CORRIGENDUM

Development 136, 347 (2009) doi:10.1242/dev.033316

# Neuropilin 1 and 2 control cranial gangliogenesis and axon guidance through neural crest cells

**Quenten Schwarz, Joaquim M. Vieira, Beatrice Howard, Britta J. Eickholt and Christiana Ruhrberg**

There was an error published in *Development* **135**, 1605-1613.

The gene *Brn3a* was incorrectly written as *Brn3b* throughout, including Fig. 8G. On p. 1611, line 12, *Brn3b* (*Pou4f2*) should read *Brn3a* (*Pou4f1*).

The authors apologise to readers for this mistake.

Development 135, 1605-1613 (2008) doi:10.1242/dev.015412

# Neuropilin 1 and 2 control cranial gangliogenesis and axon guidance through neural crest cells

**Quenten Schwarz[1], Joaquim M. Vieira[1], Beatrice Howard[2], Britta J. Eickholt[3] and Christiana Ruhrberg[1,*]**

Neuropilin (NRP) receptors and their class 3 semaphorin (SEMA3) ligands play well-established roles in axon guidance, with loss of NRP1, NRP2, SEMA3A or SEMA3F causing defasciculation and errors in growth cone guidance of peripherally projecting nerves. Here we report that loss of NRP1 or NRP2 also impairs sensory neuron positioning in the mouse head, and that this defect is a consequence of inappropriate cranial neural crest cell migration. Specifically, neural crest cells move into the normally crest-free territory between the trigeminal and hyoid neural crest streams and recruit sensory neurons from the otic placode; these ectopic neurons then extend axons between the trigeminal and facioacoustic ganglia. Moreover, we found that NRP1 and NRP2 cooperate to guide cranial neural crest cells and position sensory neurons; thus, in the absence of SEMA3/NRP signalling, the segmentation of the cranial nervous system is lost. We conclude that neuropilins play multiple roles in the sensory nervous system by directing cranial neural crest cells, positioning sensory neurons and organising their axonal projections.

**KEY WORDS: Neural crest cell, Placode, Peripheral nervous system, Sensory neuron, Axon guidance, Neuropilin, Semaphorin, Mouse**

## INTRODUCTION

The cranial ganglia of vertebrates contain sensory neurons, which are derived from neurogenic placodes and neural crest cells (D'Amico-Martel and Noden, 1983). The neurogenic placodes are focal thickenings in the head epidermis, whereas neural crest cells are a transient population of multipotent stem cells that delaminates from the neural tube and disseminates throughout the body to give rise to several other cell types in addition to sensory neurons (reviewed by Graham, 2000; Graham, 2003; Trainor, 2005). Cranial neural crest cells also contribute to cranial gangliogenesis by organising the placodally-derived sensory neurons and their axons in the chick (Begbie and Graham, 2001). Thus, hindbrain extirpation prior to cranial neural crest cell emigration causes mispositioning of placodally-derived neurons.

The emigration of cranial neural crest cells from the hindbrain follows its segmental organisation into morphologically distinct compartments termed rhombomeres (r1-r7); specifically, neural crest cells emanate in distinct streams from r2, r4 and r6, which are separated by crest-free zones at the levels of r3 and r5 (Lumsden et al., 1991; Sechrist et al., 1993). Neural crest cells actively avoid the cranial mesenchyme at r3 and r5 level, which suggests that inhibitory guidance mechanisms are present in these areas (e.g. Farlie et al., 1999; Kulesa and Fraser, 1998; Kulesa and Fraser, 2000; Sechrist et al., 1993). Members of the class 3 semaphorin family are good candidates for mediating neural crest cell guidance, as they provide repulsive cues for a number of different cell types, most notably migrating neurons and their axons (reviewed by Huber et al., 2003).

Consistent with the idea that class 3 semaphorin/neuropilin (SEMA3/NRP) signalling guides cranial neural crest cells, SEMA3F and its receptor NRP2 are expressed in a complementary pattern during cranial neural crest migration, with SEMA3F being expressed in r3 and r5 and NRP2 being expressed by the r2-derived (trigeminal) and r4-derived (hyoid) neural crest cells (Gammill et al., 2007; Eickholt et al., 1999). Moreover, cranial neural crest cells travel through the normally crest-free zone at r3 level when semaphorin function is perturbed by ectopic NRP2 expression in the chick (Osborne et al., 2005) and when NRP2 or SEMA3F are ablated by targeted gene inactivation in the mouse (Gammill et al., 2007). Like SEMA3F, SEMA3A is expressed in r3 and r5 of the chick hindbrain (Eickholt et al., 1999), and the ectopic expression of a soluble form of its receptor, NRP1, in the chick hindbrain causes invasion of neural crest cells into the normally neural crest-free territory adjacent to r3 (Osborne et al., 2005). However, it is not known whether NRP1 is a major factor controlling neural crest guidance in mammals, as conflicting data exist with respect to the role of NRP1 in neural crest guidance in the mouse.

On the one hand, it has been suggested that NRP1 is not expressed in mouse neural crest cells and that SEMA3A is not expressed in a pattern consistent with a role in neural crest cell migration (Kuan et al., 2004). On the other hand, it has been shown that antibodies specific for NRP1 recognise mouse neural crest cells in vitro, and that dorsal root ganglia are organised more loosely in *Nrp1*-null mutants, even though the same study reported that trunk neural crest cells migrate normally (Kawasaki et al., 2002).

To reconcile the conflicting information provided by previous studies, we have re-examined the expression pattern and functional requirement of NRP1 during cranial neural crest cell migration in the mouse. Contrary to previous reports, we have found that murine hyoid neural crest cells express *Nrp1*, and that SEMA3A/NRP1 signalling is essential to guide their migration. Furthermore, we have discovered that SEMA3A/NRP1 and SEMA3F/NRP2 cooperate to guide cranial neural crest cells, and that neuropilin-mediated neural crest cell patterning is essential for cranial ganglia segmentation. Based on our findings, we propose a novel and comprehensive model that explains the multiple roles of neuropilin signalling in the peripheral nervous system of the vertebrate head.

[1]Institute of Ophthalmology, University College London, Bath Street, London EC1V 9EL, UK. [2]Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK. [3]MRC Centre for Developmental Neurobiology, King's College London, London SE1 1UL, UK.

*Author for correspondence (email: c.ruhrberg@ucl.ac.uk)

Accepted 26 February 2008

## MATERIALS AND METHODS

### Animals

To obtain mouse embryos of defined gestational ages, mice were mated in the evening, and the morning of vaginal plug formation was counted as 0.5 days post-coitum (dpc). To stage-match embryos within a litter, or between litters from different matings, we compared somite numbers. Mice carrying a *Sema3a*-null (Taniguchi et al., 1997) or *Nrp1*-null (Kitsukawa et al., 1997) allele, and mice carrying mutations that disrupt semaphorin signalling through both neuropilins ($Nrp1^{sema-/-}$ $Nrp2^{-/-}$) (Gu et al., 2003) have been described. Conditional-null mutants for *Nrp1* ($Nrp1^{fl/fl}$) (Gu et al., 2003) were mated to mice expressing CRE recombinase under the control of the endothelial-specific *Tie2* promoter (Kisanuki et al., 2001) or the neural crest-specific *Wnt1* promoter (Jiang et al., 2000). The *Erbb4*-null allele has been described (Gassmann et al., 1995). Genotyping protocols are available on request.

### In situ hybridisation

In situ hybridisation was performed according to a previously published method (Riddle et al., 1993), using digoxigenin-labelled riboprobes transcribed from cDNA-containing plasmids. Plasmids encoding *Nrp1* and *Sox10* were provided by M. Fruttiger and N. Kessaris (University College, London, UK), respectively. Plasmids containing *Sema3a* or *Sema3f* were obtained from M. Tessier-Lavigne (Genentech). Plasmids containing *Phox2b*, *Ngn1* and *Brn3b* were provided by C. Goridis (INSERM, Marseille, France), F. Guillemot (National Institute for Medical Research, London, UK) and E. Turner (University of California, La Jolla, CA), respectively.

### Immunolabelling

Mouse embryos were fixed in 4% formaldehyde in PBS, washed in PBS and incubated for 2 hours in a blocking solution of PBS containing 0.1% Triton X-100 (PBT) and 10% goat serum. In some experiments, samples were subjected to in situ hybridisation prior to immunolabelling. The following primary antibodies were used: for blood vessels, rat anti-endomucin (Santa Cruz Biotechnology); for mouse neural crest cells, rabbit anti-p75 (a gift of Drs K. Deinhardt and G. Schiavo, Cancer Research UK, London); for neuronal cell bodies, mouse anti-HUC/D (Molecular Probes); for axons, rabbit anti-neurofilament (Chemicon). Samples were washed in PBT and incubated overnight at 4°C with secondary antibodies in blocking solution. Secondary antibodies used were Alexa 488-conjugated goat anti-rat, and Alexa 594-conjugated goat anti-rabbit IgGs (Molecular Probes). In some experiments, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (DAKO) was used as a secondary antibody. Neurofilament-stained samples were dehydrated in methanol and cleared in a solution containing 2:1 benzyl benzoate:benzyl alcohol. HRP-labelled samples were visualised by conventional light microscopy, fluorescently labelled samples with a LSM510 laser-scanning confocal microscope (Zeiss).

## RESULTS

### Expression pattern of *Sema3a* and *Nrp1* during cranial neural crest migration in the mouse

In the chick, trigeminal and hyoid neural crest cells express *Nrp1* (Eickholt et al., 1999). To address whether NRP1 plays a role during cranial neural crest guidance in the mouse, we determined its expression pattern during the period of neural crest migration from the hindbrain into the pharyngeal arches using wholemount in situ hybridisation. We found that *Nrp1* was not expressed in murine trigeminal neural crest cells; these were, however, clearly identified with the neural crest marker *Sox10* (Fig. 1, compare A with C and B with D). By contrast, the murine hyoid neural crest stream expressed both *Nrp1* and *Sox10* (Fig. 1, compare black arrowheads in A,B with C,D). In addition, *Nrp1* was expressed in the second pharyngeal arch mesenchyme (Fig. 1C,D, open arrowheads). Double labelling of coronal sections from an 8.5 dpc embryo by in situ hybridisation with the *Nrp1* probe and immunocytochemistry with antibodies specific for the p75 neurotrophin receptor (NGFR), a neural crest marker (Rao and Anderson, 1997), confirmed *Nrp1* expression in

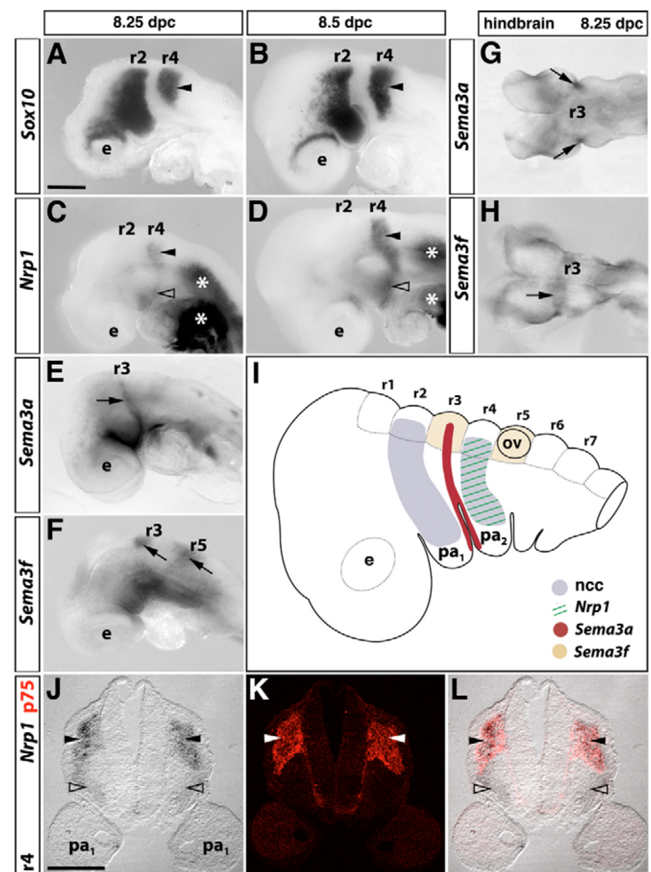


**Fig. 1. Expression pattern of *Sema3a*, *Sema3f* and *Nrp1* during cranial neural crest migration.** (**A**,**B**) *Sox10* expression in the trigeminal and hyoid neural crest streams at 8.25 and 8.5 dpc (10- and 12-somite stage, respectively); the hyoid stream is indicated by the black arrowheads. (**C**,**D**) *Nrp1* expression in the hyoid stream (black arrowheads), in the distal pharyngeal arch mesenchyme (open arrowheads), and in areas where major vessels form (asterisks). (**E**,**F**) At 8.25 dpc, *Sema3a* was expressed in a stripe between the trigeminal and hyoid neural crest streams (arrow in E). *Sema3f* was expressed in r3 and r5 (arrows in F). (**G**,**H**) Dorsal view of the hindbrain at 8.25 dpc, when it has not yet closed. *Sema3a* was expressed only weakly in the hindbrain, but prominently in the domain just outside r3 (arrows in G), corresponding to the stripe indicated with an arrow in E. *Sema3F* was expressed strongly in r3 (arrow in H). (**J**-**L**) Coronal sections at r4 level through an 8.75 dpc mouse embryo subjected to in situ hybridisation for *Nrp1* (J) and immunolabelling of the neural crest marker p75 (K). Both markers detected the same population of neural crest cells (black arrowheads in overlay, L). Open arrowheads (J,L) indicate weak *Nrp1* expression in the p75-negative distal mesenchyme. (**I**) Summary of the expression patterns for *Nrp1*, *Sema3a* and *Sema3f* during neural crest migration on embryonic day 8. ov, otic vesicle; e, eye anlage; ncc, neural crest cell; $pa_1$ and $pa_2$, pharyngeal arches 1 and 2. Scale bars: 250 μm in A for A-H, in J for J-L.

the p75-positive hyoid neural crest stream (Fig. 1J-L, black arrowheads) and in the p75-negative distal pharyngeal arch mesenchyme (Fig. 1J,L, open arrowheads).

In the chick, *Sema3a* is expressed in r3 and r5 around the time of neural crest cell delamination (Eickholt et al., 1999). In the mouse, *Sema3a* was expressed only weakly within the hindbrain during the time of neural crest cell emigration (Fig. 1G). By contrast, it was expressed prominently in a stripe between the first and second

DEVELOPMENT

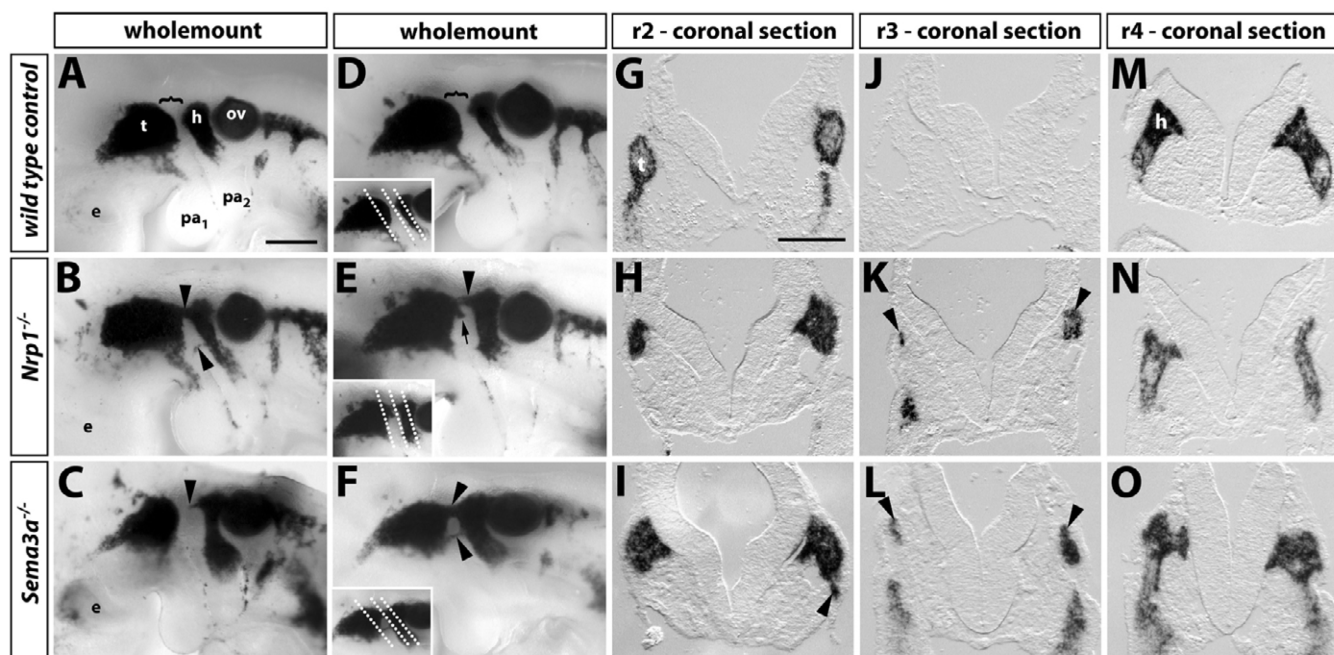


**Fig. 2. SEMA3A/NRP1 signalling guides cranial neural crest migration.** (**A-F**) Wholemount in situ hybridisation of 9.5 dpc (21- to 23-somite stage) mouse embryos with a *Sox10* probe revealed a neural crest-free zone beneath r3 (bracket) in wild-type embryos; two examples are shown (A,D). Loss of NRP1 (B,E) or SEMA3A (C,F) caused neural crest cell invasion into the territory adjacent to r3 (arrowheads) in all mutants examined (two examples are shown for each mutation). Invading neural crest cells appeared to emerge from the hyoid (h) neural crest stream (arrowhead in C) or formed bridges between the trigeminal (t) and hyoid neural crest streams (arrowheads in B,E,F). Ectopically migrating neural crest cells were usually found at the level of the dorsal neural tube, but in some mutants an additional smaller ventral stream formed (lower arrowhead in B,F). In some cases, trigeminal neural crest cells invaded the territory adjacent to r3 (arrow in E). (**G-O**) Coronal sections through the wild-type embryo shown in D, the *Nrp1*-null mutant shown in E, and the *Sema3a*-null mutant shown in F at r2 (G-I), r3 (J-L) and r4 (M-O) level; the planes of sectioning are indicated with dotted white lines in the insets (miniaturised images of the embryo) in D-F. Arrowheads in K,L,I indicate ectopic neural crest cells in the cranial mesenchyme adjacent to r3. t, trigeminal neural crest cells; h, hyoid neural crest cells. ov, otic vesicle; e, eye anlage; ncc, neural crest cell; $pa_1$ and $pa_2$, pharyngeal arches 1 and 2. Scale bars: 250 μm in A for A-F, in G for G-O.

pharyngeal arches (Fig. 1E, arrow); this expression domain originated just outside the hindbrain at the r3 level (Fig. 1G, arrows). *Sema3a* is therefore expressed in an appropriate position to prevent intermingling of migrating trigeminal and hyoid neural crest cells. In contrast to *Sema3a*, *Sema3f* was expressed strongly in the mouse hindbrain, with expression first in r3 (Fig. 1F,H, arrows), and then in r3 and r5, consistent with previous reports (Gammill et al., 2007). In addition, both semaphorins were co-expressed in the distal arch mesenchyme. Taken together, the *Sema3a* and *Sema3f* expression patterns overlap only partially in the mouse embryo head at this stage. Importantly, the expression patterns of *Sema3a* and *Nrp1* raise the possibility that SEMA3A signalling cooperates with SEMA3F to provide inhibitory cues for hyoid neural crest cells, preventing their invasion into the mesenchymal territory adjacent to r3 (Fig. 1I).

## SEMA3A and NRP1 are required to control cranial neural crest migration in the mouse

To determine whether SEMA3A signalling through NRP1 is required for the guidance of hyoid neural crest cells in the mouse, we examined mouse embryos lacking either SEMA3A or NRP1 (Kitsukawa et al., 1997; Taniguchi et al., 1997) with the neural crest marker *Sox10* (Pusch et al., 1998). At 9.5 dpc, we observed ectopic neural crest cells in the normally neural crest-free area adjacent to r3 in 9/9 *Nrp1*-null mutants (Fig. 2B,E,K, arrowheads) and in 12/12 *Sema3a*-null mutants (Fig. 2C,F,L, arrowheads). Although all mutants examined showed such defects, in 4/9 *Nrp1*-null embryos and in 6/12 *Sema3a*-null embryos only the left or the right side was noticeably affected. In all cases, ectopically migrating neural crest cells travelled between the hyoid and trigeminal neural crest streams at the level of the dorsal neural tube (Fig. 2K,L and Fig. 3C, arrowheads). In 5/12 *Sema3a*-null and 4/9 *Nrp1*-null mutants, neural crest cells were additionally crossing between the trigeminal and hyoid neural crest streams in a more ventral region (Fig. 2B,F, lower arrowheads). In 1/9 *Nrp1*-null and 4/12 *Sema3a*-null mutants, the point of origin for the neural crest cells invading the territory adjacent to r3 was clearly identifiable as r4 (for example, Fig. 2C and Fig. 3B). In rare cases (one *Sema3a*-null and one *Nrp1*-null mutant), a small subset of trigeminal neural crest cells appeared to move into the territory adjacent to r3 (Fig. 2E, arrow).

## NRP1 acts cell-autonomously to control cranial neural crest migration

NRP1 has been implicated in axon guidance as well as in blood vessel growth (Kawasaki et al., 1999; Kitsukawa et al., 1997). Accordingly, NRP1 has been suggested to play a role in the co-pattering of nerves and blood vessels, a subject of much recent interest (Carmeliet, 2003). We therefore considered the possibility that the neural crest defects were secondary to blood vessel defects in *Nrp1*-null mutants. Double labelling of 9.5 dpc embryos with the neural crest cell marker p75 and the blood vessel marker endomucin (Brachtendorf et al., 2001) revealed that cranial neural crest cells (Fig. 3A) normally migrate in close proximity to the anterior

DEVELOPMENT

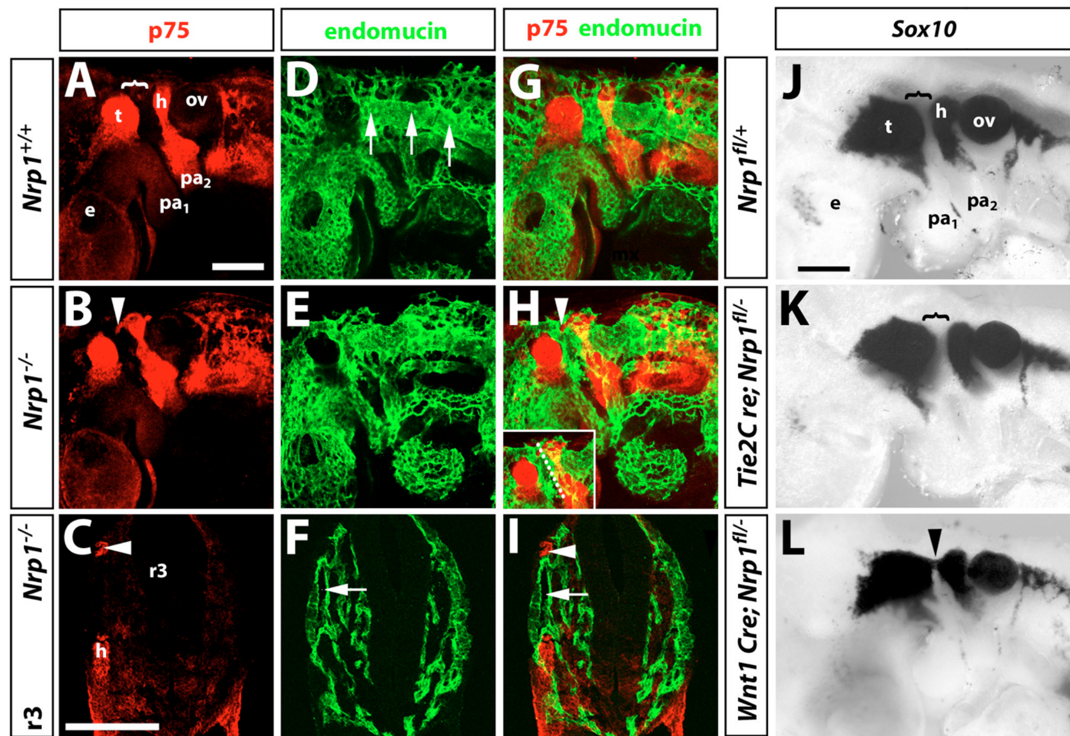


**Fig. 3. NRP1 is required for cranial neural crest migration in a cell-autonomous fashion.** (**A-I**) Double-label immunofluorescence analysis of 9.5 dpc mouse embryos (21-23 somites) with the neural crest cell marker p75 (red, A-C) and the blood vessel marker endomucin (green, D-F); (G-I) merged images. (A,D,G) In wild type, neural crest cells avoided the head mesenchyme at the level of r3 (bracket in A), but migrated ventrally in close proximity to the anterior cardinal vein (arrows in D). Vascular patterning in the head appeared disorganised in *Nrp1*-null mutants (E) compared with wild-type littermates (D). In *Nrp1*-null mutants, ectopically migrating hyoid neural crest cells travelled rostrally along the anterior cardinal vein (arrowhead in B,H). A coronal section at r3 level through the *Nrp1*-null mutant shown in B,E,H revealed that ectopic neural crest cells (arrowhead in C) migrated just dorsally of the anterior cardinal vein (arrow in F,I); the plane of sectioning is indicated with a dotted white line in the inset in H. (**J-L**) Wholemount in situ hybridisation with a *Sox10* probe at 9.5 dpc (21-23 somites) revealed a neural crest-free zone (bracket in J,K) in control embryos and in embryos lacking NRP1 in CRE-expressing blood vessel endothelium (*Tie2* promoter; K). By contrast, loss of NRP1 from CRE-expressing cranial neural crest (*Wnt1* promoter; L) caused neural crest invasion into the mesenchymal territory adjacent to r3 and bridging between the trigeminal and hyoid neural crest streams (arrowhead in L). t, trigeminal neural crest cells; h, hyoid neural crest cells; ov, otic vesicle; e, eye anlage; $pa_1$ and $pa_2$, pharyngeal arches 1 and 2. Scale bars: 250 μm in A for A,B,D,E,G,H and in J for J-L; 200 μm in C for C,F,I.

cardinal vein (Fig. 3D, arrows; Fig. 3G, overlay). Ectopic neural crest cells in *Nrp1*-null mutants also appeared to migrate in a rostral direction in close proximity to the anterior cardinal vein (Fig. 3H,I, arrowhead). We therefore used a mouse strain carrying a conditional *Nrp1*-null mutation (Gu et al., 2003) to directly address whether abnormal neural crest migration in the absence of NRP1 was secondary to defective blood vessel growth. We found that the tissue-specific ablation of NRP1 from blood vessel endothelium with CRE recombinase driven by the *Tie2* (*Tek* – Mouse Genome Informatics) promoter (Kisanuki et al., 2001) did not impair cranial neural crest guidance in any of the four mutants examined (Fig. 3K), even though this mutation perturbs blood vessel patterning (Gu et al., 2003). Rather, ablation of NRP1 specifically from neural crest cells with CRE recombinase driven by the *Wnt1* promoter (Jiang et al., 2000) phenocopied the defects seen in full *Nrp1*-null mutants (6/8 cases; compare arrowheads in Fig. 2B,E with that in Fig. 3L). These observations demonstrate that NRP1 plays a cell-autonomous role in the guidance of cranial neural crest cells.

## *Sema3a* expression is not controlled by ERBB4

In the mouse, loss of the neuregulin receptor ERBB4, which is normally expressed in r3 and r5, causes invasion of hyoid neural crest cells into the normally neural crest-free mesenchyme adjacent to r3 (Golding et al., 2002; Golding et al., 2000). It has therefore been hypothesised that ERBB4 controls the expression of a guidance cue that normally repels hyoid neural crest cells, perhaps by inducing its expression in the territory that separates the trigeminal and hyoid neural crest streams. To test the idea that ERBB4 is required for cranial neural crest cell patterning because it regulates *Sema3a* expression, we examined the expression pattern of *Sema3a* in the absence of ERBB4. We found that *Sema3a* was expressed normally in 5/5 *Erbb4*-null mutants (Fig. 4). This observation suggests that ERBB4 controls a signalling pathway that is distinct from, but cooperates with, SEMA3A/NRP1 signalling.

## NRP1 and NRP2 cooperate to guide cranial neural crest cells

NRP2 signalling has previously been reported to exclude neural crest cells from the mesenchymal territory adjacent to r3 (Eickholt et al., 1999; Gammill et al., 2007). In agreement, we found that 3/7 *Nrp2*-null mutants displayed unilateral invasion of neural crest cells into this region (Fig. 5B, arrowhead). However, the penetrance of this defect appeared lower in *Nrp2*-null than in *Nrp1*-null mutants (3/7 versus 12/12 cases, respectively). We next asked whether SEMA3F/NRP2 and SEMA3A/NRP1 signalling act synergistically during cranial neural crest cell guidance. Given

DEVELOPMENT

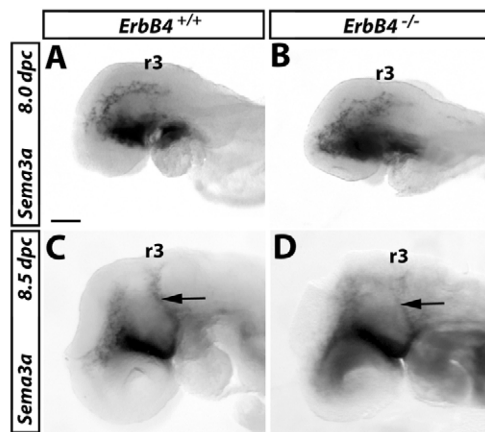


**Fig. 4. ERBB4 does not control *Sema3A* expression.** The expression of *Sema3a* in control (**A**,**C**) and *Erbb4*-null mutant (**B**,**D**) littermates was monitored by wholemount in situ hybridisation at 8.0 dpc (A,B) and 8.5 dpc (C,D). At 8.5 dpc, expression was prominent in a dorsoventral stripe that initiates below r3 (arrow in C,D). Scale bar: 100 μm.

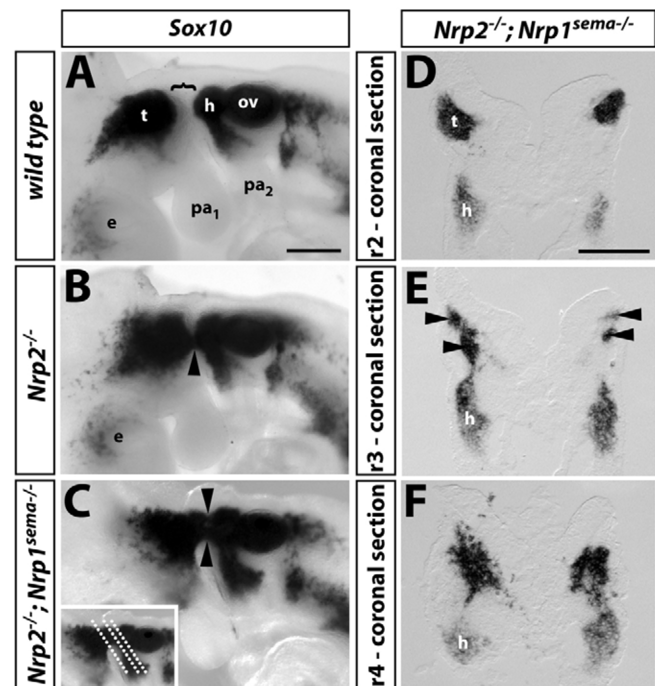


**Fig. 5. NRP1 and NRP2 cooperate to guide cranial neural crest cells.** (**A-C**) Wholemount in situ hybridisation of 9.5 dpc mouse embryos (20-somite stage) with a *Sox10* probe revealed a neural crest-free zone (bracket) between the trigeminal and hyoid neural crest streams in wild type (A), but not in stage-matched *Nrp2*-null mutants (arrowhead in B). Loss of semaphorin signalling through both NRP1 and NRP2 caused more extensive invasion of neural crest cells into the territory that normally separates the trigeminal and hyoid neural crest streams (arrowheads in C). (**D-F**) Coronal sections through the double-null mutant shown in C at r2 (D), r3 (E) and r4 (F) level; the three planes of sectioning are indicated with dotted white lines in the inset in C. Arrowheads in E indicate dorsal and ventral ectopic neural crest cell streams in the cranial mesenchyme at the r3 level. t, trigeminal neural crest cells; h, hyoid neural crest cells; ov, otic vesicle; $pa_1$ and $pa_2$, pharyngeal arches 1 and 2. Scale bars: 250 μm in A for A-C, in D for D-F.

that lack of both NRP2 and NRP1 is lethal prior to 9.5 dpc owing to severe vascular defects (Takashima et al., 2002), we took advantage of a mouse mutant that is deficient in semaphorin signalling, but not in vascular endothelial growth factor signalling through NRP1, and which therefore survives to birth (*Nrp1*$^{sema-/-}$ *Nrp2*$^{-/-}$) (Gu et al., 2003). In 3/3 mutants lacking semaphorin signalling through both NRP1 and NRP2, the territory adjacent to r3 was invaded by ectopic neural crest cells much more heavily than in single-null mutants, and the trigeminal and hyoid crest streams appeared to fuse (compare Fig. 5C with Fig. 5B and Fig. 2B,E). SEMA3A/NRP1 and SEMA3F/NRP2 therefore act synergistically to exclude neural crest cells from the mesenchyme at the r3 level and to separate the trigeminal and hyoid neural crest cell streams.

## NRP1 controls sensory neuron positioning in the head

Cranial neural crest cells contribute only a small proportion of sensory neurons to the cranial ganglia (D'Amico-Martel and Noden, 1983), but they are essential for neuronal development in the cranial ganglia by organising placodally-derived sensory neurons and their projections (Begbie and Graham, 2001). We therefore asked whether ectopic cranial neural crest cell migration in neuropilin-null mutants affected sensory neuron positioning (Fig. 6). Labelling of neuronal cell bodies at 10.5 dpc with an antibody specific for HUC/D (ELAVL3/4) proteins (Wakamatsu and Weston, 1997) identified ectopic neurons between the proximal parts of the facioacoustic and trigeminal ganglia in 5/5 *Nrp1*-null mutants; in 1/5 cases, ectopic neurons were also seen between the distal parts of the facioacoustic and trigeminal ganglia (Fig. 6H, arrowheads). The location of these ectopic neurons correlated with the position of ectopic neural crest cells at earlier stages (compare Fig. 6H with Fig. 2B,E). Similarly, ectopic neurons were found in positions prefigured by ectopic neural crest cells in *Nrp2*-null mutants (data not shown). The number of ectopic neurons increased dramatically when semaphorin signalling through NRP1 was reduced on a *Nrp2*-null background (Fig. 6, compare circled area in I with the equivalent area in H). Consequently, there was no clear separation between the trigeminal and facioacoustic ganglia in 3/3 compound mutants (Fig. 6C,F). This dramatic defect in gangliogenesis reflected the greater severity of the neural crest phenotype in compound mutants compared with single mutants at earlier stages (compare Fig. 6A with Fig. 2B, and Fig. 6C with Fig. 5C). Taken together, our observations suggest that abnormal cranial neural crest migration in neuropilin mutants directly affects sensory neuron localisation.

## Altered sensory neuron position in neuropilin mutants contributes to axonal mispatterning

Loss of NRP1 or NRP2 results in ectopic projections and defasciculated nerve tracts in the peripheral nervous system (Giger et al., 2000; Kitsukawa et al., 1997). These axonal defects are generally attributed to the loss of semaphorin signalling at the axonal growth cone (e.g. Luo et al., 1993). We observed that the abnormal positioning of sensory neurons in neuropilin mutants also contributed to the disorganisation of sensory projections (Fig. 6). Thus, ectopic neurons in *Nrp1*-null mutants (Fig. 6H, arrowheads) extended axons between the trigeminal and facioacoustic ganglia (Fig. 6K, arrowheads). Similarly, ectopic neurons in *Nrp2*-null mutants also extended aberrant axons (data not shown). The axon tracts of compound mutants, with their

DEVELOPMENT

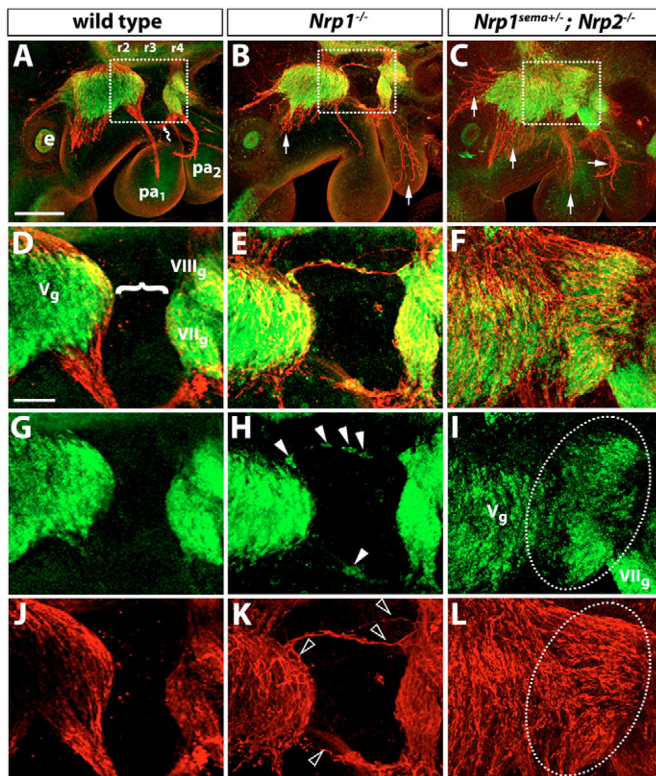


**Fig. 6. Neuropilin mutants contain ectopic neurons and extend misprojecting axons.** Wholemount immunochemistry at 10.5 dpc for HUC/D-positive neuronal cell bodies (green) and neurofilament-containing axons (red) in wild-type mouse embryos (**A**,**D**,**G**,**J**), in *Nrp1*-null mutants (**B**,**E**,**H**,**K**), and in *Nrp2*-null mutants with reduced semaphorin signalling though NRP1 (**C**,**F**,**I**,**L**). Defasciculated axon bundles (white arrows) extended from the trigeminal (Vg) and facioacoustic (VII/VIIIg) ganglia in *Nrp1*-null (B) and in compound mutants (C). The wavy arrow in A indicates the greater superficial petrosal nerve. Ectopic axon tracts extended between the trigeminal (Vg) and facioacoustic (VII/VIIIg) ganglia in single and compound mutants (boxed area in B and C, respectively; shown at higher magnification in D-F and as single labels in G-I and J-L, respectively). Note absence of neurons in the area between the trigeminal and facioacoustic ganglia in wild-type embryos (bracket in D), and presence of ectopic neurons between the trigeminal and facioacoustic ganglia in single and compound mutants (arrowheads in H and circled in I). Misprojecting axons extend from ectopic neurons (arrowheads in K, circled in L). $V_g$, $VII_g$, $VIII_g$, cranial ganglia; e, eye anlage; $pa_1$ and $pa_2$, pharyngeal arches 1 and 2. Scale bars: 200 μm in A for A-C; 50 μm in D for D-L.

greater number of ectopic neurons, were even more disorganised than those of single mutants: in areas where ectopic sensory neurons were situated in compound mutants (circled in Fig. 6I), a large number of axons extended through the normally axon-free space between the trigeminal and facioacoustic ganglia (circled in Fig. 6L). In addition, axon guidance defects within the pharyngeal arches were more severe in compound than in single mutants (Fig. 6, compare A with C).

The disorganisation of cranial sensory axons was also obvious in 11.5 dpc wholemount neurofilament stains (Fig. 7). Specifically, the trigeminal ganglion appeared to project posteriorly towards the geniculate ganglion in 4/5 mutants lacking NRP2 (Fig. 7B), and the trigeminal and facioacoustic ganglion appeared fused in 2/2 mutants lacking semaphorin signalling through both NRP1 and NRP2 (Fig. 7C). Taken together, our findings are consistent with the idea that NRP1 and NRP2 play essential and non-redundant roles during growth cone guidance and axon fasciculation.

## Ectopic neurons in *Nrp1*-null mutants are of placodal origin

The role of neural crest cells in guiding placodal sensory neurons was previously demonstrated in the chick by surgical ablation of hindbrain segments prior to neural crest emigration (Begbie and Graham, 2001). Even though this experiment was consistent with the idea that cranial neural crest cells play a key role in the guidance of placodal neurons and their axons, it had technical limitations; specifically, the removal of whole hindbrain segments might have disturbed gangliogenesis indirectly by impairing communication between hindbrain tissue and head mesenchyme. Because neural crest cells are misrouted in *Sema3a* and *Nrp1* mutants in the absence of gross alterations to other head structures, they present an ideal model system with which to test the hypothesis that neural crest cells organise placodal sensory neurons. Moreover, they allow us to determine the placodal origin of the ectopic neurons, as specific molecular markers exist for the different placodes in the mouse.

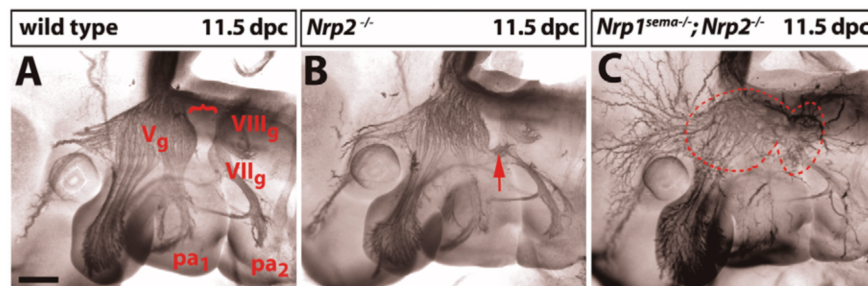


**Fig. 7. SEMA3/NRP control axon guidance during cranial gangliogenesis.** (**A-C**) Wholemount immunolabelling of neurofilament-containing axons in stage-matched 11.5 dpc mouse embryos shows that the trigeminal ganglion ($V_g$) and facioacoustic ganglion complex ($VII_g$ and $VIII_g$) are well separated in the wild type (red bracket, A), but not in mutants lacking NRP2 (B); note the presence of ectopic projections that appear to extend from the trigeminal ganglion towards the geniculate ganglion (arrow, B). The trigeminal and facioacoustic ganglia appeared completely fused in mutants lacking semaphorin signalling through both NRP1 and NRP2 (outlined in red, C). Scale bar: 250 μm.

DEVELOPMENT

The ectopic neurons in *Nrp1*-null mutants are located between the trigeminal ganglion, which receives neurons from the trigeminal placode, and the facioacoustic ganglion complex, which comprises the vestibuloacoustic ganglion in its proximal part and the geniculate ganglion in its distal part. Whereas the vestibuloacoustic ganglion receives neurons from the otic placode, the geniculate ganglion receives neurons from the first epibranchial placode (Graham and Begbie, 2000). To identify the placodal origin of the ectopic neurons, we therefore used *Ngn1* (*Neurog1* – Mouse Genome Informatics) as a marker for trigeminal placode-derived neurons (Ma et al., 1998), *Phox2b* as a marker for geniculate neurons (Fode et al., 1998), and *Brn3b* (*Pou4f2*) as marker for otic placode-derived neurons (Eng et al., 2004; Huang et al., 2001) (Fig. 8C,E,G). In addition, we used the general neuronal marker *Isl1* (Begbie et al., 2002) to demonstrate that ectopic sensory neurons could be identified by in situ hybridisation (Fig. 8A,B).

The ectopic neurons did not express the geniculate neuron marker *Phox2b* (Fig. 8D), suggesting that they were not derived from the first epibranchial placode. Moreover, they were not labelled with the *Ngn1* probe (Fig. 8F), suggesting that they were not derived from the trigeminal placode either. As *Ngn1* additionally labels neurons derived from trigeminal neural crest cells (Ma et al., 1998), this experiment also ruled out the possibility that ectopic neurons in *Nrp1*-null mutants had differentiated from trigeminal neural crest cells, which had moved caudally. Subsequent neurofilament staining confirmed that ectopic bridges were present between the trigeminal and facioacoustic ganglia in all mutants examined with *Phox2b* and *Ngn1* (data not shown). Thus, ectopic neurons were likely to be derived from the otic placode; consistent with this idea, the *Brn3b* marker was expressed by the ectopic neurons (Fig. 8H). Even though this marker also labels trigeminal neurons, the lack of *Ngn1* expression implied that the ectopic neurons must have been derived from the otic placode. Taken together, the data presented here demonstrate that NRP1-dependent neural crest cells control the positioning of placodal sensory neurons.

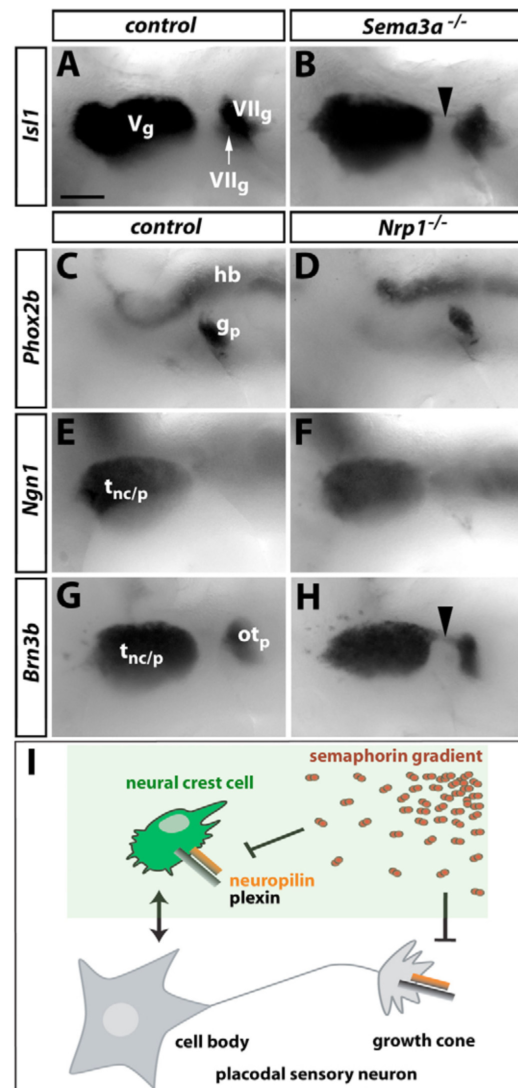


**Fig. 8. Ectopic neurons in *Nrp1* mutants have placodal identity.** In situ hybridisation of *Nrp1*-null mutants and heterozygous control littermates at 10.5 dpc with probes that detect (**A**,**B**) all cranial sensory neurons (*Isl1*) or (**C-H**) subpopulations of placodal neurons (*Phox2b*, *Ngn1* and *Brn3b*). Ectopic neurons could be clearly identified by in situ hybridisation (arrowhead in B). At 10.5 dpc, *Phox2b* marked neurons from the geniculate placode ($g_p$, C), whereas *Ngn1* was expressed by neurons derived from the trigeminal neural crest stream and trigeminal placode ($t_{nc/p}$, E). However, neither marker labelled the ectopic neurons in any of the *Nrp1*-null mutants examined (compare B with D,F). *Brn3b* labelled the same population of trigeminal neurons as *Ngn1* and, in addition, neurons derived from the otic placode ($ot_p$, G); this marker was expressed by the ectopic neurons (arrowhead in H). Scale bar: 100 μm. (**I**) Working model for the multiple roles of semaphorin signalling in cranial sensory neuron patterning. By acting on neuropilin/plexin receptors, gradients formed by the class 3 semaphorins SEMA3A and SEMA3F synergise to repel cranial neural crest cells and the axonal growth cones of sensory neurons. Neural crest cells in turn determine the position of cell bodies from placodal sensory neurons through an unknown molecular interaction (bi-directional arrow).

## DISCUSSION

SEMA3A and NRP1 have previously been implicated in neural crest patterning in the chick. However, it was generally assumed that this role was not evolutionary conserved (Gammill et al., 2006; Kawasaki et al., 2002; Kuan et al., 2004). The data presented here demonstrate that this assumption was incorrect, as SEMA3A/NRP1 signalling plays a key role in cranial neural crest patterning in the mouse. Specifically, SEMA3A/NRP1 signalling excludes hyoid neural crest cells from the cranial mesenchyme at the level of hindbrain rhombomere r3 and thereby prevents the intermingling of the trigeminal and hyoid neural crest streams (Fig. 2). SEMA3A is likely to act as a repulsive cue for hyoid neural crest cells because: (1) *Sema3a* is expressed in an area between the trigeminal and hyoid streams (Fig. 1); (2) hyoid neural crest cells express *Nrp1* (Fig. 1); and (3) hyoid neural crest cells require *Nrp1* in a cell-autonomous fashion (Fig. 3).

The cranial neural crest defect of *Nrp1*-null mouse mutants resembles the defect seen in chick embryos when a vector expressing soluble NRP1 protein was electroporated into the hindbrain prior to neural crest cell emigration (Osborne et al., 2005). There are, however, several important differences between our previous chick and current mouse analyses. Firstly, the chick experiments did not attempt to demonstrate a specific role for SEMA3A, whereas the analysis of *Sema3a* mouse mutants established that it is indeed the repulsive signal detected by the NRP1-expressing cranial neural crest cells. Secondly, the *Sema3a* expression pattern during cranial neural crest cell migration differs in chick and mouse: whereas *Sema3a* and *Sema3f* are expressed in an overlapping pattern in r3 and r5 during neural crest delamination in the chick, *Sema3a* is expressed only weakly

DEVELOPMENT

in the mouse hindbrain, but prominently in the periphery at this stage. These differences in the *Sema3a* and *Sema3f* expression patterns in the mouse might reflect an evolutionary divergence in the mechanisms that control cranial neural crest streaming in these two organisms. Importantly, *Sema3a* is expressed in the mouse in the correct spatiotemporal fashion to directly affect migrating neural crest cells at a short distance. This observation therefore eliminates the need to postulate a long-range SEMA3A gradient emanating from the hindbrain beyond the perineural membranes into the head mesenchyme. In addition, the overlapping expression patterns of *Sema3a* and *Sema3f* in the distal pharyngeal arch mesenchyme might present a barrier to neural crest migration as they invade the arches.

The non-overlapping expression patterns of *Sema3a* and *Sema3f* in conjunction with the expression pattern of their neuropilin receptors suggested that SEMA3A/NRP1 and SEMA3F/NRP2 act in a synergistic fashion to prevent the mixing of the trigeminal and hyoid neural crest cell streams. Consistent with this idea, compound mutants lacking semaphorin signalling through both NRP1 and NRP2 had a more severe defect than single mutants, with extensive intermingling of the trigeminal and hyoid neural crest streams in the mesenchyme at r3 level (Fig. 5).

Both cranial neural crest cells and neurogenic placodes give rise to the sensory neurons in the cranial ganglia (e.g. D'Amico-Martel and Noden, 1983), and their development must therefore be coordinated. Previous experiments suggested that cranial neural crest cells play a key role in the guidance of placodal neurons and their axons in the chick (Begbie and Graham, 2001). We have now substantiated the concept that cranial neural crest cells orchestrate cranial gangliogenesis and have provided a molecular mechanism for neural crest cell positioning upstream of cranial gangliogenesis. Thus, in the absence of either NRP1 or NRP2, ectopic neurons were found in locations that were prefigured by the position of ectopic neural crest cells (compare Figs 2 and 5 with Fig. 6). Using a collection of different molecular markers for placodal neurons, we then established that the ectopic neurons of *Nrp1*-null mutants had placodal identity (Fig. 8).

Importantly, mutants lacking semaphorin signalling through both NRP1 and NRP2 (Figs 6, 7) displayed an even more extensive disorganisation of the cranial sensory nervous system, with a lack of separation between the trigeminal and facioacoustic ganglia and extensive misprojections between both ganglia; these defects occurred in addition to the extensive axon guidance defects within the pharyngeal arches that we had anticipated based on the reported phenotypes of the single mutants (Giger et al., 2000; Kitsukawa et al., 1997). Owing to the abnormal positioning of sensory neurons, the organisation of the trigeminal and facioacoustic ganglia no longer reflected the segmental nature of the hindbrain and pharyngeal arches. Based on the severity of these defects, we predict that more than one type of placodal neuron will be affected in double mutants. Even though the paucity of double mutants (frequency of 1:16 in an average litter size of <8 embryos) has precluded us from performing a placodal marker analysis similar to that which we carried out for single *Nrp1* mutants, the observation that *Brn3b* is also expressed by ectopic neurons in 2/4 *Nrp2*-null mutants (data not shown) provides further support for the idea that NRP1 and NRP2 pathways cooperate to organise the position of placodal sensory neurons.

In conclusion, our study confirms that the neuropilin-mediated guidance of cranial neural crest cells is an essential prerequisite for the ordered positioning of cranial sensory neurons in the head and for the spatial separation of the cranial ganglia. We therefore propose a new working model to explain the role of class 3 semaphorin/neuropilin signalling in the sensory nervous system (Fig. 8I): by acting on neuropilin/plexin receptors, SEMA3A and SEMA3F synergise to repel axonal growth cones (Huber et al., 2003) and cranial neural crest cells (this study; indicated by the green box in Fig. 8I). As cranial neural crest cells in turn determine the position of sensory neuron cell bodies (Begbie and Graham, 2001), semaphorin/neuropilin signalling plays a novel role in patterning both cell bodies and axons in the peripheral nervous system.

We thank Drs Hajime Fujisawa, Masahiko Taniguchi, Andrew McMahon, Masahi Yanagisawa, Dorothy Reimert, David D. Ginty and Alex L. Kolodkin for mouse strains; Drs Marcus Fruttiger and Nicoletta Kessaris for advice and reagents; Drs Anthony Graham and Matthew Golding for invaluable discussions; the staff of the Biological Resources Unit for help with mouse husbandry; and Dr John Greenwood for support. This research was funded by a project grant of the Medical Research Council to C.R. and a PhD studentship from the Fundação para a Ciência e Tecnologia (SFRH/BD/17812/2004) to J.M.V.

## References

**Begbie, J. and Graham, A.** (2001). Integration between the epibranchial placodes and the hindbrain. *Science* **294**, 595-598.

**Begbie, J., Ballivet, M. and Graham, A.** (2002). Early steps in the production of sensory neurons by the neurogenic placodes. *Mol. Cell. Neurosci.* **21**, 502-511.

**Brachtendorf, G., Kuhn, A., Samulowitz, U., Knorr, R., Gustafsson, E., Potocnik, A. J., Fassler, R. and Vestweber, D.** (2001). Early expression of endomucin on endothelium of the mouse embryo and on putative hematopoietic clusters in the dorsal aorta. *Dev. Dyn.* **222**, 410-419.

**Carmeliet, P.** (2003). Blood vessels and nerves: common signals, pathways and diseases. *Nat. Rev. Genet.* **4**, 710-720.

**D'Amico-Martel, A. and Noden, D. M.** (1983). Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. *Am. J. Anat.* **166**, 445-468.

**Eickholt, B. J., Mackenzie, S. L., Graham, A., Walsh, F. S. and Doherty, P.** (1999). Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. *Development* **126**, 2181-2189.

**Eng, S. R., Lanier, J., Fedtsova, N. and Turner, E. E.** (2004). Coordinated regulation of gene expression by Brn3a in developing sensory ganglia. *Development* **131**, 3859-3870.

**Farlie, P. G., Kerr, R., Thomas, P., Symes, T., Minichiello, J., Hearn, C. J. and Newgreen, D.** (1999). A paraxial exclusion zone creates patterned cranial neural crest cell outgrowth adjacent to rhombomeres 3 and 5. *Dev. Biol.* **213**, 70-84.

**Fode, C., Gradwohl, G., Morin, X., Dierich, A., LeMeur, M., Goridis, C. and Guillemot, F.** (1998). The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron* **20**, 483-494.

**Gammill, L. S., Gonzalez, C., Gu, C. and Bronner-Fraser, M.** (2006). Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling. *Development* **133**, 99-106.

**Gammill, L. S., Gonzalez, C. and Bronner-Fraser, M.** (2007). Neuropilin 2/semaphorin 3F signaling is essential for cranial neural crest migration and trigeminal ganglion condensation. *Dev. Neurobiol.* **67**, 47-56.

**Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G.** (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-394.

**Giger, R. J., Cloutier, J. F., Sahay, A., Prinjha, R. K., Levengood, D. V., Moore, S. E., Pickering, S., Simmons, D., Rastan, S., Walsh, F. S. et al.** (2000). Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. *Neuron* **25**, 29-41.

**Golding, J. P., Trainor, P., Krumlauf, R. and Gassmann, M.** (2000). Defects in pathfinding by cranial neural crest cells in mice lacking the neuregulin receptor ErbB4. *Nat. Cell Biol.* **2**, 103-109.

**Golding, J. P., Dixon, M. and Gassmann, M.** (2002). Cues from neuroepithelium and surface ectoderm maintain neural crest-free regions within cranial mesenchyme of the developing chick. *Development* **129**, 1095-1105.

**Graham, A.** (2000). The evolution of the vertebrates-genes and development. *Curr. Opin. Genet. Dev.* **10**, 624-628.

**Graham, A.** (2003). The neural crest. *Curr. Biol.* **13**, R381-R384.

**Graham, A. and Begbie, J.** (2000). Neurogenic placodes: a common front. *Trends Neurosci.* **23**, 313-316.

**Gu, C., Rodriguez, E. R., Reimert, D. V., Shu, T., Fritzsch, B., Richards, L. J., Kolodkin, A. L. and Ginty, D. D.** (2003). Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev. Cell* **5**, 45-57.

**Huang, E. J., Liu, W., Fritzsch, B., Bianchi, L. M., Reichardt, L. F. and Xiang, M.** (2001). Brn3a is a transcriptional regulator of soma size, target field

DEVELOPMENT

innervation and axon pathfinding of inner ear sensory neurons. *Development* **128**, 2421-2432.
**Huber, A. B., Kolodkin, A. L., Ginty, D. D. and Cloutier, J. F.** (2003). Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annu. Rev. Neurosci.* **26**, 509-563.
**Jiang, X., Rowitch, D. H., Soriano, P., McMahon, A. P. and Sucov, H. M.** (2000). Fate of the mammalian cardiac neural crest. *Development* **127**, 1607-1616.
**Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T. and Fujisawa, H.** (1999). A requirement for neuropilin-1 in embryonic vessel formation. *Development* **126**, 4895-4902.
**Kawasaki, T., Bekku, Y., Suto, F., Kitsukawa, T., Taniguchi, M., Nagatsu, I., Nagatsu, T., Itoh, K., Yagi, T. and Fujisawa, H.** (2002). Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system. *Development* **129**, 671-680.
**Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson, J. A. and Yanagisawa, M.** (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev. Biol.* **230**, 230-242.
**Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku, Y., Yagi, T. and Fujisawa, H.** (1997). Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. *Neuron* **19**, 995-1005.
**Kuan, C. Y., Tannahill, D., Cook, G. M. and Keynes, R. J.** (2004). Somite polarity and segmental patterning of the peripheral nervous system. *Mech. Dev.* **121**, 1055-1068.
**Kulesa, P. M. and Fraser, S. E.** (1998). Neural crest cell dynamics revealed by time-lapse video microscopy of whole embryo chick explant cultures. *Dev. Biol.* **204**, 327-344.
**Kulesa, P. M. and Fraser, S. E.** (2000). In ovo time-lapse analysis of chick hindbrain neural crest cell migration shows cell interactions during migration to the branchial arches. *Development* **127**, 1161-1172.
**Lumsden, A., Sprawson, N. and Graham, A.** (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113**, 1281-1291.
**Luo, Y., Raible, D. and Raper, J. A.** (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* **75**, 217-227.
**Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J. L. and Anderson, D. J.** (1998). neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* **20**, 469-482.
**Osborne, N. J., Begbie, J., Chilton, J. K., Schmidt, H. and Eickholt, B. J.** (2005). Semaphorin/neuropilin signaling influences the positioning of migratory neural crest cells within the hindbrain region of the chick. *Dev. Dyn.* **232**, 939-949.
**Pusch, C., Hustert, E., Pfeifer, D., Sudbeck, P., Kist, R., Roe, B., Wang, Z., Balling, R., Blin, N. and Scherer, G.** (1998). The SOX10/Sox10 gene from human and mouse: sequence, expression, and transactivation by the encoded HMG domain transcription factor. *Hum. Genet.* **103**, 115-123.
**Rao, M. S. and Anderson, D. J.** (1997). Immortalization and controlled in vitro differentiation of murine multipotent neural crest stem cells. *J. Neurobiol.* **32**, 722-746.
**Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C.** (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.
**Sechrist, J., Serbedzija, G. N., Scherson, T., Fraser, S. E. and Bronner-Fraser, M.** (1993). Segmental migration of the hindbrain neural crest does not arise from its segmental generation. *Development* **118**, 691-703.
**Takashima, S., Kitakaze, M., Asakura, M., Asanuma, H., Sanada, S., Tashiro, F., Niwa, H., Miyazaki, J., Hirota, S., Kitamura, Y. et al.** (2002). Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc. Natl. Acad. Sci. USA* **99**, 3657-3662.
**Taniguchi, M., Yuasa, S., Fujisawa, H., Naruse, I., Saga, S., Mishina, M. and Yagi, T.** (1997). Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. *Neuron* **19**, 519-530.
**Trainor, P. A.** (2005). Specification of neural crest cell formation and migration in mouse embryos. *Semin. Cell Dev. Biol.* **16**, 683-693.
**Wakamatsu, Y. and Weston, J. A.** (1997). Sequential expression and role of Hu RNA-binding proteins during neurogenesis. *Development* **124**, 3449-3460.

DEVELOPMENT