglia are fused (Kalcheim and Teillet, 1989). However, dorsal root ganglia are also fused in *Uncx4.1* and *Tbx18* mutants, where anteroposterior somite polarity is abolished but physical somites still form (Bussen et al., 2004; Leitges et al., 2000; Mansouri et al., 2000). Together, these data suggest that somite polarity creates positional information at the somite boundary that impacts upon the segmentation of the peripheral nervous system. Migrating neural crest cells normally maintain filopodial contact across the posterior somite and can even cross over between adjacent streams (Kasemeier-Kulesa et al., 2004), thus the somite boundary could normally curtail this movement as dorsal root ganglia condense.

The sympathetic ganglia also are not dependent upon the pattern of neural crest migration for their segmented organization. By imaging actively migrating neural crest cells, Kasemeier-Kulesa and colleagues (Kasemeier-Kulesa et al., 2004) showed that, once they have passed through the somites, neural crest cells no longer maintain their segmental position and can migrate as far as two segments anteriorly or posteriorly. The mechanisms that ultimately result in the aggregation of these cells into individualized sympathetic ganglia are likely to be similar to those we propose for the formation of metameric dorsal root ganglia in *Npn2* mutants. Interestingly, Npn1 and Sema3A are required for localization and condensation of sympathetic precursors as well (Kawasaki et al., 2002).

The overriding message is that the segmental migration of neural crest cells through the somites itself is not requisite for the creation of a segmented peripheral nervous system, despite what has been assumed for 20 years (reviewed by Kuan et al., 2004). Although the dorsal root ganglia eventually segment in the *Npn2* mutants, they are more closely spaced than normal. Thus, the pattern of neural crest migration is important, but not essential for the formation of segmented dorsal root ganglia. This may not be surprising given the regulative nature of vertebrate embryos, which may have 'back-up' mechanisms for formation of important organ systems in the event that primary mechanisms are perturbed.

Functional validation of the neural crest gene expression profile

We originally identified *Npn2a1* in a screen for genes upregulated in response to neural crest induction (Gammill and Bronner-Fraser, 2002). Our current analysis of Npn2 function has several implications. First of all, the importance of Npn2 for neural crest migration validates our neural crest gene expression profile and demonstrates that our collection of genes contains true regulators of neural crest development. This conclusion is supported by the demonstration that Laminin-α5, another gene identified in our screen, is also important for proper emigration of neural crest cells (Coles et al., 2005).

In addition, although we screened for genes expressed in premigratory neural crest (Gammill and Bronner-Fraser, 2002), Npn2 is required for neural crest migration and apparently not for specification, as no differences were noted in the expression of early neural crest markers *Sox10*, *Pax3* and *FoxD3* in the neural folds and dorsal neural tube of the mouse. We cannot, however, rule out the possibility of an early role for chick Npn2 in neural crest specification, as it is expressed earlier and at higher levels in this organism. Regardless, genes crucial for migration are clearly expressed in premigratory neural crest as a consequence of neural crest induction. This supports our model that early neural crest development entails a sequential activation of migratory potential, with a signal to migrate activating this potential in a subset of premigratory neural crest cells (Gammill and Bronner-Fraser, 2002). Further analysis of our neural crest gene collection promises to reveal the roles of additional genes in this process.

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