

In vivo evidence for short- and long-range cell communication in cranial neural crest cells

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Accepted 8 October 2004

Development 131, 6141-6151

Published by The Company of Biologists 2004

doi:10.1242/dev.01534

Summary

The proper assembly of craniofacial structures and the peripheral nervous system requires neural crest cells to emerge from the neural tube and navigate over long distances to the branchial arches. Cell and molecular studies have shed light on potential intrinsic and extrinsic cues, which, in combination, are thought to ensure the induction and specification of cranial neural crest cells. However, much less is known about how migrating neural crest cells interpret and integrate signals from the microenvironment and other neural crest cells to sort into and maintain the stereotypical pattern of three spatially segregated streams. Here, we explore the extent to which cranial neural crest cells use cell-to-cell and cell-environment interactions to pathfind. The cell membrane and cytoskeletal elements in chick premigratory neural crest cells were labeled *in vivo*. Three-dimensional reconstructions of migrating neural crest cells were then

obtained using confocal static and time-lapse imaging. It was found that neural crest cells maintained nearly constant contact with other migrating neural crest cells, in addition to the microenvironment. Cells used lamellipodia or short, thin filopodia (1–2 μm wide) for local contacts (<20 μm). Non-local, long distance contact (up to 100 μm) was initiated by filopodia that extended and retracted, extended and tracked, or tethered two non-neighboring cells. Intriguingly, the cell-to-cell contacts often stimulated a cell to change direction in favor of a neighboring cell's trajectory. In summary, our results present *in vivo* evidence for local and long-range neural crest cell interactions, suggesting a possible role for these contacts in directional guidance.

Key words: Chick, Neural crest, Cranial, Filopodia, Cell guidance, Confocal, Time-lapse imaging

Introduction

The cranial neural crest is a highly invasive subpopulation of cells that pathfind from the neural tube to the branchial arches during development. The neural crest contributes to many peripheral structures, including the facial skeleton and the nervous system. Intriguingly, cranial neural crest cells do not invade all areas lateral to the neural tube, but rather sort into and migrate along stereotypical routes, creating a striking pattern of three segregated streams (Lumsden and Keynes, 1989). The segregation of the streams is thought to prevent cell mixing and cranial ganglionic fusions to ensure proper establishment of anteroposterior structures in the branchial arches (Kontges and Lumsden 1996; Graham and Begbie, 2000). Failure of the cranial neural crest cells to reach the branchial arches can lead to a wide range of facial birth defects (Mooney and Siegel, 2002; Helms and Schneider, 2003), making it an important motivating factor for studying neural crest cell guidance mechanisms.

Because neural crest cells arise, undergo extensive migration and contribute to many different derivatives (LeDouarin and Kalchiem, 1999), this system is an excellent model for studying induction, cell guidance and cell differentiation (Santagati and Rijli, 2003). Here, our focus is on the formation of the cranial neural crest cell migratory pattern, which in vertebrates is a consistent, stereotypical pattern of three

segregated streams (Kulesa et al., 2004). From many cell labeling, tissue transplant and molecular studies, several distinct hypotheses have emerged on how neural crest cell streams form, but most agree that the pattern develops from a combination of intrinsic and extrinsic factors (Trainor and Krumlauf, 2000; Halloran and Berndt, 2003; Graham et al., 2004). However, despite advances in identifying molecules that induce premigratory cells and specify the fate of a cranial neural crest cell (Knect and Bronner-Fraser, 2002; Anderson, 2000; Santagati and Rijli, 2003), relatively little is known about the molecular mechanisms that direct migrating cranial neural crest cells. Thus, one crucial step in analyzing the pattern formation is to determine the extent to which cranial neural crest cells interact with each other and the environment along the migratory routes *in vivo*.

Cranial neural crest cells migrate along a dorsolateral route and, for the most part, within one of three streams of cells that develop between the neural tube and the branchial arches. The subregions of the neural tube from which the majority of the neural crest cells emerge correlate with specific segments [rhombomeres (r)] of the hindbrain, namely r1+r2, r4, and r6 (Lumsden and Keynes, 1989). Early studies suggest that after receiving a particular molecular identity at the neural tube, neural crest cells emerge and target a peripheral region with a similar molecular identity (Hunt et al., 1991), carrying cues for the patterning of the arch from neural-tube-derived signals

(Noden, 1983). The need for individual neural crest cell pathfinding in this scenario is simplified if the neural tube controls cell exit points (Lumsden et al., 1991) such that cells diffuse laterally from high populations to low populations in segregated streams (LeDouarin, 1982; Newgreen et al., 1979; Rovasio et al., 1983). Studies in chick (Graham et al., 1993; Smith and Graham, 2001) and other amniotes (Knabe et al., 2004) show increased levels of cell death in specific rhombomeres, particularly r3 and r5. However, there is no apoptosis specific to r3 and r5 in *Xenopus*, zebrafish, and mouse (Schilling and Kimmel, 1994; Ellies et al., 1997; Hensey and Gautier, 1998; Del Pino and Medina, 1998; Kulesa et al., 2004), suggesting that cell death is not solely responsible for segregating the streams. Thus, in a prepattern-type model the neural tube is thought to endow destination instructions and control exit points such that there are few directional cues necessary to produce the cranial neural crest cell pattern.

In contrast to a prepattern hypothesis, studies of cell migratory behaviors and the local environment adjacent to the neural tube suggest that the cranial neural crest cell pattern emerges when cells encounter and respond to environmental cues and interactions with other neural crest cells. Novel culture and imaging techniques, combined with Nomarski optics and labeling of premigratory neural crest cells, has allowed cell migratory behaviors to be visualized in tissue culture (Abercrombie, 1970; Bard and Hay, 1975; Erickson et al., 1980; Krull et al., 1995), in 2D and 3D gel substrates (Newgreen et al., 1979; Rovasio et al., 1983; Thomas and Yamada, 1992), in whole embryo culture (Spieth and Keller, 1984; Kulesa and Fraser, 1998), and in ovo (Kulesa and Fraser, 2000). Analyses of cell movements suggest the mechanisms that sculpt the pattern are more complex than would be expected from a purely directed diffusion model and may include cell-cell and cell-environment interactions in the form of chemotaxis, contact inhibition, contact guidance and haptotaxis. From cell labeling studies in a variety of animal model systems, it was learned that cranial neural crest cells emerge and emigrate from all rhombomeres, rather than preferentially exiting from only the even-numbered ones (Sechrist et al., 1993; Schilling and Kimmel, 1994; Birgbauer et al., 1995; Trainor et al., 2002). Regions lateral to r3 and r5 inhibit neural crest cell movements; cells stop and collapse filopodia or dramatically change direction into a neighboring stream (Kulesa and Fraser, 1998). Avian grafting experiments suggest the microenvironment adjacent to the neural tube may be important for maintaining the proper segregation of the neural crest cell streams (Farlie et al., 1999). When even-numbered quail rhombomeres are grafted lateral to and adjacent to chick r3, cells from the transplant diverge toward the even-numbered streams rather than migrate further laterally. There are clues that the repulsion is caused by a secreted factor at the neural tube midline. When either the r3 chick neuroepithelium or r5 surface ectoderm is removed, neural crest cells invade the area immediately adjacent to r3 and r5, respectively (Golding et al., 2002; Golding et al., 2004). Thus, in a self-organizing model, the neural crest cell pattern emerges from multiple factors and regional differences.

The following study was guided by our interest in learning more about the nature of neural crest cell pathfinding. Here, we take advantage of the accessibility of chick embryos to perform direct observations. The proximity of the migratory

routes (just underneath the surface ectoderm and relatively short time period over which neural crest cells migrate to the branchial arches) permit us to use a whole embryo explant culture method and perform hi-resolution static and confocal time-lapse imaging of individual cell migratory behaviors. We examined in detail to what extent neural crest cells interact with each other and the environment and whether this may influence cell directionality. Using a set of fusion protein constructs targeted to the cell membrane and nucleus, we were able to distinguish the lamellipodia and filopodia of individual neural crest cells. The time-lapse movies capture the dynamics of the cell-cell interactions and outline a chronology of the downstream movements of individual neural crest cells within the migratory streams exiting from r4 and r6. Some of the cell-cell interactions demonstrate obvious opportunities for cell-cell communication and directional guidance. Our data reveal an exciting level of detail to in vivo neural crest cell pathfinding and suggest that local and long-range cell-cell interactions play an important role in cell guidance.

Materials and methods

Embryos

Fertile white leghorn chick eggs were acquired from a local supplier (Ozark Hatchery) and incubated at 38°C until approximately the 5-8 somite stage (ss) of development. Eggs were rinsed with 70% alcohol and 3 ml of albumin was removed before cutting a window in the shell. A solution of 10% India ink (Pelikan Fount; PLK 51822A143) in Howard Ringer's solution was injected below the blastodisc to visualize the embryos. Embryos were staged according to the criteria of Hamburger and Hamilton (Hamburger and Hamilton, 1951), denoted as stage 10, for example; in other cases, the embryos were staged by their number of somites, denoted as '10 ss'.

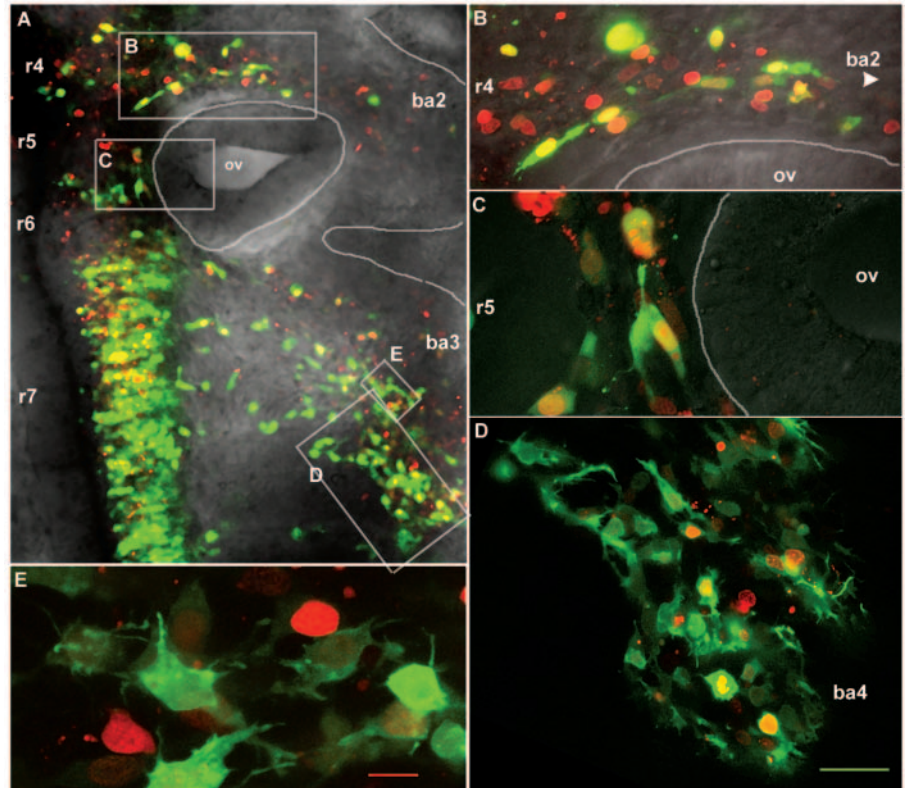
Cell labeling

Embryos at 5-8 ss were injected with fusion protein constructs [GAP-43 EGFP and H2B-MRFP (Okada et al., 1999) (gifts from Dr Rusty Lansford/Caltech)] to label premigratory cranial neural crest. After windowing the eggshell, a hole was cut in the vitelline membrane above the neural tube at the cranial end using a fine tungsten needle. In embryos labeled for fluorescence imaging, constructs were injected into the lumen of the neural tube, filling the hindbrain region, using a Picospritzer III (Parker Hannfin Corporation). A small amount (10 mg/ml) of Fast Green FCF (Fisher 42053) was added to the construct for easier visualization during injection. The eggs were then electroporated using platinum electrodes and an Electro Square Porator ECM 830 (BTX, a division of Genetronics) with 20 V, 55 milliseconds pulse length, 5 pulses, at 1 second intervals. Injected eggs were resealed with adhesive tape and re-incubated at 38°C. For static imaging, embryos were evaluated after 12-15 hours. Embryos for time-lapse imaging were evaluated after 6 hours. A fluorescence dissecting microscope (Leica MZFL III) was used to evaluate each embryo for health, uniformity of labeling and brightness.

Static imaging

For static imaging, individual embryos were removed from the egg with paper rings (Whatman #1), cleansed in Ringer's solution and placed dorsal side up within a thin ring of high vacuum grease (Dow Corning 79810-99) on 22×75 mm microslides (VWR 48312-024). The embryo did not come in contact with the vacuum grease. A small amount of Ringer's solution was pipetted away from the embryo and the embryo positioning was adjusted with forceps. A 22×22 mm glass coverslip (VWR 48312-024) was placed on top of the ring of grease, creating a sealed, humidified chamber. The embryos were then imaged

Fig. 1. Typical confocal sections through a chick embryo show the dense streams of cranial neural crest cells and various features of cell shapes and extensions within the migratory streams. (A) This typical embryo was co-injected with Gap43-tagged EGFP (green) and MRFP (red) to label the cell plasma membranes and nuclei, respectively, and re-incubated for 15 hours. Neural crest cells are seen along the migratory routes in dense streams emanating from r4 and r6, and individual cells leaving from r7. Many of the cells have nearly reached the destination branchial arches (ba2-ba4). The individual boxes highlight regions that are magnified in the remainder of the figure. (B) Cells within the r4 stream are relatively densely packed. Some of the individual Gap43-labeled cells reveal filopodial extensions, which overlap with neighboring MRFP-labeled cells. The Gap43-labeled cells also show the difference in potential extent to which an individual cell has contacts with other neural crest cells in the stream. (C) Interestingly, some neural crest cells from r5 travel laterally to the edge of r5 and then move in the anteroposterior direction toward a neighboring migratory stream along the edge of the otic vesicle. (D) Neural crest cells that have nearly reached the branchial arch destinations are very closely packed within the migratory stream. The protruding extensions of the cells appear to stretch out from the cell body such that an individual cell may contact a cell that is not within its local neighborhood. (E) A closer look at individual cells within the stream reveals that an individual cell may extend many thin filopodial protrusions. Scale bars: 50 μ m in D; 10 μ m in E. ov, otic vesicle.



using one of three microscopes (Zeiss Axiovert 200M, Zeiss LSM 510 META, and Zeiss LSM 5 PASCAL) and a wide range of objectives.

Time-lapse confocal imaging

Whole-embryo culture preparation

Whole-embryo explant cultures were prepared according to the method described in Krull and Kulesa (Krull and Kulesa, 1998). Briefly, embryos were removed from the egg by placing an oval ring of filter paper (Whatman #1) around the circumference of the embryo and then cutting around the outer edges of the ring. The ring with the embryo attached was placed in Ringer's solution for rinsing. The paper rings are approximately 1.5 cm along the major axis with holes wide enough to provide ample space between the inner side of the rings and the embryos. This method leaves the entire embryo, as well as the surrounding blastoderm, intact. The excised embryos were cleansed of yolk platelets and India ink by gently pipetting Ringer's solution across them with a P-200 pipetman.

Short-term, hi-resolution time-lapse imaging

For short-term (<5 hours), hi-resolution (>20 \times), in vivo time-lapse imaging, embryos were mounted directly dorsal side down into glass bottom microwell dishes (MatTek corporation, P35G-0-14-C). An embryo's blastoderm was spread out using fine forceps. Excess Ringer's was removed to stabilize the embryo while maintaining its 3D morphology. Once positioned, a Teflon membrane ring was sealed on top of the embryo with a bead of silicone grease. The membrane is designed to keep the embryo moist while allowing gas exchange. The membrane ring was made from a Teflon membrane (YSI incorporated, 97L0038) stretched over a plastic ring (OD=1 inch, ID=7/8 inch) and sealed in place using beeswax.

To maintain embryos at temperature, a single dish was placed on a

heating plate (Lyon Electric, TX7115-020) kept at 38°C using a temperature controller (Cell Temp Bionomic Controller, BC-100). A heat sink compound (#276-1372A-RadioShack) was spread on the heating plate prior to the microwell dish placement to help heat convection to the plastic dish and its contents. Imaging was performed on an LSM 510 META (Carl Zeiss) or an Axiovert 200M inverted fluorescence compound microscope (Carl Zeiss) using a 40 \times (NA=0.75) plan-neofluar or 40 \times C-Apochromat W objective (Carl Zeiss). The GFP proteins were excited with the 488 nm laser line using the filter set (Chroma) intended for GFP. The RFP proteins were excited with the 543 nm laser line using the filter set (Chroma) intended for rhodamine. Images were recorded every 1-5 minutes and analyzed using either the AIM (Carl Zeiss) or Axiovision software (Carl Zeiss).

Long-term, low resolution time-lapse imaging

For long-term (>5 hours), low resolution (<20 \times), in vivo time-lapse imaging, cultures were set up using Millicell culture inserts (Millipore PICM 030-50) and six-well culture plates (Falcon 3046), similar to the protocol described in Krull et al. (Krull et al., 1995). The culture insert membranes were precoated with 200 μ l of fibronectin (Sigma F-2006, diluted 1:50 in phosphate buffer) with the excess pipetted away. The dorsal surface of the embryos was placed on the coated culture insert, leaving the ventral surface exposed to the atmosphere. Excess Ringer's solution was pipetted from the membrane surfaces at the rostral and caudal ends of the explants, such that the flow of solution straightened the rostrocaudal axis of the embryos. This naturally spread the explants without flattening the embryos and mimicked the tension of the blastoderm normally created by the stretching of the yolk sac. Each explant covered approximately two-thirds (~2.8 cm²) of the area of a culture insert. Each individual culture

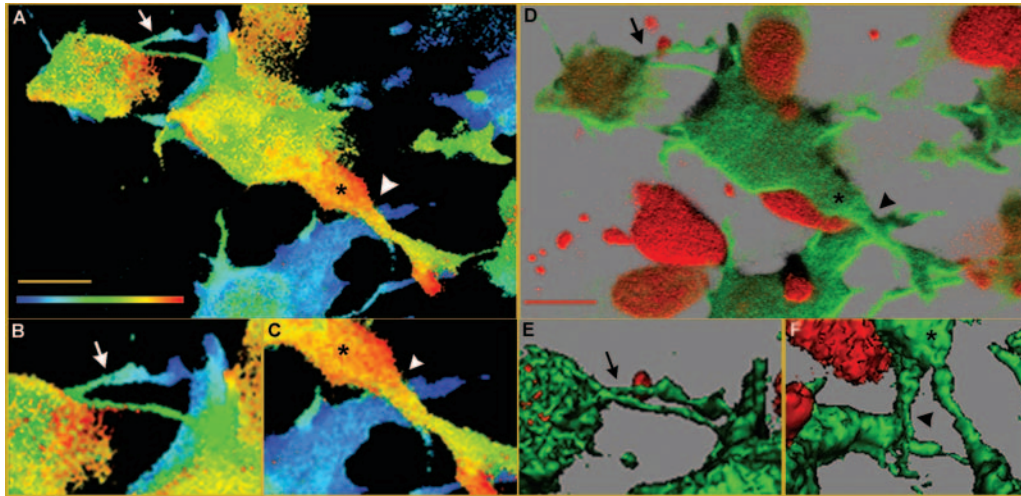


Fig. 2. Depth coding and 3D reconstruction of neighboring neural crest cells reveals contacts between neighboring cells. (A-C) Depth coding (in z -height) reconstruction of a confocal z -stack through neighboring neural crest cells in a live fluorescently-labeled (Gap43-EGFP and H2B MRFP) chick embryo emphasizes the filopodial connections between cells in a migrating r6 stream near the third branchial arch (ba3). The z -stack represents the neighboring cells shown in Fig. 1E. (A) A migrating cell (asterisk) has connections with two different migrating cells (arrow and arrowhead). The depth code shows the depth to the filopodia and the different parts of the cell, and z -height differences between each filopodium. The connections are very different; one cell is making a double connection with two filopodia (arrow; top left corner) while the other cell is stretching out a thicker filopodium, which is making a side connection with a neighboring cell (arrowhead; bottom right corner). (B) A magnified view reveals that the contacts are in the same z -plane (green-to-green and light blue-to-green) between the two cells. (C) A magnified view of the side connection (arrowhead) between the two neighboring cells shows that the two cells may not be in contact with one another, but that there may be as much as 14 μm in z -height difference. The color code ranges from 0 (blue) to 14 μm (red). (D-F) 3D rendering of the same confocal z -stack. (D) The main cell (asterisk) has multiple filopodial connections between two different cells while migrating. The first connection (arrow) shows two outstretched filopodia contacting a neighboring neural crest cell. The thick connection (arrowhead) shows a possible connection between the neighboring cells toward the bottom right corner. (E) A magnified view reveals the local contacts (arrow). (F) Surprisingly, the 3D reconstruction and rotation of the data stack shows there is a separate branch of the extending filopodium that is definitely in contact with the neighboring cell (arrowhead). This contact was not visible in either the flat projection or the depth coding of the z -stack. Scale bars: 10 μm .

insert was then placed in separate wells of a six-well plate. The membranes were underlain with a Neurobasal medium (Gibco 21103-031), supplemented with B27 (Gibco 17504-036) and 0.5 mmol/l L-glutamine (Sigma G-3126). Sterile water was added to the any unfilled wells to minimize dehydration during time-lapse acquisition. The edges of the six-well plate were then sealed with parafilm.

Fluorescently-labeled explants were visualized using an inverted confocal microscope (LSM 510 META or LSM 5 Pascal; Carl Zeiss Inc.) using a 10 \times Neofluar (NA=0.30) lens with a zoom=2. This allowed observation of the entire r6 and r7 streams. For better image resolution, plastic was removed from the optical path by making holes in the bottom of the wells into which round 22 mm glass coverslips (VWR 48380-080) were sealed using silicone grease (Dow Corning 79810-99). The membrane of the Millipore culture insert becomes transparent when moist.

The microscope is surrounded by an incubating box fashioned from cardboard (4 mm thick) and covered with thermal insulation (Reflectix Co.; 5/16 inch thick). An enclosed heater (Lyon Electric Co. 115-20) maintained the cultures at 38°C for the duration of the time-lapse acquisition, with only mild temperature fluctuations. Images were recorded every 5 minutes and analyzed using the AIM software (Carl Zeiss).

Data analysis

Several features from the AIM software were used, including 2D and 3D visualization, projection and depth coding (Carl Zeiss). The depth code feature provided the ability to recognize which focal planes the cells were in throughout a z -stack by assigning a color code to the pixel intensity as a function of the z -depth. This was important for determining whether two cells were in contact with one another.

Stacks of images were manipulated for analysis using VisArt (Carl Zeiss), Volocity (Improvision) and Image J (NIH).

Results

To determine the extent to which neural crest cells interact with each other and the environment during migration to the branchial arches, we investigated intact cranial neural crest cells within living chick embryos. Previously, fine cellular processes, such as filopodia (~10-20 μm in length) and lamellipodia of neural crest cells, were detected by scanning electron microscopy (Tosney, 1978) and in organ culture (Bard and Hay, 1975). Using a set of fusion-protein-expressing constructs targeted to the cell membrane and nucleus, we electroporated the constructs into young chick embryos to label premigratory cranial neural crest cells. Compared with the conventional methods of vital dye labeling, DiI or non-targeted cytoplasmic GFPs, constructs targeted to specific cytoskeletal elements permit surprising neural crest cell features to be visualized in vivo, including very long and spiny filopodial extensions and thin cellular processes. Through a series of detailed static images, we describe the features of intact neural crest cells in terms of cell shape and number and length of filopodia and lamellipodia. We show how these aspects vary depending on the position of a cell within a migratory stream. We analyze the entire extent of the cranial neural crest cell migratory routes from the dorsal neural tube to the branchial

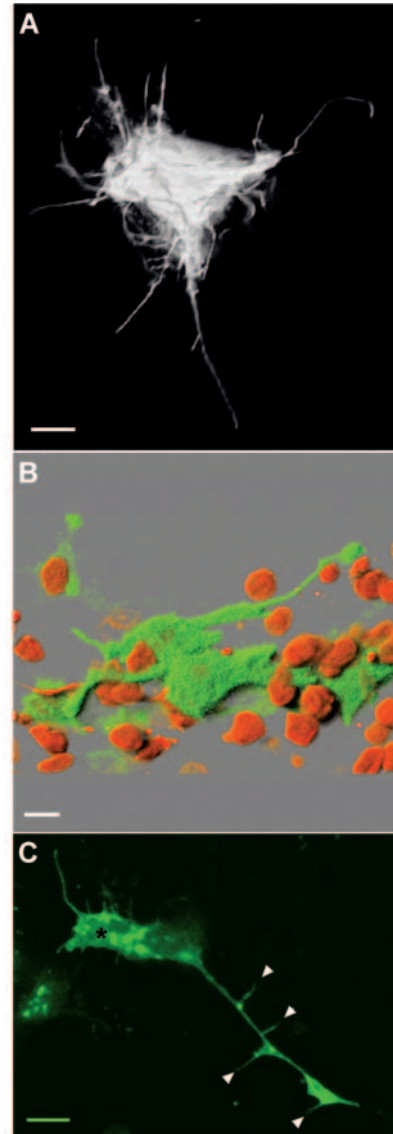
Fig. 3. Neural crest cells display a wide variety of cell shape and number and length of filopodial and lamellipodial extensions. Static confocal images of typical neural crest cells within migratory streams show different features, including numerous long and short filopodia extending from all areas around the neural crest cell and shorter lamellipodial protrusions of various shapes extending from the cell body. (A) A typical hairy cell displays many long and short filopodia. The extensions course off in many different directions and cover a wide region in the environment near the cell body. The length of a filopodium may extend up to 100 μm . The embryo was injected with Gap43-EGFP, electroporated and re-incubated for 12 hours. (B) A typical bipolar cell within a neural crest cell stream (r4 stream). The cell displays a long, forward extending filopodium that intertwines around local and non-local neighboring neural crest cells. The trailing filopodium may extend as long as the forward protrusion. The embryo was co-injected with Gap43-EGFP (green) and H2B-MRFP (red) and electroporated to label the cell plasma membranes and nuclei, respectively, and re-incubated for 12 hours. (C) The neural crest cell displays an outstretched filopodium in the direction of the destination branchial arch along the flow of the migratory stream (r6 stream). The filopodium on this cell has many substructures along its length that resemble spines. These shorter protrusions are directed perpendicularly from the main filopodial branch. The embryo was injected with Gap43-EGFP, electroporated and re-incubated for 12 hours. Scale bars: 10 μm .

arches, focusing mainly on streams emanating from the mid- and caudal-hindbrain. We demonstrate the dynamics of the lamellipodia and filopodia and the extent to which neural crest cells contact one another and the environment during pathfinding in vivo from time-lapse confocal recordings.

Neural crest cells within dense streams make numerous contacts with neighboring cells

Neural crest cells that traveled within the dense r4 and r6 streams maintained numerous contacts with neighboring cells (Fig. 1). The r4 stream was composed of cells originating from r3-r5 and extended laterally to r4 around the anterior portion of the otic vesicle (Fig. 1A). The r4 stream was densely packed with neural crest cells, as evidenced by the number of nuclear stained cells (Fig. 1B). Many of the cells within the r4 stream had a bipolar shape with two processes extending in opposite directions, one directed toward the branchial arch destination (Fig. 1B). Cells closer to the front of the r4 stream had many more filopodia extended in a variety of directions (described in a separate section below). Cells from the r5 region are known to migrate and join the r4 and r6 streams. Near the mid-r5 region, cells migrated laterally and then turned in either the anterior or posterior direction and moved in a perpendicular fashion to the r4 or r6 streams until making contact with cells in those streams (Fig. 1C).

In a similar manner, the r6 migratory stream was as dense as the r4 stream with as many neural crest cells in close contact with each other (Fig. 1A,D). By contrast to the bipolar shape of the cells within the stream, cells closer to the migratory fronts had a more polygonal shape (Fig. 1D; distal part of the stream). Individual cells tended to be in contact with at least one other cell and often were in contact with several neighbors (Fig. 1D). In higher resolution, neural crest cells indeed had several processes extended in many different directions, such that one cell simultaneously contacted several neighboring neural crest cells (Fig. 1E).



An in-depth look at individual cells within migratory streams revealed the extent to which an individual cell had several contacts with neighboring cells (Fig. 1E, Fig. 2; see Movie 1 in the supplementary material). Color coding cellular features based on z -depth helped to confirm whether filopodial extensions between cells were in the same z -plane (i.e. in contact) with neighboring neural crest cells (Fig. 2). For example, two neighboring neural crest cells were in contact (Fig. 2A,B; arrow), but other filopodia may have actually been separated in z -height (Fig. 2A,C; arrowhead). This contact could not be determined precisely from a projected image (Fig. 1E). The ability to rotate and render a 3D z -stack of confocal images around different axes of rotation (Fig. 2D-F) revealed that there may be other filopodial processes emanating from cells that were not visible in 2D projections. For example, an individual neural crest cell may have been in contact with at least two neighboring fluorescently-labeled cells (Fig. 2D). The first contact is clearly visible (Fig. 2D-E, arrow). The second point of contact is via a branch of the filopodium (Fig. 2F, arrowhead) that is not visible in either the

projected image (Fig. 1E) or the depth coding (Fig. 2C, arrowhead).

There are differences in cell shape and the number and length of filopodia, depending on the position of the neural crest cell in the migratory stream

Filopodia may be distributed around the entire circumference of the cell body

By contrast to the single or bipolar neural crest cells, numerous neural crest cells displayed filopodia around the entire circumference of the cell body (Fig. 3A; see Movie 2 in the supplementary material). The filopodia did not appear to be distributed or aligned in any specific direction and consisted of both long and short lengths, ranging from approximately 20 μm to 100 μm (Fig. 3A). Cells with filopodia distributed around their entire circumference were usually located at or near the fronts of dense migratory streams (r4 or r6 streams) or adjacent to the neural tube in sparsely populated regions (near r1 or r7). Near the stream fronts, no labelled neural crest cells were usually observed distal to the hairy cells.

Bipolar neural crest cells have forward extending and trailing filopodia

Many neural crest cells had a smaller number of filopodia that extended from the cell body in two different directions (Fig. 3B). These extended processes were usually aligned in the direction along the cell's trajectory, toward the branchial arches (at the front of the cell), or in the reverse direction toward the neural tube (at the back of the cell). Within a typical migratory stream, the filopodia may extend and weave around neighboring neural crest cells to contact non-local neighbors (Fig. 3B; see Movie 3 in the supplementary material). The filopodium itself may have a distinct shape (Fig. 3C). The process may be thick near the cell body ($\sim 5 \mu\text{m}$ in diameter) while tapering away from the cell to approximately 1 μm in diameter. Forward extending filopodia varied in length, typically 50–60 μm , but could extend up to 100 μm in length. Along the length of the filopodium were multiple short processes (5–10 μm in length) that extended in orthogonal directions from the filopodium (Fig. 3C; arrowheads). These shorter protrusions were distributed at random points along a filopodium, maintaining spacing with each other (Fig. 3C) and extended at different angles to the filopodium (Fig. 3C). Some filopodia had a wider fan-shape along the length or at the end of the filopodium (Fig. 3C, bottom right corner).

Bipolar neural crest cells are found near the middle of migratory streams, hairy cells at the front

To determine whether there are differences in the proximal (back of the stream) to distal (front of the stream) distribution of hairy versus bipolar cells, we analyzed cell shapes in typical neural crest cell streams ($n=8$). We then plotted the percent of hairy versus bipolar cells at the front, middle and back of typical neural crest cell streams as a percentage of the total number of cells in the stream (Fig. 4A). We found that a larger percentage of hairy cells tend to be at the stream front (distal) (Fig. 4A). In a typical stream, there were approximately three times as many hairy cells near the front compared with bipolar cells. At the back portion of a stream, the percentage of hairy

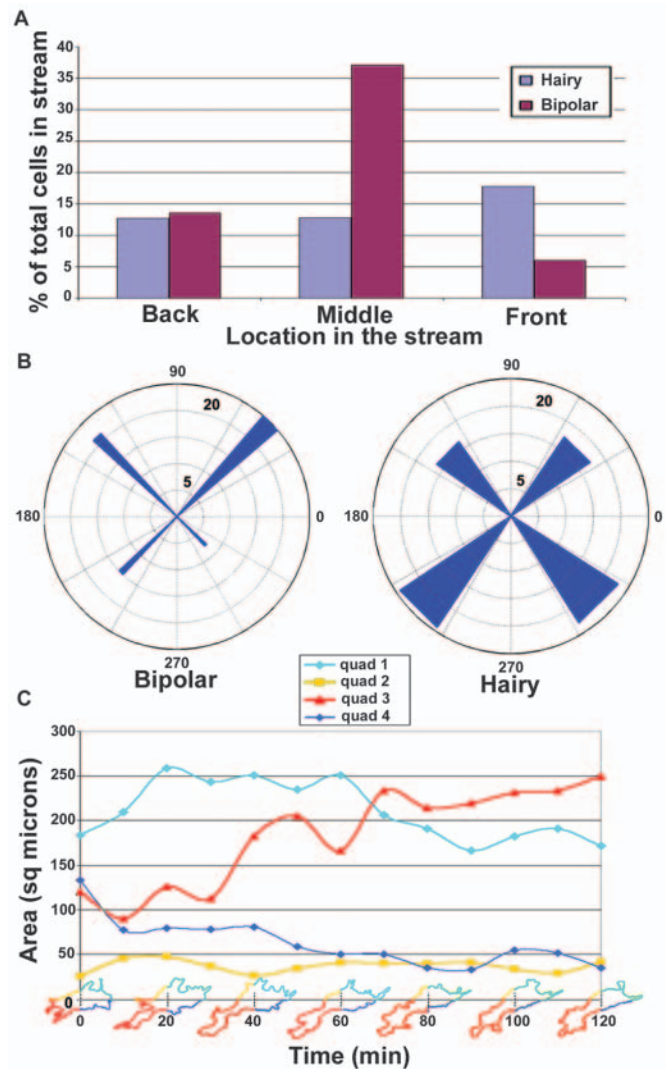


Fig. 4. Quantitative analysis. (A) Quantitative analysis of typical neural crest migratory streams ($n=8$) shows that different cell shapes (bipolar versus hairy) are distributed in different positions along the stream. The graph plots the percent of hairy versus bipolar cells at the front (distal), middle and back (proximal) of typical neural crest cell streams as a percentage of the total number of cells in the stream. The majority of bipolar cells are located in the middle of the migratory stream, while there are almost three times as many hairy cells versus bipolar cells at the front of the stream. At the back portion of a stream, the percentage of hairy and bipolar cells are nearly equal. (B) Further quantitative analysis of the directional distribution and length of the filopodia on hairy versus bipolar cells is shown in the second set of graphs. A sample of hairy and bipolar cells ($n=16$) within typical migratory streams were selected and the filopodia lengths and spatial distributions were measured. Bipolar cells have the feature of having few long filopodia that typically extend into the first two quadrants, forward (toward the branchial arch) and trailing (toward the neural tube). In comparison, hairy cells have a large number of filopodia extending from nearly all aspects of the cell's circumference, seen in thick lines in all four quadrants. The filopodia are slightly longer in the directions toward the branchial arches and the neural tube. (C) A typical migrating neural crest cell was analyzed for the dynamic cell shape changes. The quantitative analysis of the area of a trailing cell, plotted as a function of time, reveals that the area of the cell does not increase monotonically in the direction of motion, but oscillates slightly.

and bipolar cells was nearly equal. By contrast, a much larger percentage of bipolar cells was found in the midstream region; there were three times as many bipolar cells as hairy cells in a typical migratory stream (Fig. 4A). Thus, a typical neural crest cell stream has the striking feature of hairy cells at the front and bipolar cells distributed throughout the middle of the stream.

To determine whether there is a bias to the directional distribution and length of the filopodia on hairy versus bipolar cells, we measured the average filopodial lengths and spatial distributions on a sample of hairy and bipolar cells ($n=16$) within typical migratory streams. Bipolar cells had the feature of having few long filopodia that typically extended into only two quadrants (Fig. 4B). The forward extending filopodium stretched in the direction of travel (toward the branchial arch destination) and the trailing filopodium extended parallel, but in the opposite direction (Fig. 4B). In comparison, hairy cells had a large number of filopodia extending from nearly all aspects of the cell's circumference (Fig. 4B). The filopodia were longer in the directions toward the branchial arches and the neural tube.

Time-lapse analysis reveals that filopodia play a role in cell directionality

Contact with a lead cell often results in directional guidance by the follower cell

Long, extended filopodial processes at the leading edge of cells appeared to act as active cell-cell contacts that influenced the direction in which a cell subsequently moved. There were two typical neural crest cell migratory behaviors that occurred when a follower cell contacted a downstream cell. In one case, the filopodium made contact with the downstream cell, and then retracted before the trailing cell began to move in the direction of the downstream cell (Fig. 5). The sequence of events was as follows. First, a cell extended numerous filopodia in different directions throughout the local microenvironment [Fig. 5 ($t=0$); red cell]. Second, an extended filopodium made contact with a neighboring cell (Fig. 5; $t=1$ hour), and elicited a response (Fig. 5; $t=1$ hour 5 minutes). The trailing cell then retracted its filopodium (Fig. 5; $t=1.5$ hours) and began to move in the direction of the contact with the lead cell (Fig. 5; $t=3$ hours).

By contrast to this, a neural crest cell filopodium may contact and track the position of a downstream cell (see Movie 4 in the supplementary material). In this sequence of events, a cell first extended a filopodium and contacted a downstream cell. The filopodium remained extended and followed the direction of the back of the downstream cell. At the back of the trailing cell, filopodia continued to be extended in the reverse direction toward the neural tube and to a small extent into other directions adjacent to the cell. As the lead cell continued to migrate further downstream, the filopodium grew as more of the body of the trailing cell followed. Interestingly, quantitative analysis of the area of the trailing cell, plotted as a function of time, revealed that the area of the follower cell did not increase monotonically in the direction of motion, but oscillated slightly (Fig. 4C). The oscillation of cell area versus time appears to result from filopodia that continued to extend in the reverse direction (back toward the neural tube) as the cell body moved in the forward direction (see Movie 4 in the supplementary material).

Long, thin cellular processes retain a contact between individual cells

As described previously, neural crest cells can be connected by thin, long filopodia that are only 1–3 μm in diameter, but extend up to 100 μm in length (Fig. 6; see Movie 5 in the supplementary material). These long cellular processes typically did not lie in a single focal plane, but transcended

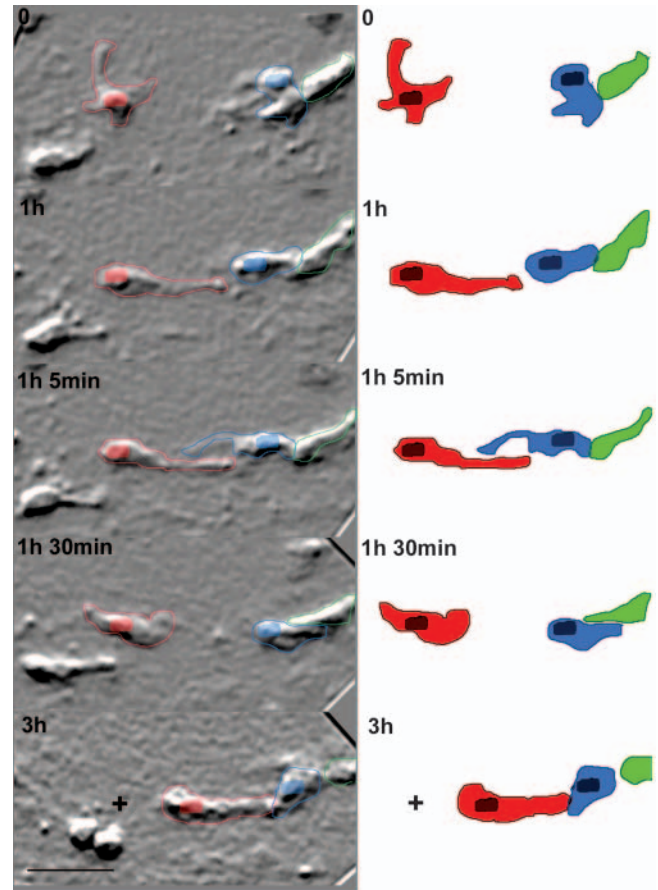


Fig. 5. Neural crest cell-cell contact via filopodia may alter the trajectory of a trailing cell. Selected images from a typical time-lapse imaging session show neural crest cells within the r6 stream. The direction of migration is to the right. Three individual neural crest cells are highlighted (green, red and blue), with the blue cell and the green cell positioned downstream (distal) to the red cell. First, the trailing cell (red) senses the local environment with short filopodial extensions ($t=0$). The downstream cell ahead in the r6 stream (blue) is migrating toward the branchial arch. The red cell extends a filopodium in the direction of the blue cell ($t=1$ hour). The blue-colored cell moves slightly backward. Then, the filopodium from the red cell makes contact with the blue cell, and elicits a response from the blue cell in the form of a short filopodial extension in the reverse direction of travel ($t=1$ hour 5 minutes). Both the red and blue cells retract their filopodia ($t=1$ hour 30 minutes). The red cell then begins to migrate in the direction of the point of contact with the blue cell ($t=3$ hours) and migrates further downstream to catch up with the blue cell. The images in the left column have been embossed from the raw data in Adobe Photoshop 7.0 to bring out the edges of the cells. The images in the right-hand column are tracings over the cells from the raw data. The + sign ($t=3$ hours) marks the original location of the red cell before contacting and migrating toward the blue cell. Scale bar: 30 μm .

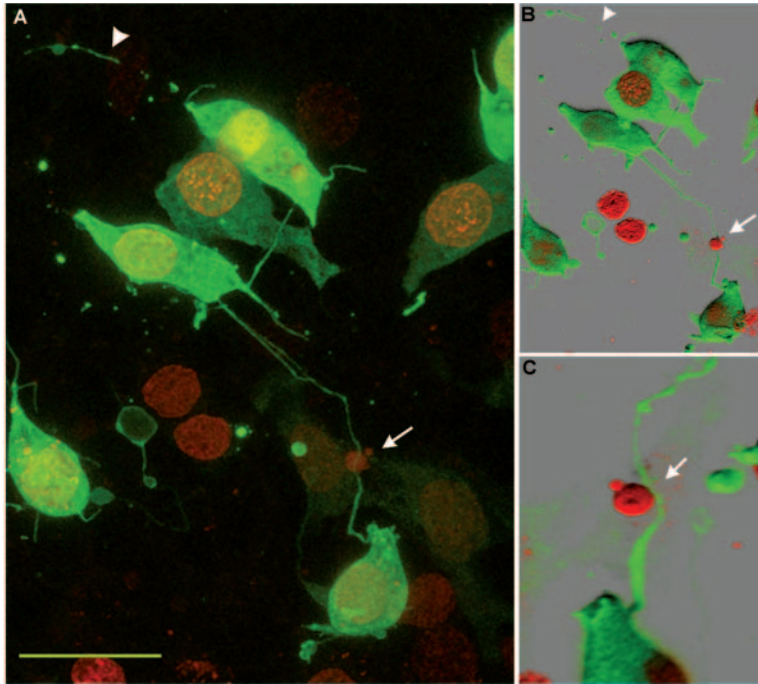


Fig. 6. Neural crest cells within a stream may be connected by long, thin cellular processes. In a typical cell migration stream, many neural crest cells appear to be connected by long, thin cellular processes. (A) Two neural crest cells appear to be linked by a long, thin cellular process (arrow) that stretches between two cells and intertwines around a cell. A fragmented cellular process lies adjacent to one of the cells (arrowhead). This image represents the projection of 30 confocal slices at 1 μm intervals. (B) A 3D rendering of the confocal z-stack reveals the long, thin cellular link (arrow) and short-range contacts. The fragments of a broken cellular process are also shown (arrowhead). (C) A close-up of the 3D rendering of the z-stack and rotation reveals the actual physical connection of the cellular process between the two cells (arrow). The z-stack has been turned by approximately 30 degrees around the y-axis. The embryo was injected with Gap43-EGFP (green) and H2B-MRFP (red) into the neural tube, electroporated and re-incubated for 12 hours. Scale bar: 30 μm .

large x , y and z distances, such that two cells within a migratory stream, although located far apart, may have been connected (Fig. 6B,C). Intriguingly, the long filopodial connections wound in between neighboring neural crest cells (Fig. 6B,C; arrow). An individual cell may have maintained several concurrent connections between neighboring cells, with connections of various lengths (Fig. 6).

The long, thin filopodial connections were visually distinguishable when two neighboring cells moved apart from one another or when one cell divided and one of the progeny moved away. The sequence of how this occurred is demonstrated in Fig. 7. Time-lapse analysis shows a typical pair of neighboring cells in contact (Fig. 7). As the lead cell moved away, a thin process between the cells became visible (Fig. 7). As the distance increased, the process lengthened until it broke at an arbitrary point along the process (Fig. 7). Remnants of a thin filopodium were visible along the cell's route [Fig. 6A,B (arrowhead); Fig. 7]. This behavior was most often observed as cells were just exiting the neural tube or between two progeny of a recently divided cell.

Migrating cells often change shape and are not always bi-directional

As mentioned before, neural crest cells can have a varied number of filopodia that extend and retract in multiple directions. A single cell can undergo numerous phenotypic changes as it migrates. For example, a cell may start out with a bi-directional shape, extend filopodia in numerous directions and then regain a bi-directional shape while moving forward. These cells typically had few (<5) lengthy (50–60 μm) filopodial extensions, some of which remained extended while the cell migrated; the filopodia responded to the cell's forward movements. Interestingly, although the lamellipodial extensions may be numerous and spread through the local environment, these processes appeared to be confined mostly

to the xy -plane of migration, rather than completely around the cell body. In this way, neural crest cells appeared fairly flat in the z -plane (<30 μm) in comparison to the tremendous spatial spread in the z -plane (up to 100 μm in certain directions) of the filopodia.

Discussion

Cranial neural crest cells undergo an extensive migration in three stereotypical streams to pattern peripheral structures of the face and neck. Over the past 30 years, research on neural crest cell migration has focused on elucidating the molecular mechanisms underlying the directional migration cues. Culture and imaging techniques have allowed neural crest cell migratory behaviors to be visualized in cell and tissue culture, 3D matrices, and in intact embryos. However, the lack of resolving fine cellular structures, such as lamellipodia and filopodia, on individual migrating neural crest cells in a living embryo has limited the ability to test the relative roles of potential guidance cues. The approach used here provides insight into the structure and dynamics of neural crest cell interactions in live chick embryos. We found that cranial neural crest cells interact extensively with each other and the local environment through remarkably dynamic short- and long-range lamellipodia and filopodia. The number and directionality of the cell's extensions change dynamically; however, at specific locations within a migratory stream, cells share similar shape features. Long filopodial protrusions can extend up to 100 μm in length and wind in between neighboring cells to contact more distant, downstream neural crest cells. Some of the filopodial connections persist between neighboring cells that move apart. Time-lapse confocal imaging data reveal the spatiotemporal dynamics of the neural crest cell interactions with each other and the local environment.

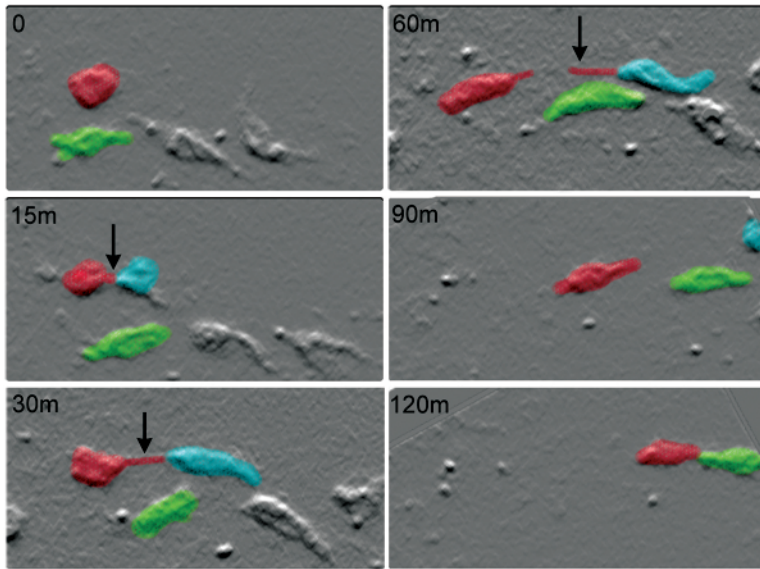


Fig. 7. The neural crest cell-cell long-range contact develops as two neighboring cells move apart. Selected images taken from a typical time-lapse confocal imaging session showing the interaction between two neighboring neural crest cells that move apart and continue to maintain contact. Initially ($t=0$), the red cell is migrating within the stream, moving laterally from r6 (not shown, but to the left in the figure). The red cell undergoes cell division to produce a progeny (blue cell) ($t=15$ minutes). As the blue cell moves away ($t=15$ minutes), a thin cellular process is maintained (arrow). As the blue cell continues to move laterally ($t=30$ minutes), the length of the cellular process increases (arrow). Also, the green neighboring cell continues to migrate in the lateral direction. The contact between the red and blue cells lengthens until it breaks at an arbitrary point ($t=60$ minutes), leaving remnants (arrow). The red cell begins to move toward the blue cell. The blue cell continues to move in the lateral direction and is nearly out of the field of view ($t=90$ minutes). The green cell migrates near to the location of the previous position of the blue cell and ($t=120$ minutes) the red cell makes contact with the green cell. The diameter of the red cell at ($t=0$) is about $10\ \mu\text{m}$.

The observations of lamellipodial and filopodial dynamics between migrating neural crest cells suggest a role for the cell-cell contacts in directional guidance. Short, wide lamellipodial and short, thin filopodial contacts were often exchanged between neighboring neural crest cells. A typical filopodial extension from a trailing neural crest cell contacted, and tracked the back end of a downstream cell (see Movie 4 in the supplementary material), or retracted after contact and then re-extended, with the cell body migrating toward the position of the contact (Fig. 5). These extensions appeared to provide a

feedback to inform the cell body what is in the local environment. The cell may then integrate the information and alter its trajectory. We did not see any evidence of a trailing cell nudging a lead cell forward, as judged by the lack of blebbing at the front of the lead cell. Evidence of nudging has been reported in deep cells of the fish *Fundulus* (Tickle and Trinkaus, 1976). Our data support the growing evidence for the role of filopodia in directional guidance in other systems. During *Drosophila* neuromuscular synapse formation, embryonic muscles at the target site extend dynamic actin-

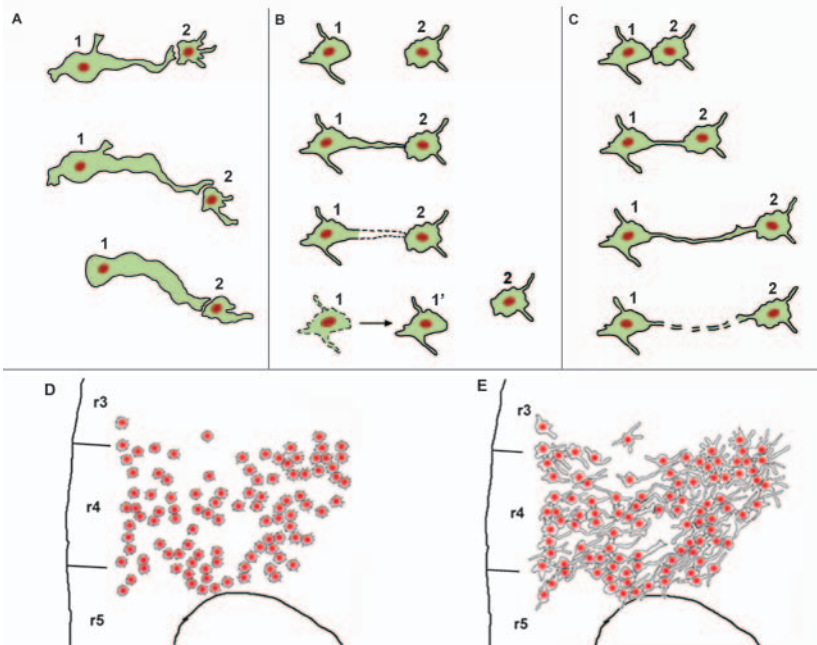


Fig. 8. Schematic of the neural crest cell streams and different forms of contact between two neural crest cells, which lead to directional movement. (A) Tracking a lead cell. Two neural crest cells (1 and 2) within a migratory stream may have short filopodia around the circumference of the cell. The contact is initiated when one of the cells (1) extends a filopodium in the direction of the other cell (2) and makes contact. The filopodium from the trailing cell then tracks the position (back end) of the downstream cell as the rest of the trailing cell body follows. (B) Contact, retraction, forward movement. Two migrating neural crest cells within a stream may come into contact with each other when a filopodium of the trailing cell (1) extends and contacts a downstream cell (2). The filopodium retracts and then the trailing cell moves forward to a new position (1') near the location of the contact as the downstream cell (2) moves away. (C) Tethered contact. Two neighboring cells (1 and 2) may begin to move apart from each other. As the cells move apart, or, as shown here, one of the cells (2) moves away, the cells maintain a filopodial connection. As

the length of the filopodium grows, it breaks at an arbitrary point, leaving fragments in the extracellular matrix. The trailing cell (1) may stay in its location or move in the direction of the former neighboring cell (2). (D) Our previous view of neural crest cells within migratory streams (for example, the stream forming adjacent to r4) based on DiI cell labeling revealing round neural crest cells with short filopodia. (E) Our working model of neural crest cells within migratory streams based on imaging of cells with fusion protein expressing constructs targeted to the plasma membrane and cytoskeletal elements to reveal multiple cell-cell connections with short and long filopodia.

based filopodia (myopodia) toward invading motoneurons (Ritzenthaler and Chiba, 2003). The myopodial contacts are thought to lure the motoneurons to the proper synaptic targets. Also in *Drosophila*, during dorsal closure the lead cells of an epithelial sheet send out numerous filopodia that contact cells on the opposite side. When the assembly of protrusions is inhibited, the adhesion and fusion of the opposing epithelial partners is prevented, leading to segment misalignments (Jacinto et al., 2000). Thus, our data indicate that local cell-cell contacts influence the migration of neural crest cell direction. It will be interesting to test to what extent the neural crest cells can pathfind when the protrusive activity of the filopodia is modulated.

The long, thin filopodia that stretch between two neighboring neural crest cells as the cells move apart suggests a role for filopodia in long-range cell communication. We presented evidence that some neighboring cells maintain a contact as one of the cells moves away (Figs 6, 7; see Movies 5, 6 in the supplementary material). The process between the cells lengthens until it breaks. In some cases, the trailing cell migrates toward the direction of the fragmented contact, although we did not determine whether the cell precisely follows the trail of the broken contact. Neural crest cells have been shown to make numerous short (10–20 μm) filopodial contacts with neighboring neural crest cells during migration in the chick cornea (Bard and Hay, 1975) and in the mouse gut (Young et al., 2004). In this case, the cell contacts are thought to play a role in mediating the collective migration of the cells in chains. Our evidence of cell-cell contact in neural crest cell streams includes much longer connections (up to 100 μm) and is similar to the growing evidence in other embryonic systems that non-neighboring cells form long-range contacts (Rorth, 2003; Cohen, 2003). The discovery and analysis of long cellular extensions (cytonemes) in the *Drosophila* imaginal disc revealed that cytonemes project from distant cells toward the signaling centers of the disc (Ramirez-Weber and Kornberg, 1999). The cytonemes are thought to transport, deposit or retrieve signaling molecules and play a role in pattern formation. This idea of long-range cell communication was strengthened with recent work reporting that during *Drosophila* sense organ development, long filopodia on a single precursor cell convey signals at a distance to non-local neighbors to pattern the field (De Jossineau et al., 2003). Previously, it had been thought that the signal to pattern the sense organ was conveyed through the long-distance secretion of a signaling protein. Thus, it will be interesting to investigate the intracellular dynamics within the neural crest lamellipodia and filopodia during cell-cell contact for the possible role in cell communication.

Our studies have shown that there is extensive detail in neural crest cell pathfinding in the form of short- and long-range cell-cell contacts in vivo, pointing to a diverse set of directional guidance mechanisms for neural crest cells (Fig. 8). The number and length of the contacts, in the form of lamellipodia and filopodia, vary depending on the cell's position within a migratory stream. Time-lapse confocal imaging reveals that the long-range cell-cell contacts, mediated by filopodial extensions, play a role in directional guidance by allowing trailing cells to follow downstream leaders. Short-range contacts between neighboring migrating neural crest cells appear to inform the cells of the position

and number of neighboring cells. Long, thin filopodial connections allow two neighboring cells to remain in contact as the cells move apart. Our results support the hypothesis that a combination of intrinsic and extrinsic cues sculpts the cranial neural crest cell migration pattern, but that lamellipodia and filopodia play a critical role in neural crest cell pathfinding in the local microenvironment. Our evidence of neural crest cell short- and long-range cell communication parallels with data in *Drosophila*, mediated through myopodia and cytonemes, and opens several exciting lines of investigation. The neural crest cell-cell contacts may involve signaling to communicate positional information or allow cells of a similar fate to maintain a relationship. By contrast to our previous view of neural crest cell streams consisting of compactly shaped cells with lamellipodia and relatively short filopodia, our new perspective is that neural crest cell streams are very densely packed with lamellipodia and filopodia in constant cell contact, intertwined around local and non-local migrating cells. Further dissection of the function of the cell-cell and cell-microenvironment interactions will probably bring unexpected insights into how neural crest cells navigate.

The authors would like to kindly thank Rusty Lansford (Caltech) for his generosity in providing the fusion protein constructs. We kindly thank Paul Rupp and Paul Trainor (Stowers Institute) for their careful reading of the manuscript.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/131/24/6141/DC1>

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