Cranial neural crest cells form corridors prefiguring sensory neuroblast migration

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SUMMARY
The majority of cranial sensory neurons originate in placodes in the surface ectoderm, migrating to form ganglia that connect to the central nervous system (CNS). Interactions between inward-migrating sensory neuroblasts and emigrant cranial neural crest cells (NCCs) play a role in coordinating this process, but how the relationship between these two cell populations is established is not clear. Here, we demonstrate that NCCs generate corridors delineating the path of migratory neuroblasts between the placode and CNS in both chick and mouse. In vitro analysis shows that NCCs are not essential for neuroblast migration, yet act as a superior substrate to mesoderm, suggesting provision of a corridor through a less-permissive mesodermal territory. Early organisation of NCC corridors occurs prior to sensory neurogenesis and can be recapitulated in vitro; however, NCC extension to the placode requires placodal neurogenesis, demonstrating reciprocal interactions. Together, our data indicate that NCC corridors impose physical organisation for precise ganglion formation and connection to the CNS, providing a local environment to enclose migrating neuroblasts and axonal processes as they migrate through a non-neural territory.

KEY WORDS: Neural crest, Placode, Cranial sensory ganglia

INTRODUCTION
Sensory ganglia in the head and trunk are generated from migratory populations of cells. In the trunk, they develop from neural crest cells (NCC) that migrate into the rostral somite. A subpopulation of these NCCs stop lateral to the neural tube and coalesce, before undergoing neurogenesis to form the dorsal root ganglia (DRG) (George et al., 2010). The imposition of this migration pattern by the somites leads to the regular, segmental organisation of the DRG and their innervation of the central nervous system (CNS) (Gammill and Roffers-Agarwal, 2010).

The cranial sensory ganglia (CSG) are more complex in organisation and embryonic origin. The CSG can be subdivided into proximal and distal ganglia relating to location on the associated cranial nerve. Neurons of the proximal CSG are generated from NCCs (D’Amico-Martel and Noden, 1983; Thompson et al., 2010). However, in the distal CSG, NCCs mainly generate the non-neuronal components, whereas the majority of neurons originate in ectodermal neurogenic placodes (D’Amico-Martel and Noden, 1983; Harlow et al., 2011; Thompson et al., 2010). With the exception of the ophthalmic trigeminal placode, which generates postmitotic neurons, neurogenic placodes produce neuroblasts that delaminate and actively migrate towards the hindbrain (Begbie et al., 2002; Blentic et al., 2011; Graham et al., 2007; McCabe et al., 2009). In the head, unlike the trunk, there is no architectural framework to guide CSG formation; so how is neuroblast migration controlled to pattern the CSG and their accurate innervation of the hindbrain?

Reports across a range of species demonstrate that NCCs play a role in organising the CSG. Our studies in chick show that loss of cranial NCCs either by physical ablation or by ectopic neuropilin expression, affects accurate CSG formation (Begbie and Graham, 2001; Osborne et al., 2005). Similarly, zebrafish mutants lacking a subpopulation of NCCs show defective ganglion formation (Culbertson et al., 2011). Complementary studies in mice show that ectopic NCC migration is associated with ectopic cranial sensory neuron migration (Chen et al., 2011; Schwarz et al., 2008). Analysis of the molecular interactions between the two populations has mainly been carried out in the developing trigeminal ganglion, and shows roles for Robo and Wnt signalling pathways (Shiau and Bronner-Fraser, 2009; Shiau et al., 2008; Shigetani et al., 2008). Here, we examine the cellular relationship between the NCCs and placodal neuroblasts underlying the organisation of the CSG, and address how NCCs are positioned to play this role.

MATERIALS AND METHODS

Animals
Fertilised hens’ eggs (Winter Egg Farm, UK) were incubated in a humidified chamber at 38°C to the required Hamburger Hamilton stage (Hamburger and Hamilton, 1992). The mouse line used was Wntlr:R26RYFP (Danielian et al., 1998; Srinivas et al., 2001). Embryos from both species were fixed overnight at 4°C in 1×MEMFA, washed with 1×PBS, stored in 1×PBS + 0.02% azide before antibody staining. Mouse work was approved by the King’s College London Ethical Review Process and was performed in accordance with UK Home Office Project Licence 70/7441 (K.J.L.).

Antibody staining and in situ hybridisation
Whole-mount antibody staining was carried out as described previously (Thompson et al., 2010). Primary antibodies were: mouse anti-HNK1, 1:200 (Sigma); mouse anti-neurofilament medium chain, 1:5000 (RMO-270, Zymed, Invitrogen); mouse anti-islet 1/2, 1:1000 (a kind gift from Ivo Lieberam, King’s College London, UK); rabbit anti-GFP, 1:500 (Invitrogen). Secondary antibodies were: Alexa 488-conjugated anti-mouse IgG; Alexa 488-conjugated anti-rabbit IgG; Alexa 568-conjugated anti-
mouse IgM; Alexa 647-conjugated anti-mouse IgM used at 1:1000 (Invitrogen Molecular Probes).

Single and double whole-mount in situ hybridisation was carried out as described previously (Begbie et al., 1999). For triple labelling, whole-mount in situ hybridisation was carried out using the FastRed substrate (Sigma), and then whole-mount antibody staining carried out as above. The FastRed signal was visualised at 568 nm and Alexa-conjugated antibodies at 488 nm and 647 nm by confocal.

**Cell labelling**

*In ovo* electroporation was used to introduce chick β-actin GFP (2 μg/μl) into presumptive placodal ectoderm at HH15 using 4×5 msecond 10 V pulses, or hindbrain crest at HH9 using 5×5 msecond 10 V pulses (Graham et al., 2007).

**In vitro culture**

Placodes

For neural crest cultures, midbrain and hindbrain segments from HH9 (6–9 somite) embryos were cultured for 24 hours to allow NCCs to establish, then neural tubes were removed. Lateral plate mesoderm caudal to the last somite was taken from the same embryos and cultured for 24 hours prior to addition of placode. Placodes were taken at HH17 from GFP electroporated embryos targeted at HH14 using flame-sharpened tungsten needles. Placodes were plated on top of neural crest, mesoderm or FN, in F12+N2, allowed to settle for 15 minutes before cultured for 24 hours at 37°C in 5% CO2.

Neural tube

Neural tubes from diencephalon to first somite level were excised from HH9 (6–9 somite) embryos using tungsten needles. Neural tubes were carefully positioned on fibronectin-coated coverslips (10 μg/ml) and cultured for 22 hours in F12+N2 at 37°C in 5% CO2.

**Targeted ablation of placodal neurons**

The diphtheria toxin α-chain expression vector (CMV-TdTomato-2A-DtA) was produced as follows. The expression vector pCMV-tdT-2A-MCS was cloned products were confirmed by sequencing. Bsr Shankar Srinivas, University of Oxford, UK) with Nhe(Xba)/Bsr NotI. Co-translational cleavage of the 2A sequence was validated to exceed 0.95 with various insert. Clones from pMR26-ires-tdTomato was cloned into backbone pCMV-tdT-2A-MCS using BsrG1 and NotI. All cloning products were confirmed by sequencing.

*In ovo* electroporation was used to introduce CMV-TdTomato-2A-DtA (4 μg/μl) unilaterally into placodal ectoderm at HH13-14 as above and embryos incubated to HH16-18.

**Embryo visualisation**

Embryos after whole-mount *in situ* hybridisation were imaged using a Zeiss Stereolumar stereomicroscope. Confocal analysis of whole embryos was performed at ×10 and ×20 magnification acquiring optical sections at 2 μm intervals (Zeiss LSM710). For 75 μm transverse vibratome sections, embryos were embedded in 15% gelatin:15% sucrose:PBS and fixed overnight (MEMFA). Confocal analysis of sections was performed at ×20 and ×40 magnification, acquiring optical sections at 1 μm intervals (Zeiss LSM710). Velocity visualisation software (Perkin-Elmer) was used for 3D reconstruction.

**RESULTS AND DISCUSSION**

**Cranial neural crest cells assemble into corridors delineating sensory ganglion formation**

Three-dimensional reconstruction of HNK1- (glycan epitope labelling NCC) and NFM- (neurofilament medium chain; neuroblasts) stained chick embryos revealed that early hindbrain NCC streams were maintained, forming robust structures between hindbrain and pharyngeal arches (Fig. 1A,B; supplementary material Movie 1). These NCC structures were associated with the migrating neuroblasts (Fig. 1A,B), with HNK1 staining localised peripherally to the neuroblast population in all of the CSG examined (Fig. 1A,C,D). This tube-like appearance suggests that NCCs form a corridor delineating the path of the neuroblasts from their birthplace in the placodal epithelium towards the hindbrain. To confirm the NCC origin of these structures, we labelled pre-migratory NCCs by *in ovo* GFP electroporation. As predicted, the GFP+ NCC encircled the migrating neuroblasts (Fig. 1E).
To address neuroblast behaviour within the HNK1+ NCC corridor, we visualised individual GFP-labelled neuroblasts in whole embryos (Fig. 1F; supplementary material Movies 2, 3). In all embryos analysed (n=4), the neuroblasts were located deep within the corridor, and neither the GFP+ cell bodies nor their processes crossed the HNK1+ NCCs. This suggests that the HNK1+ NCCs provide a physical barrier constraining neuroblast migration. Three-dimensional reconstruction showed that at stages of peak neuroblast migration, HNK1+ NCCs extended directly to the edges of the corridor, we visualised individual GFP-labelled neuroblasts in migration, HNK1+ NCCs extended directly to the edges of the placode (asterisk in Fig. 1F), enclosing the neuroblasts as they delaminate from the epithelium (supplementary material Movie 3). We propose that the NCCs form a physical corridor through a non-neural territory, funnelling the neuroblasts inwards as they exit from the surface ectoderm.

**Neural crest corridors are conserved in chick and mouse**

To determine whether the NCC corridor is also present in mice, we used the Wnt1Cre:R26RYFP mouse line, genetically labelling NCCs (Danielian et al., 1998; Srinivas et al., 2001). As in the chick, YFP+ NCCs formed a structure extending between the hindbrain and the top of the pharyngeal arches (Fig. 1G). Confocal analysis showed YFP+ NCCs encircling the NFMy+ neuroblasts (Fig. 1H). Studies in mice show that mutations affecting NCC patterning are reflected by defects in CSG patterning, but that genetic NCC ablation has less effect (Chen et al., 2011; Coppola et al., 2010; Golding et al., 2000; Schwarz et al., 2008). We suggest that the NCC corridor provides a physical constraint for neuroblasts with an inherent ability to migrate, rather than an active guidance mechanism. As such, neuroblasts would still migrate in the absence of NCC, albeit less accurately, whereas a NCC patterning defect causing a misplaced corridor would have more effect on CSG patterning.

**In vitro behaviour of placodal neuroblasts on NCC versus mesoderm**

Our hypothesis favours physical constraint versus active guidance by NCCs. We tested this by comparing neuroblast behaviour from isolated placodes cultured on NCCs with those cultured on fibronectin (FN) alone, or on lateral plate mesoderm. Explants cultured on NCC showed cells exiting together and extending long processes with small growth cones (n=6) (Fig. 2A; growth cone, arrowhead in 2A’ and ×40 inset). These explants were similar to explants cultured on FN alone (n=4) (Fig. 2B), suggesting that NCCs do not provide active guidance to the neuroblasts. By comparison, mesoderm provided a less favourable substrate for migration. In these explants the neuroblast processes were less straight and shorter with fewer projections exiting from the ×10 field (Fig. 2C,D) and larger growth cones (n=4) (arrowhead, Fig. 2C’ and ×40 inset). This reinforces the idea that the NCC corridor provides a physical setting for inherent neuroblast migration and axon extension through a less-permissive mesodermal environment between placode and neural tube.

**Neural crest corridor development**

We used Isl1 and Sox10 double in situ hybridisation to examine how these NCC structures assemble with respect to epibranchial placode neuroblast production. At HH13 there is a clear dorsal Sox10+ stream (arrowheads in Fig. 3A,A’), but this does not extend to the first neuroblasts of the geniculate placode (asterisk in Fig. 3A,A’). As neurogenesis becomes established, the Sox10+ NCC corridor extends towards the placodes (Fig. 3B,B’)

and by HH17 there is a clear extension of Sox10+ NCCs surrounding the neuroblasts delaminating from the placode (Fig. 3C,C’). We compared this with HNK1+ NCC corridor development, combining Sox10 staining with HNK1 and ISL1 antibody labelling. HNK1 is a carbohydrate epitope added to many different glycolipids and glycoproteins that has been shown to recognise NCC (Tucker et al., 1984), whereas Sox10 is one of the earliest NCC-specifying genes that later is maintained and required for non-ectomesenchymal lineages (Sauka-Spengler and Bronner-Fraser, 2008). At HH13, most NCC were Sox10/HNK1 double positive (white in Fig. 3E). By HH14+, HNK1 staining (magenta) was restricted to the mesodermal interface, encircling a core of Sox10+ NCCs (yellow) (Fig. 3F). At the peak of placodal neuroblast migration (HH17), the HNK1 staining (magenta) formed a sheath around the Sox10+ NCCs (yellow), which in turn surrounded the ISL1+ neuroblasts (Fig. 3D,G). The peripheral localisation of HNK1 staining compared with Sox10+ NCCs, and the extension to the placode are clear in 3D embryo reconstruction (see supplementary material Movie 3; Fig. 1F; Fig. 3J). We examined HNK1 staining in GFP+ NCC-labelled chick embryos at HH14, prior to CSG formation. Coronal sections showed that HNK1 specifically labelled GFP+ cells at the interface with the mesoderm (white arrowhead, Fig. 3H). This is before significant peripheral neurogenesis, suggesting that the neuroblasts do not play a crucial role in initial establishment of the NCC corridors. Interestingly, YFP+ NCC corridors were also seen in mouse embryos with few neuroblasts (E9, Fig. 3I).
Together, these data suggest that the initial establishment of NCC corridors adjacent to the neural tube occurs in the absence of placodal neurogenesis, but that the extension to the placode requires the production of neurons.

Neural crest cells organise into corridors in vitro
Previous work in the axolotl has shown that cranial NCCs will form streams in vitro (Cerny et al., 2004). To test whether chick NCCs could form streams and associated HNK1+ corridors in vitro, we cultured HH9 cranial neural tubes (hindbrain to diencephalon) upright on fibronectin (FN). After 22 hours, distinct HNK1+ staining was seen at discrete intervals along the hindbrain explant (12/12). In a small number of cases (3/12), HNK1+ NCCs formed distinct streams directly on the FN which showed little three-dimensionality. However, in 9/12 cases, 3D HNK1+ NCC structures were seen extending from the dorsal hindbrain down to the FN (Fig. 3K,K’). Confocal analysis of these samples showed that the HNK1+ staining was restricted to the periphery (Fig. 3L,L’). This demonstrates that organisation into 3D structures with peripheral HNK1 staining is intrinsic to the NCCs.

Interactions between the neural crest cells and placodal neuroblasts are required for correct NCC corridor formation
Our expression analysis suggested that the extension of the NCC corridor to the placode requires the presence of placodal neuroblasts. To test this, the diphtheria toxin α-chain (DTA) was electroporated into ectoderm to eliminate the placode with minimal mechanical disruption. Isl1 and Phox2b staining showed a severe reduction in neurons in the DTA-targeted region compared with the contralateral side: Isl1 (15/17; 88%; Fig. 4A) and Phox2b (5/6: 83%; Fig. 4B). Sox10 staining showed that the NCC corridor did not form correctly on the DTA-targeted side, losing the extension to the placode (17/22; 77%; Fig. 4C). Isl1 and Sox10 double labelling showed a clear correlation between neuroblast ablation and the failure of Sox10+ NCCs to reach the placode (25/38: 66%; Fig. 4D). Although analysis was mainly carried out at rhombomere 4 (r4) (Fig. 4), the effect was also seen at r6 (supplementary material Fig. S1). Control GFP-electroporated embryos were normal for all markers analysed (n=15; 10; Phox2b: 5; Sox10: 11; double Isl1/Sox10: 5). Earlier Isl1/Sox10 analysis was carried out to determine whether Sox10+ NCCs reached the placode before being lost, and showed that the Sox10+ NCCs were still absent beneath the ectoderm (DTA, n=3/3; GFP, n=2/2; Fig. 4E). Furthermore, analysis of apoptosis using lysotracker showed cell death in NCCs, but no observable increase following placode ablation compared with contralateral side (DTA, n=12; Fig. 4F), or control electroporation (GFP, n=11; data not shown).

Our data show that although the NCC corridor is required to facilitate the correct neuroblast migration and axon projection inwards, the neuroblasts themselves are necessary for the correct formation of the NCC corridor. We suggest that the extension to the placode occurs by migration of Sox10+ NCCs; however, our results cannot confirm this. Interestingly, in Drosophila, peripheral glia form tubes required for accurate sensory axon entry to the CNS, and studies have described similar reciprocal interactions between the two cell types (Sepp and Auld, 2003; Sepp et al., 2001).

Conclusions
Our results demonstrate that cranial NCC streams transform into corridors that are subsequently associated with migrating sensory neuroblasts in chick and mouse. We propose that the NCC corridor

Fig. 3. Neural crest corridor development. (A–C) Lateral view of Sox10/Isl1 double-labelled chick embryos showing NCC corridor development in relation to developing CSG. (A–C) Transverse sections reveal extension of Sox10+ NCCs (yellow arrowheads indicate ventral point) to placode (asterisks) with increasing neurogenesis. (D) Transverse section of triple-labelled HH17 chick (D) shows HNK1+ NCCs (magenta; white arrowhead) and FoxD3+ NCCs (yellow; yellow arrowhead), surrounding ISL+ neuroblasts (green) (confocal stack projection). (E–G) Coronal sections show restriction of HNK1 staining to periphery of NCC corridor (confocal stack projection). At HH13, NCCs are Sox10/HNK1 double positive (D), but by HH14+ (F) HNK1 staining (magenta; white arrowhead) is localised to the periphery of Sox10+ NCCs (yellow; yellow arrowhead). At HH17+, ISL+ neuroblasts can be seen at core of NCC (G). (H) Coronal section of GFP NCC electroporated chick confirms restriction of HNK1 staining (magenta; white arrowhead) to periphery of total GFP+ NCCs (green; blue arrowhead) at HH14 prior to robust neurogenesis (single optical confocal section). (I) Analysis in mouse at 18 ss also shows YFP+ NCCs (magenta; orange arrow) forming corridors containing few NF-M processes (green; white arrow). (J) Schematic representation of the NCC corridor with HNK1+ NCCs (magenta) surrounding Sox10+ NCCs (yellow), extending to the placode (asterisk). Neuroblasts (blue) migrate at the NCC corridor centre. Arrow indicates direction of migration. (KK’) Chick hindbrain explants demonstrate in vitro streamed NCC migration with DIC (K) and HNK1 (K’). (LL’) Confocal analysis reveals 3D nature of in vitro HNK1+ NCC corridor (L), whereas digital re-section reveals peripheral HNK1 staining (L’). Asterisks indicate placode; NT, neural tube; OV, otic vesicle; PAI, PAII, pharyngeal arches I and II. Scale bar: 100 μm.
provides structural organisation for the CSG, bridging the mesodermal domain between the placodal epithelium and the CNS, for the inward migration of neuroblasts and their axonal extensions (Fig. 3J). In chick, an anatomical structure funnelling cells in from the surface ectoderm was first suggested as long ago as 1893 (Goronowitsch, 1893), and an HNK1 scaffold associated with developing cranial nerves in 1991 (Layer and Kaulich, 1991). Here, we have confirmed that NCCs generate a structural corridor and have shown that it is present in mice. Within the CNS, physical corridors for neuronal migration and axon guidance have been described in the form of astrocytic tubes promoting neuroblast migration in the rostral migratory stream and tangentially migrating corridor cells guiding thalamocortical axons in the telencephalon (Lois et al., 1996; López-Bendito et al., 2006). Interestingly, however, although these structures facilitate migration within the CNS, the NCC corridor described here traverses a non-neural territory.

The use of corridors for neuronal migration in peripheral nervous system (PNS) development has not been described before. It is possible that, in the trunk, NCC corridors also provide a physical cue for control of migration and axon guidance. However, as trunk sensory neurons themselves are derived from NCCs this is difficult to determine. In support of the hypothesis, we can turn to studies of axon regeneration in the adult trunk after injury. These show that endoneurial tubes constrain axon growth following nerve crush (Nguyen et al., 2002), whereas following transection Schwann cells dedifferentiate and form bridges that act as guides for regenerating axons (Parrinello et al., 2010). Furthermore, recent studies have shown that olfactory ensheathing cells, which enhance the correct re-establishment of axonal connections, are also derived from neural crest cells (Barraud et al., 2010). The cranial NCC corridors are neural crest cells as opposed to Schwann cells, but suggest an exciting developmental parallel to the regeneration scenario.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
Experiments and analysis were carried out by S.F., S.J.F. and J.B.; the DTA construct was designed and generated by R.F.; mouse embryos were provided by K.J.L.; experimental design and manuscript preparation were by J.B.

Supplementary material
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References

Fig. 4. Placode ablation affects neural crest corridor formation. (A–C) Sections at r4 level of DTA-electroporated chick embryos at HH18. Isl1 (88%; n=17) (A,A’/H11032) and Phox2b (83%; n=6) (B,B’/H11032) both demonstrate reduction in neuroblasts migrating from targeted placodes (asterisks) on electroporated side (ep) compared with unelectroporated side (control). (C,C’) Sox10 reveals failure of NCC corridor extension (arrowheads) to targeted placodes (77%; n=22). (D,D’) Sox10 Isl1 confirms reduction in NCC corridor (arrowhead) correlates with loss of neuroblasts (asterisks) (66%; n=38), seen clearly in transverse sections comparing DTA (ep) with GFP (control). (E,E’) Earlier Sox10 Isl1 staining (HH16) still demonstrates Sox10+ NCC deficiency at the targeted placode; compare DTA (ep) with GFP (control). (F) Lysotracker staining for apoptosis shows no significant increase in NCC cell death in ep compared with control. nt, neural tube; ov, otic vesicle; g, n, p, t, geniculate, nodose, petrosal and trigeminal ganglia; r, rhombomere. Scale bar: 100 μm.


