Imaging neural crest cell dynamics during formation of dorsal root ganglia and sympathetic ganglia

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Accepted 29 October 2004

Development 132, 235-245
Published by The Company of Biologists 2005
doi:10.1242/dev.01553

Summary

The neural crest is a migratory population of cells that produces many diverse structures within the embryo. Trunk neural crest cells give rise to such structures as the dorsal root ganglia (DRG) and sympathetic ganglia (SG), which form in a metameric pattern along the anterior-posterior axis of the embryo. While static analyses have provided invaluable information concerning the development of these structures, time-lapse imaging of neural crest cells navigating through their normal environment could potentially reveal previously unidentified cellular and molecular interactions integral to DRG and SG development. In this study, we follow fluorescently labeled trunk neural crest cells using a novel sagittal explant and time-lapse confocal microscopy. We show that along their dorsoventral migratory route, trunk neural crest cells are highly motile and interact extensively with neighboring cells and the environment, with many cells migrating in chain-like formations. Surprisingly, the segregated pattern of crest cell streams through the rostral somite is not maintained once these cells arrive alongside the dorsal aorta. Instead, neural crest cells disperse along the ventral outer border of the somite, interacting extensively with each other and their environment via dynamic extension and retraction of filopodia. Discrete sympathetic ganglia arise as a consequence of intermixing and selective reorganization of neural crest cells at the target site. The diverse cell migratory behaviors and active reorganization at the target suggest that cell-cell and cell-environment interactions are coordinated with dynamic molecular processes.

Key words: Chick, Neural crest, DRG, SG, Cell migration, Time-lapse imaging

Introduction

The neural crest is a highly migratory population of cells that gives rise to many diverse cell derivatives within the body. Neural crest cells emerge from the dorsal region of the fusing neural tube, migrate along stereotypical pathways through the embryo and differentiate into the vast majority of the cell types and tissues that comprise the facial skeleton, aspects of the cardiovascular system, and the peripheral nervous system (PNS) (Weston, 1970; LeDouarin, 1982; Bronner-Fraser, 1986). Neural crest cells arise at all axial levels of the embryo, and emigrate in a temporal gradient anterior to posterior, with migration initiated in the cranial region. Trunk neural crest cells give rise to melanocytes, glia and neurons of the dorsal root ganglia (DRG) and autonomic ganglia, including sympathetic ganglia (SG) and parasympathetic ganglia (Weston, 1963; LeDouarin, 1982; Teillet et al., 1987; Lallier and Bronner-Fraser, 1988), which form in a metameric pattern along the anterior-posterior (AP) axis of the embryo. Although static studies have elegantly characterized the lineage of individual trunk neural crest cells (Fraser and Bronner-Fraser, 1991; Frank and Sanes, 1991; Zirlinger et al., 2002), time-lapse imaging of live, motile neural crest cells navigating through their normal environment could potentially identify unknown cellular and molecular interactions not apparent by analysis of static images (Lichtman and Fraser, 2002). However, because trunk neural crest cells migrate ventrally deep within the embryo, very little is known about the cellular mechanisms that mediate their migration, aggregation and differentiation as they form the metameric array of DRG and SG.

The routes navigated by migrating trunk neural crest cells have been well characterized: those neural crest cells that take a ventromedial route migrate through the somatic mesoderm, stop midway along and lateral to the neural tube, coalesce and give rise to the DRG. A subpopulation of trunk neural crest cells that continue ventrally past the formation site of DRG accumulate dorsolateral to the dorsal aorta and give rise to the SG. Neural crest cells that migrate dorsolaterally underneath the ectoderm become melanocytes (Tosney, 1978; Erickson et al., 1980; Theiry et al., 1982; Loring and Erickson, 1987). Neural crest cell migration through the somites is patterned: cells enter the rostral half but avoid the caudal half of the somite (Keynes and Stern, 1984; Rickman et al., 1985; Bronner-Fraser, 1986), and this stereotypy generates segregated streams of cells, which form the basis for the metameric pattern of the DRG, SG and spinal motor axons (Bronner-Fraser, 1986; Lallier and Bronner-Fraser, 1988; Oakley and Tosney, 1993). Experimental evidence implicates extrinsic environmental cues, localized in the somites, in

guiding trunk neural crest cells. When presumptive somites are rotated by 180°, neural crest cells and motor axons migrate through the (now) caudal somite (Bronner-Fraser and Stern, 1991). This segregation of neural crest cells to follow a specific migratory route or corridor suggests that the function of the operative molecular mechanisms is to generate segregated lateral structures, such as the DRG and SG.

Studies investigating the expression patterns of candidate molecules influencing the migratory patterns of neural crest cells in vitro (Erickson and Perris, 1993) and in vivo (Bronner-Fraser, 1986) have implicated neuregulins, bone morphogenetic proteins (BMPs), semaphorins/collapsins and the Eph/ephrins family of molecules (Krull, 2001; Graham, 2003). Upon entering the sclerotome, Eph-family receptors and their ephrin ligands mediate repulsive interactions between neural crest and caudal half-sclerotome cells, thereby restricting neural precursors to specific territories in the developing nervous system (Krull et al., 1997; Wang and Anderson, 1997). In addition to avoiding the actively inhibitory cues present in the caudal half of each somite, there is evidence for the presence of attractive, positive molecular interactions that influence neural crest cell migration through the rostral half of each somite (Koblar et al., 2000; Krull, 2001). The stop signals that regulate the cessation of migration at sites of DRG formation are less characterized, however; an intact β-catenin signaling pathway within neural crest cells is required for the formation of DRG anlagen (Hari et al., 2002) and DRG often form in aberrant locations in the absence of sonic hedgehog signaling (Fedtsova et al., 2003). Sympathetic precursors respond to the local secretion of BMP-4 from the dorsal aorta, which is necessary for inducing differentiation of mature sympathetic ganglia, although its exact role in the migration of neural crest cells has not been elucidated (Reissmann et al., 1996; Shah et al., 1996; Schneider et al., 1999; McPherson et al., 2000). Targeted deletion of neuregulin, erbB2 or erbB3 genes all result in a marked hypoplasia of the primary chain of SG (Britsch et al., 1998). In these mutants, neural crest cells emigrate normally from the neural tube but fail to migrate ventrally toward the dorsal aorta and hence to contribute to SG anlagen formation; instead their migration is arrested dorsally in the vicinity of the DRG anlagen. Thus, these data implicate neuregulin and its receptors in the ventral migration of neural crest cells, although the mechanisms by which they effect migration remain incompletely characterized. Another guidance molecule, Sema3A and its receptor neuropilin-1 are required for the arrest and aggregation of sympathetic neural precursors at their normal site dorsolateral to the dorsal aorta (Kawasaki et al., 2002; Bron et al., 2004), although the cellular mechanisms mediating these steps have not been elucidated. Interestingly, it has been shown that trunk neural crest cells do not respond to guidance cues known to influence the cranial neural crest (Bronner-Fraser, 1993), indicating differences in either the environmental guidance cues present at the two levels and/or in the guidance receptors expressed by cranial versus trunk neural crest.

Due to their dorsal-to-ventral migration to deep within the embryo, the cell-cell and cell-environment interactions that mediate DRG and SG formation have been difficult to analyze in intact developing embryos. Cell tracking studies of fluorescently labeled cranial neural crest cells revealed a rich set of cell migratory behaviors, including collective chain-like cell arrangements, suggesting that a sophisticated set of underlying patterning mechanisms and extrinsic cues play a role in sculpting the migration pattern (Kulesa and Fraser, 1998; Teddy and Kulesa, 2004). By contrast, there is a paucity of knowledge on the migratory behaviors of neural crest cells in the trunk region. A very useful trunk explant technique designed to image the early sorting of trunk neural crest cells into streams (Krull et al., 1995) is insufficient for imaging later events, due to complications with tissue thickness and the beating of the developing heart. Several groups have used transverse slice explants to study early events in the development of the PNS (Hotary et al., 1996; Krull and Kulesa, 1998). However, this system is not ideal for imaging structures that develop along the vertebrate rostrocaudal axis, such as the DRG and SG.

In this study, we investigated the cellular dynamics that mediate DRG and SG formation. We followed fluorescently labeled trunk neural crest cells using a novel sagittal explant technique and time-lapse confocal microscopy (Kasemeier et al., 2004). To our knowledge, this is the first study in which DRG and SG formation has been described in spatiotemporal detail. We show that along their dorsoventral migratory route, trunk neural crest cells are highly motile and dynamically interact with neighboring cells and the environment via an elaborate extension and retraction of filopodia. Some cells migrate collectively, forming chain-like arrangements that stretch from the DRG to the SG. Surprisingly, the segregated pattern of neural crest cell streams is not maintained once cells arrive at the presumptive SG sites. Instead, cells disperse and intermix along the anterior-posterior axis and contact cells in the neighboring SG sites. Here we document this segregation process in detail and reveal for the first time the highly dynamic filopodial activity that transforms an initially continuous stream of cells into discrete, segregated SG. By imaging crest cells we also found that rerouting of neural crest cells between developing DRG and SG is temporally regulated in that early migrating cells, but not later migrating cells, can reverse their direction of migration once they have arrived in their target ganglion (DRG versus SG). The diverse cell migratory behaviors and active reorganization at the target sites suggest that cell-cell and cell-environment interactions are coordinated with dynamic molecular processes to ultimately sculpt the organization of the PNS.

Materials and methods

Embryos

Fertilized White Leghorn chicken eggs (Spafas Avian products; North Franklin, CT) were placed in a rocking incubator at 37°C (Kuhl, Flemington, NJ). Eggs were rinsed with 70% alcohol and 3 ml albumin was removed. Eggs were windowed and embryos staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos at HH stage 10 (Hamburger and Hamilton, 1951) were injected with a GFP-encoding vector, pMES, 4.3 μg/ml or pCAX (kind gift of Dr Cathy Krull, U. Michigan) or pLZRS.gap43.GFP plasmid (kind gift of Dr Rusty Lansford, Caltech). Fast Green FCF (Sigma, F-7252) at 10 mg/ml was added 1:5 to the injection needle to visualize injection of the construct in ovo. The GFP-plasmid was microinjected into the lumen of the neural tube using a borosilicate glass capillary pulled needle (World precision instruments, MTW100-4) until the region of the neural tube between the forelimbs and hind limbs was filled. Constructs were

electroporated into pre-migratory neural crest cells using gold-coated Genetrode electrodes (Fisher, BTX512) and an electroporator (Genetronics, San Diego, CA). Eggs were resealed with adhesive tape and incubated at 38°C for 2 days. After this incubation period we evaluated each embryo prior to generating sagittal explants for brightness and uniformity of GFP label using a Zeiss Axiovert microscope with a 10× objective, and selected those embryos that were well labeled and developing normally.

Preparation of sagittal explants

Embryos were removed from the egg using a paper ring (Whatman, #1001185), cleaned in warmed Ringer's solution, and the surrounding membranes were carefully removed using forceps. Sagittal explants of embryos ranging from HH stage 17-22 were prepared by using the trunk sagittal explant technique described in Kasemeier et al. (Kasemeier et al., 2004) and also briefly described below. A trunk explant was made from the region between the forelimbs and hind limbs. A cut along the midline of the spinal column was made with a tungsten needle to splay open the spinal cord. A razor blade was then inserted into the incision made with the tungsten needle (A-M Systems, 717000) and sliced through the embryo to produce two sagittal explants that can be cultured medial (cut) or lateral side down.

A Millipore culture plate insert (Millipore, PICMORG50) was prepared by coating with 20 μg/ml of fibronectin (Gibco, 33016-015). To increase image resolution, we used a modified six-well dish with a hole in the center of the bottom of the well, and a coverslip sealed with vacuum grease over the hole (Kulesa and Fraser, 1998). The sagittal explant was then transferred onto the Millipore filter with a few drops of neural basal medium (Gibco, 21103-049) supplemented with B27 (Gibco, 17504-044). The filter with explant was then transferred into the coverslipped well filled with supplemented neural basal media. Sufficient media was added so that the level of the Millipore filter was reached (for media exchange through the filter to the tissue) but not too much so that the filter floated in the well. The other wells were filled with sterile water and the dish was covered and sealed with parafilm to create a humidified chamber to place on the heated microscope stage.

Time-lapse video microscopy

GFP-labeled explants were visualized using a laser scanning confocal microscope (Zeiss LSM). Optical thickness was set between 10 and 20 μm in *z*-height with a 10× Neofluar (NA=0.30) lens. This optical thickness was optimal for the observation and tracking of the maximal number of cells as they migrated ventrally over longer time periods. The microscope was surrounded with an incubator composed of a snug-fitting cardboard box surrounded by thermal insulation (Reflectix, BP24025) and a tabletop incubator (Lyon Electric, 950-107) fed into one side of the box (Kasemeier et al., 2004). The fluorescent GFP plasmid was excited with the 488 nm laser line using the FITC filter. Time-lapse images were recorded every 10 minutes for an average of between 24 and 36 hours. Images were digitally collected and analyzed using Zeiss AIM software and ImageJ v1.30 software (developed at NIH and available on the Internet at http://rsb.info.nih.gov/ij/). Static image analysis and rendering (depth-coding and embossing) was done using Adobe photoshop 7.0. In total, 12 embryonic explants were imaged for >24 hours and five for between 6 and 24 hours. For higher resolution imaging, explants were mounted on a coverslip [as described in Kulesa and Fraser (Kulesa and Fraser, 2002)] and imaged for 4-6 hours with a 40× LD-Achroplan, Zeiss objective on a laser scanning confocal microscope (Zeiss LSM Pascal on a Zeiss Axiovert).

Results

To characterize the cellular dynamics during DRG and SG formation, premigratory neural crest cells were fluorescently labeled with a GFP encoding vector and followed using time-lapse confocal microscopy. After allowing sufficient time for GFP expression, transfected neural crest cells were imaged from a medial (Fig. 1B) or lateral (Fig. 1C) perspective. Imaging from the exposed medial surface in a sagittal explant allows for visualization of neural crest cells as they migrate ventrolaterally en route to their final destination adjacent to the dorsal aorta (Fig. 1B). Imaging from the lateral perspective offers the ability to follow neural crest cells from the moment they emerge from the neural tube, form the DRG, and migrate through the rostral half of somites (Fig. 1C). Normal morphology is maintained in the sagittal explant and SG and DRG develop normally through 72 hours in culture. The uniqueness of the sagittal explant allowed us to follow the complete migratory route of trunk neural crest cells from the neural tube to their target destinations as they formed the DRG and primary chain of SG (Fig. 1A; see Movie 1 in the supplementary material). By analyzing time-lapse confocal imaging sequences, we were able to identify many of the cellular dynamics integral to DRG and SG formation. Here, we describe the spatiotemporal aspects of DRG and SG formation and neural crest cell migratory behaviors not previously discerned from static images.

DRG formation

The close juxtaposition of the DRG to the neural tube (both GFP labeled) can obscure the resolution of the cellular morphological events integral to DRG formation; however, this problem can be largely obviated by imaging the lateral surface of the sagittal explant, which provides a relatively clear view of the aggregation of neural crest cells as they form the DRG anlagen (Fig. 2A). Although the neural crest cells that give rise

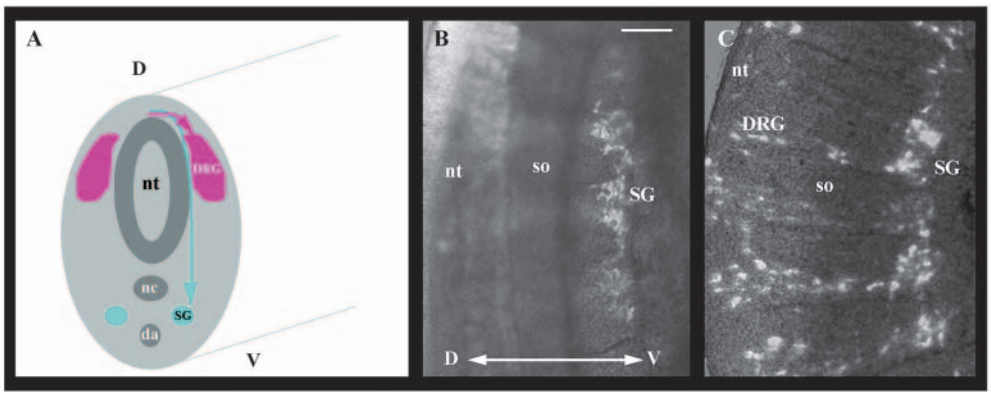

Fig. 1. Imaging perspectives in sagittal explants. (A) Schematic showing the migratory routes of neural crest cells from the dorsal neural tube to the DRG (pink) and SG (blue). (B,C) Typical views of a sagittal explant containing GFP-labeled neural crest cells. (B) Medial view; (C) lateral view. Scale bar: 25 μm. D, dorsal; da, dorsal aorta; DRG, dorsal root ganglia; nc, notochord; nt, neural tube; SG, sympathetic ganglia; so, somite; V, ventral.

to the DRG travel a much shorter distance than those that give rise to the SG, we identified a few subtle intercellular interactions that mediate DRG formation using time-lapse analysis of sagittal explants. As observed during SG formation (see following results on SG), crosstalk existed between adjacent DRG anlagen as they formed, but to a much lesser extent than occurred during SG condensation (Fig. 2B). As cells emigrated from the neural tube, they could extend filopodia across the caudal somite and thereby be in contact with cells from two different DRG anlagen. Once the cells committed to one of the condensing DRG, they often continue to reorient and move but to a lesser extent than observed within and between SG anlagen. Cells were observed moving from one DRG anlagen to the neighboring one (Fig. 2C-E) but it was difficult to determine whether cells moved more than one axial level while in the periphery, due to the abundance of cells that were labeled. Cells that switched to the next most caudal or rostral DRG did so while maintaining contact with DRG cells. Cells along the perimeter of the incipient DRG exhibited more movement than cells within the central core. These cells on the perimeter actively extended and retracted filopodia into the surrounding environment. Teillet et al. (Teillet et al., 1987), using chick: quail chimeras, also described the repositioning of neural crest cells along the rostrocaudal axis of the neural tube during DRG formation; here we extend those observations by imaging the dynamic cell-cell interactions and relocation of cells that occurs once cells have exited the neural tube and are forming the DRG anlagen.

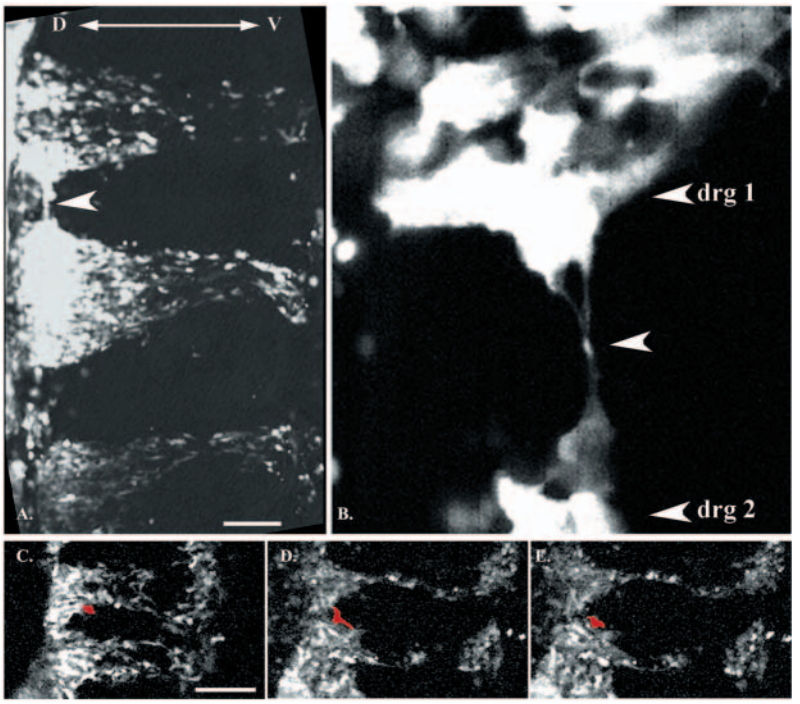

Fig. 2. Aggregating neural crest cells as they form the DRG anlagen. Embryos were injected at HH stage 10 with GFP coding vector pMES and sagittal explant mounted at embryonic day 3.5. (A) Neural crest cells migrating from three axial levels through the rostral somite, with arrowhead indicating cells between two DRG anlagen. Double-ended arrow indicates dorsal (D) and ventral (V) directions. (B) High magnification of area indicated by arrowhead in (A). Cells from DRG1 and DRG2 are in contact with each other. (C-D) Cell tracking of a single neural crest cell that initially resided in one DRG and then migrated to the DRG immediately posterior. Scale bars: 20 μm in A; 40 μm in C-E.

Neural crest cells migrate in chains through the somites

Neural crest cells migrating through the rostral sclerotome formed streams of cells interconnected by extensive filopodia and adopted a chain-like formation as they made their way toward the dorsal aorta (Fig. 3). Even though only a subset of neural crest cells were transfected with the GFP-encoding plasmid, and therefore visible using fluorescence microscopy, entire chains of labeled neural crest cells could be resolved that extended from the ventral edge of the DRG, through the sclerotome, to the SG anlagen (Fig. 3B-D). Nearly every fluorescently labeled cell imaged was a member of a chain, indicating that chain behavior is an integral feature of neural crest migration through the sclerotome. Cells within a chain extended one filopodium toward a cell in the direction of the SG (ventral) and one filopodium back (dorsally) toward the neural tube (Fig. 3C,D). These filopodia interconnecting a chain of neural crest cells could change in length as cells migrated, but were always maintained as crest cells migrated toward the dorsal aorta. Virtually all cells observed migrating through the sclerotome maintained filopodial contact with, at minimum, one other cell throughout their migration. Depth coding analysis indicates that the filopodial connections were within a narrow focal plane, ranging from 5-10 μm with entire chains comprising a focal plane of less than 40 μm. These chains have been imaged previously at earlier stages in neural crest cell migration (Krull et al., 1995) but not as cells migrate deep ventrally toward the dorsal aorta.

Migration and reorganization of neural crest cells as they form the DRG and SG anlagen

The retention of filopodial contact between neural crest cells in a chain appeared to be critical for maintaining normal neural crest cell migration. By contrast to what has been observed in static images, we find that occasionally neural crest cells are not completely inhibited from entering and exploring the nonpermissive caudal somite. A small percentage of neural crest cells en route to the dorsal aorta could break from a chain and extend filopodia into the caudal somite environment (Fig. 4). While exploring the caudal somite, if such cells maintained their original filopodial contacts with their neighbors in the chain, they would continue to migrate in a directed fashion ventrally. These filopodial contacts functioned to reorient cells that had attempted to break from the chain, apparently by pulling them back into alignment. In rare cases, as shown in the sequence of images in Fig. 4B, a neural crest cell could completely break free from the chain. These cells moved extensively in all directions near the chain in an undirected manner, and did not re-establish themselves within the chain; nor did they localize to a DRG or SG. As not all neural crest cells were GFP+, we could not ascertain whether those cells

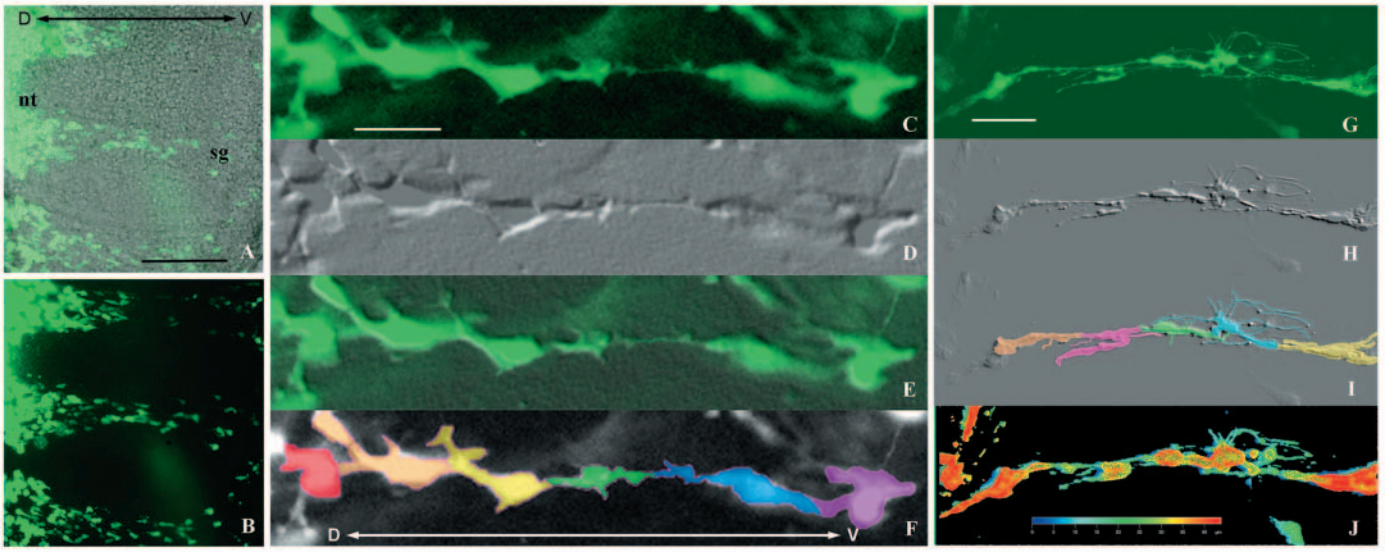

Fig. 3. Migrating neural crest cells forming chains through the rostral sclerotome, extending from the DRG to the dorsal aorta. (A) Lateral view of sagittal explant of pMES fluorescently labeled neural crest cells overlaid on brightfield image. (B) Fluorescence of labeled neural crest cells. (C-F) Single chain extending from neural tube to dorsal aorta. (C) GFP-labeled neural crest cells. (D) Embossed image of chain. (E) Fluorescence and embossed overlay. (F) Individual cells within chain colored separately to define cell boundaries. (G-J) Lateral view of sagittal explant of eGFP fluorescently labeled neural crest cells. (G) Fluorescence of single chain. (H) Embossed image of chain. (I) Individual cells colored separately and overlaid on embossed image. (J) Depth coding of entire chain. Arrow indicates dorsal (D) and ventral (V) directions. Scale bars: 50 μm in A,B; 20 μm in C-F; 20 μm in G-J. nt, neural tube; sg, sympathetic ganglia.

that broke from the visible chain were still in contact with other migrating neural crest cells. However, we show here that a visible separation from chain neighbors dramatically altered neural crest cell behavior.

At early stages, reorganization of the cellular composition of the DRG and SG anlagen could occur while crest cells were migrating ventrally. A subpopulation of cells migrating in chains toward the dorsal aorta (i.e. the site of SG formation) could reverse its ventral trajectory and migrate dorsally (i.e. toward the site of DRG formation; Fig. 5A-D). These cells maintained contacts with their chain neighbors while traveling in the opposite direction. Early on, these previously ventrally positioned cells that had reversed their trajectory could join the DRG anlagen. However, as development ensued, spatial restrictions were imposed on the migratory pattern of the crest on the dorsoventral path. Later migrating cells that reversed their ventral trajectory were repelled by an apparent boundary positioned between the DRG and SG anlagen, and were subsequently redirected ventrally toward the SG anlagen (Fig. 5E-G). Simultaneously, some cells within the DRG anlagen that attempted to migrate further ventrally through the somite were also repelled at the same spatially located boundary and restricted from further migration toward the dorsal aorta (Fig. 5H). Thus spatial constraints within the environment restricted the further exchange of cells between the developing SG and DRG. The developmental stage at which cells could no longer

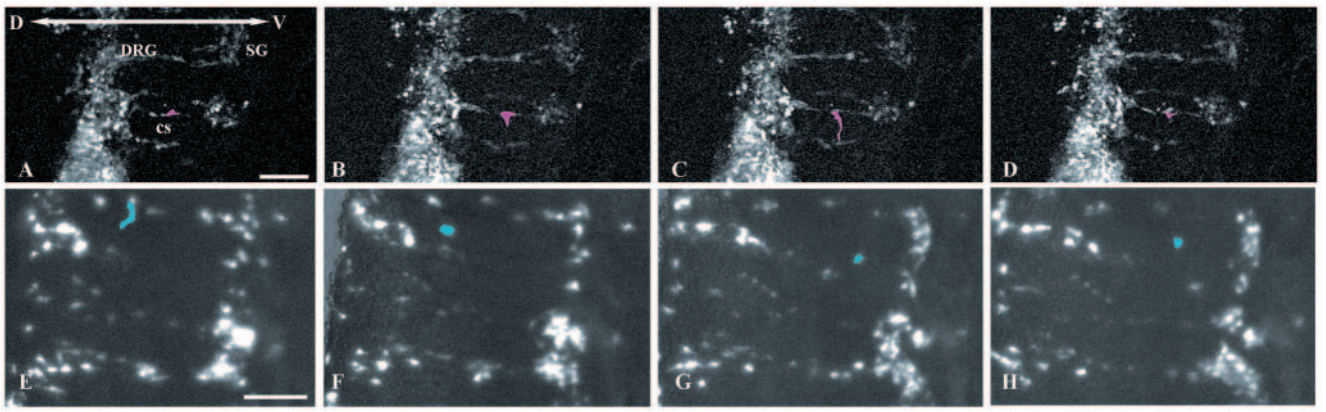

Fig. 4. Filopodial contact is important for neural crest migration through the rostral somite. (A-D) A cell positioned midway in a chain extends filopodia into the caudal somite and touches a cell in a chain in a different axial level. The cell maintains filopodial contact with its chain neighbors and returns to its original chain. (E-H) A cell attempts to extend a filopodium into the caudal somite but all connections are lost with its neighbors in the chain. This cell roams in a disoriented, undirected manner through the rostral and caudal somite. Scale bars: 30 μm in A-D; 30 μm in E-H. cs, caudal somite; D, dorsal; DRG, dorsal root ganglia; SG, sympathetic ganglia; V, ventral.

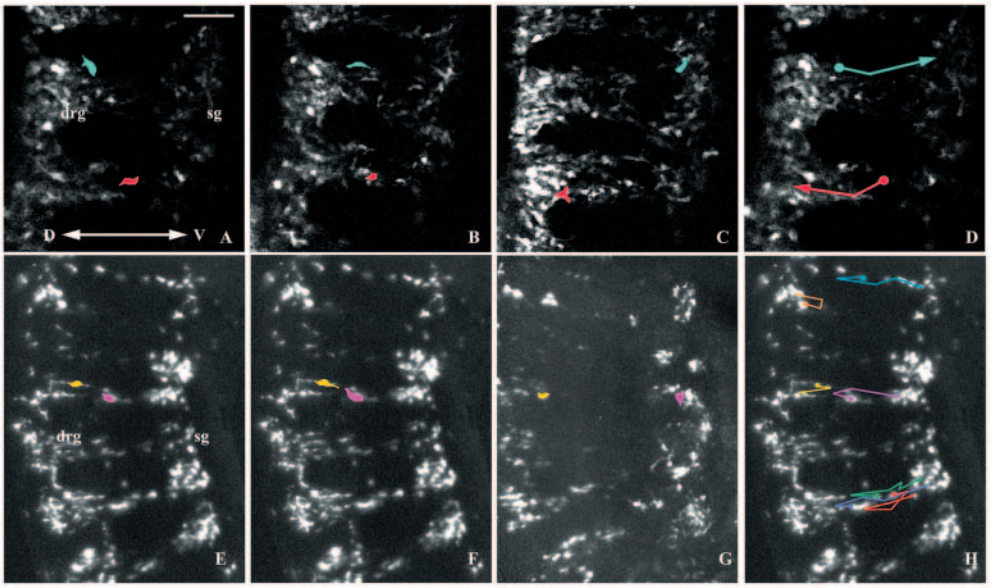

Fig. 5. Early migrating neural crest cells, but *not* later migrating cells, can reorient and reverse their initial direction of movement. (A-C) Single neural crest cells tracked in teal and red, were able to reverse their original direction through the rostral sclerotome and populate a different structure than that they were originally headed toward. (D) Summary of their pathways are shown using vector diagrams. (E-H) Cells tracked in yellow and pink try to reverse their direction but are forced back in their original direction. (F) Vector diagrams show seven individually tracked cells that were unable to reverse their migration direction. Scale bar: 30 μm. D, dorsal; drg, dorsal root ganglia; sg, sympathetic ganglia; V, ventral.

exchange locations dorsally and ventrally corresponded roughly to stage 20/21 and was observed in the trunk between the levels of the forelimbs and hind limbs.

Cells that exhibited this reorientation behavior first extended a filopodium in the opposite direction from which they had been migrating. Once that extension was established, the rest of the cell body moved in the same direction as this new extension. We observed cells that reoriented after these extensions were in contact with other GFP+ cells and cases where we could not determine whether a cell was contacted or not due to lack of GFP expression in the region contacted by the reorienting cell. The movement is analogous to that of a caterpillar inching along, in which the head crawls forward and the body then 'catches up'. At later time points, when cells were incapable of reversing their trajectory, the same cellular behavior was initiated, i.e. extension of a filopodium in the reverse direction, followed by cell movement in that new direction. However, what then ensued was an extension of a new filopodium in the original direction of movement and the subsequent reorientation of the cell back toward its original destination. Interestingly, the speed of cell movement increased as cells resumed their original direction of movement.

Neural crest cells arrive adjacent to the dorsal aorta and disperse rostrally and caudally

After neural crest cells successfully traversed the somite, they dispersed along a thin corridor rostrally and caudally, bordered on one side by the ventral, outer, sclerotome edge and on the other side by the dorsal aorta, (Fig. 6A; see Movie 2 in the supplementary material). The spatial restriction of neural crest cells to the rostral sclerotome was maintained only until the cells approached the ventral edge of the sclerotome. Once through the sclerotome, neural crest cells deviated from the metameric pattern that dictated their migration through the somites and instead spread contiguously rostrally and caudally away from their axial level of origin. By tracking individual cells we found that crest cells could move at least two segments rostrally or caudally, in agreement with previously published analysis of static images (data not shown) (Yip, 1986). However, in previous studies, it was not determined how cells ended up at axial segments other than their site of origin: i.e, by moving along the neural tube (site of origin) or the dorsal aorta (target). While our imaging analysis did not focus on neural crest cell behavior while still in the neural tube, our studies clearly indicate extensive migration and reorganization of neural crest cells once they arrive alongside the dorsal aorta.

Discrete SG arise as a result of active segregation of neural crest cells

We observed two sources of cells that comprised the SG. One population derived from chains of crest cells that diverted from their directed path in the rostral somite, made filopodial contact with cells adjacent to the dorsal aorta and integrated directly into the interganglionic space between incipient SG anlagen. The second population was derived from chains of crest cells

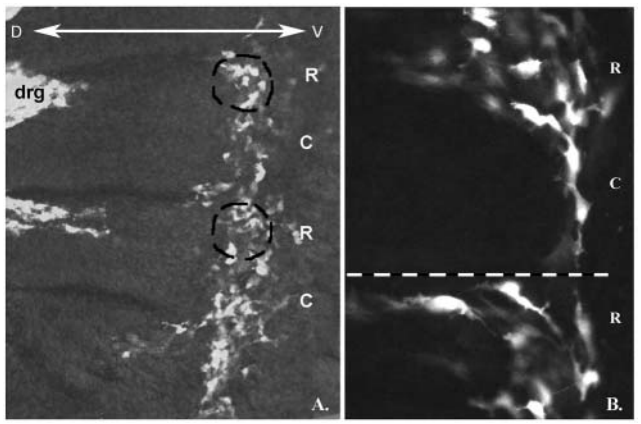

Fig. 6. Distribution of neural crest cells as they arrive in the vicinity of the dorsal aorta in a medial explant. (A) Cells initially distributed adjacent to the dorsal aorta. Double-ended arrow indicates dorsal (D) and ventral (V) directions. Dotted circles indicate where SG anlagen will form. (B) High magnification view of cells between developing SG at a later time point in this explant. Dashed line indicates somite boundary. Scale bars: 25 μm. C, caudal somite; R, rostral somite.

from the neural tube that directly targeted the SG anlagen, but then moved into the surrounding rostral and caudal environment (data not shown). However, in each case, filopodial connections allowed cells to enter the interganglionic space that might have been considered inhibitory, based on the known inhibitory properties of the caudal sclerotome (Figs 6 and 7).

The extensive cellular movement adjacent to the dorsal aorta eventually resulted in the segregation of neural crest into discrete SG anlagen (Fig. 7; see Movies 3 and 4 in the supplementary material). While resettling, many cells not only altered their direction once, but also reoriented themselves and changed their direction of movement numerous times before finally coalescing with their SG anlagen of origin, or a neighboring SG anlagen (Fig. 7B-G'). We analyzed the location of crest cells during this active period of reorganization and found that roughly 20% of the labeled neural crest cells were located between neighboring SG (21.1±3.12 s.d.; 289 cells counted) versus within an incipient SG. While between SG, cells maintained filopodial contact at all times with other neural crest cells. By analyzing cell dynamics in Movies 3 and 4, we found that initially neural crest cells in the interganglionic space extended short filopodia from the entire 360° of cell circumference. However, those filopodia that did not contact a cell in the neighboring SG were much more dynamic and rapidly retracted then those filopodia that contacted cells in neighboring SG anlagen. Gradually, those filopodia that were not extended toward either of the neighboring SG anlagen become shorter and less frequent than those pointed toward the neighboring SG. In fact those filopodia that extended in the direction of the SG and contacted a cell there tended not to retract and instead thickened and widened as the cell elaborated its contact with the SG cell. While in extensive cell contact with a cell in a neighboring SG, a neural crest cell's morphology underwent rapid phenotypic changes, and in the process altered the extent of its membrane apposition with its neighbors (Fig. 8). We quantified the series of morphological changes undergone by a given cell in the interganglionic space by measuring the extent of its membrane contact with a cell in a neighboring SG compared with its membrane juxtaposition with another cell in the interganglionic space (Fig. 8B). This analysis revealed that as a cell's membrane association was increased with a cell in the neighboring SG, its membrane apposition with a cell in the interganglionic space was decreased. These morphological changes were not accompanied by 'collapse' of filopodia; rather, filopodia were dynamically extended and retracted. Following additional filopodial extensions and contact with cells in the neighboring SG, the cell finally relocated and migrated toward and adhered to the SG while withdrawing its connections with cells in the interganglionic space. Fig. 9 depicts a series of images of a cell migrating from its original axial level, to populate the immediately caudal SG. Sufficient contact seems to be important for cells in this space to be permanently 'pulled' into a particular SG. Therefore, constant intercellular communication mediated by contact, seems to be a key component of the complex sequence of events that lead to the establishment of discrete SG.

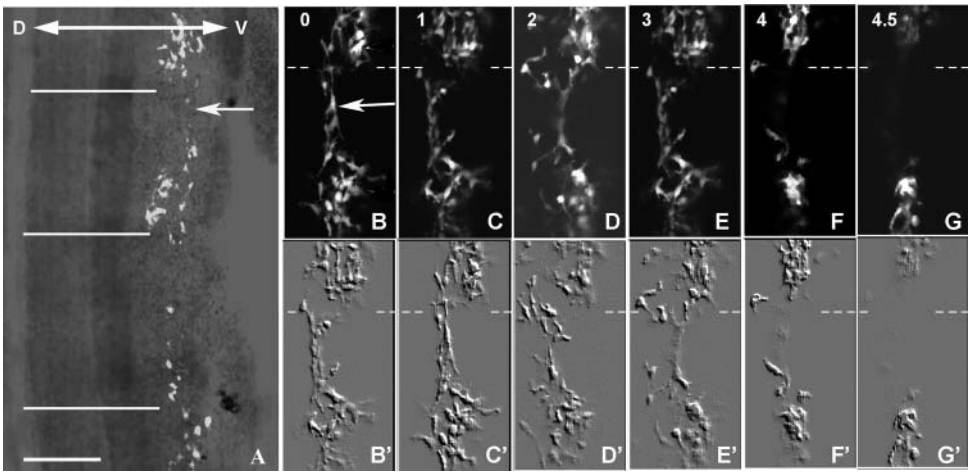

Fig. 7. Time-lapse analysis of SG formation in chick trunk sagittal explant imaged medially. Fluorescently labeled neural crest cells in a sagittal explant mounted at embryonic day 3.5. (A) Medial image of neural crest cells dispersed along dorsal aorta forming the SG anlagen. Unbroken lines indicate somite borders; double-ended arrow indicates dorsal (D) and ventral (V) directions. The behavior of cells in the interganglionic space (arrow) between two adjacent SG is illustrated in B-G and B′-G′. (B-G) Segregation of neural crest cells dispersed adjacent to the dorsal aorta into discrete SG anlagen. B'-G' are embossed images of B-G. Broken lines indicate somite borders. All time is in hours. Scale bar: 50 μm.

Discussion

The goal of this study was to characterize the behavior of trunk neural crest cells as they migrate and give rise to the DRG and SG anlagen. By labeling premigratory trunk neural crest cells with a GFP tag, we followed individual cell trajectories from the dorsal neural tube into the ventral periphery in sagittal explants using time-lapse confocal microscopy. With this approach, we have identified several novel and compelling events integral to the formation of DRG and SG that have not been previously described, including: the prevalence of chain formations as crest cells migrate ventrally and their role in maintaining directionality; the fact that crest cells interact extensively and dynamically with neighboring cells as they migrate; and that filopodial contact between neighboring neural crest cells appears to be critical for maintaining normal migratory routes. Furthermore, we showed that at early stages cells located in the DRG and SG anlagen can reverse their migratory direction and switch locations and ganglion type. However, within hours, this plasticity is lost, and cells become incapable of rerouting their trajectory and switching target locations. Most strikingly, we found that the metameric pattern of migration through the rostral somite breaks down once cells reach the ventral outer border of the sclerotome. At this location cells disperse contiguously rostrally and caudally

within a narrow corridor between the outer sclerotome border and the dorsal aorta, and over the next few hours, through a dynamic process of filopodial extensions/retractions and cell movements, neural crest cells sort into discrete sympathetic ganglia.

Chain formation is a common configuration adopted by migrating cells in the developing nervous system

Time-lapse analyses confirmed previously published observations that neural crest cell trajectories maintain a metameric pattern while navigating through the anterior portion of the somites (reviewed by Krull, 2001; Kuan et al., 2004) and that, while migrating, neural crest cells display collective movements, forming chain-like arrays. Furthermore, the behavior of crest cells while in the chain, versus those that apparently break from the chain, suggests that cell-cell and cell-environment contacts play a key role in cell guidance to the DRG and SG. Neural crest cells sampled the posterior region of the somite, but maintained stereotypical migratory patterns. Along the migratory route, cells displayed active filopodia in the direction of travel and toward the posterior region of the somite. All cells maintained two long, prominent filopodial extensions parallel to the direction of travel, connecting them to their chain neighbors ahead and behind. These trunk crest cell migratory behaviors of chain migration are reminiscent of those exhibited by migrating cranial neural crest and gut neural crest (Kulesa and Fraser, 1998; Young et al., 2004a) and have not been reported in the ventral migration of trunk neural crest cells as they project toward the dorsal aorta. The chains tended to stretch from the dorsal to ventral edge of the somites and consisted on average of approximately five to six cells. Chain migration of neuronal precursors appears to be a common mechanism of cell migration, exhibited prominently by subventricular zone neurons migrating to the adult olfactory bulb in mice (Lois et al., 1996).

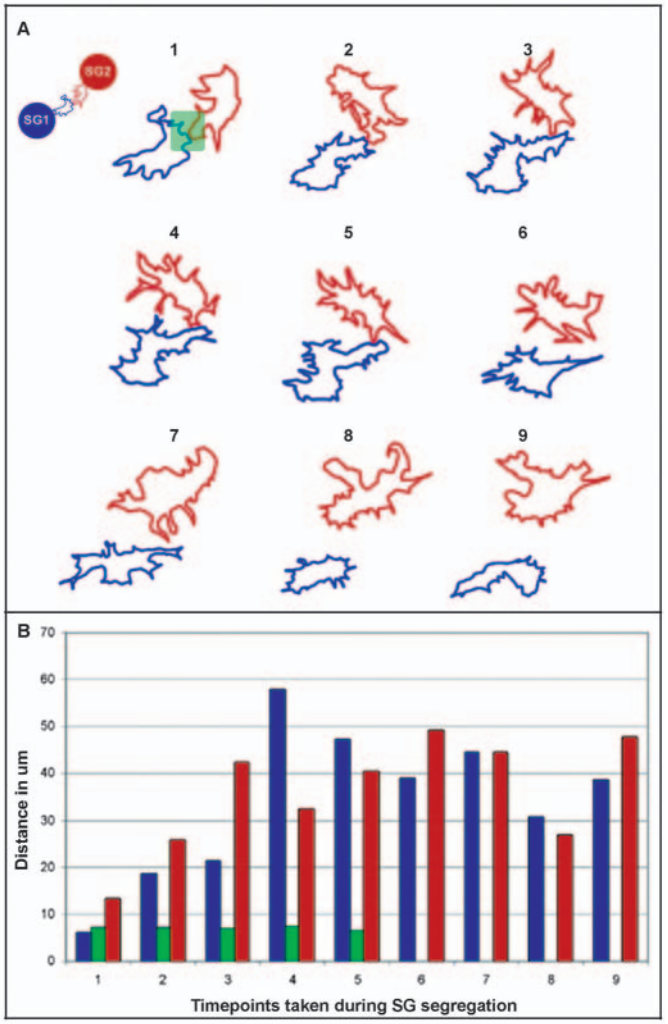

Fig. 8. High-resolution analysis of single cell dynamics during SG segregation. (A) Static outline of cells as they segregate into separate SG. Red and blue outlines represent two cells imaged at high resolution situated in the interganglionic space during SG segregation. The red and blue cells separate and join two separate SG. Inset shows orientation of cells between SG1 and SG2. Green highlighted region corresponds to the area in which the green bar measurements were taken for B. (B) Quantification of the extent of cell-cell contact during the segregation process. Blue bars represent the amount of contact (length of cell membrane in μm) the blue cell has with SG#1; green bars represent the amount of contact between the red and blue cell in the interganglionic space; red bars represent the amount of contact the red cell has with SG#2.

Neural crest cells reorganize as they migrate

Reorganization of cells destined for the DRG and SG not only occurs at the target locations but also while they are en route, as evidenced by our finding of cells being able to reorient ventrally or dorsally from their original location at early time points and contribute to the other ganglion type (i.e. DRG versus SG; Fig. 5). These data support those of Goldstein and Kalcheim (Goldstein and Kalcheim, 1991), which indicated the existence of a common pool of neural crest cells at each axial level that gave rise to both the SG and DRG at that level. Using grafts of exclusively rostral somites, DRG size could be increased, which resulted in a corresponding decrease in the SG at the same axial level. Thus, at least a subpopulation of migrating neural crest cells has the capacity to populate such functionally diverse structures as the DRG and SG, a finding that has also been shown in grafting experiments in ovo (Schweitzer et al., 1983). Reorientation of neural crest cells was also found in the cranial crest by Kulesa and Fraser (Kulesa and Fraser, 2000), who showed that hindbrain neural crest cells can reroute their migratory pathways and compensate for missing neural crest cells in a neighboring population.

Why do these cells deviate from their neighbors and change their target site? This subpopulation may remain in a more pluripotent, plastic stage longer than its neighbors and hence not be fully committed to one particular ganglion fate. In fact, a temporal pattern is observed in which early on crest cells can change their location from SG to DRG and vice versa, but later are prevented from doing so. As development ensues, the SG and DRG become sufficiently differentiated that a ‘boundary’ is established between the DRG and SG, such that crest cells traveling in reverse of their initial migration direction can no longer cross this boundary between the structures and are forced back in their original directions. This may reflect an actual physical boundary, perhaps composed of the known inhibitory proteoglycans that surround the notochord (Tosney and Oakley, 1990; Landolt et al., 1995; Perris and

Perissinotto, 2000), and/or be the manifestation of the differentiation of distinct sensory and sympathetic precursors that no longer respond to the local cues in the other ganglion type's environment and/or become repulsed by cues in the other (now aberrant) environment. Specific members of the bHLH class of transcription factors have been elegantly shown to regulate the differentiation of sensory (*Ngn 1* and *Ngn2*) versus sympathetic progenitors (*Mash1*), indicating a pre-specification of subsets of migrating neural crest cells (Parras et al., 2002; Zirlinger et al., 2002; Luo et al., 2003). Evidence also indicates a role for Wnt-1 in specifying sensory precursor fate (Lee et al., 2004; Bronner-Fraser, 2004) and for BMPs in inducing the differentiation of sympathetic precursors (Reissmann et al., 1996; Shah et al., 1996; Schneider et al., 1999; McPherson et al., 2000). However, the exact time and place of fate restriction of migrating neural crest cells remains an important question that could be resolved by combining molecular marker methods with time-lapse image analysis.

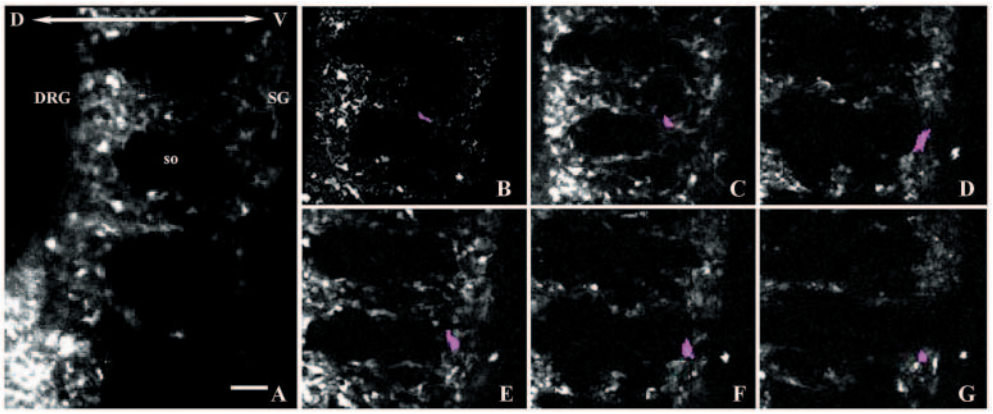

Fig. 9. Neural crest cells migrate through the sclerotome and can change axial levels once at the dorsal aorta. (A) Medial view of migrating neural crest cells shown at higher resolution in B-G. (B-C) Single cell, tracked through time, that migrates through the somite at one axial level. (D-E) This cell establishes filopodial contact with a cell in the SG anlagen immediately caudal to its axial level. (F) Cell moves caudally in the direction of the established contact. (G) This cell ends up populating the SG anlagen caudal to its original axial level of migration. Scale bar: 20 μm. D, dorsal; DRG, dorsal root ganglia; SG, sympathetic ganglia; so, somite; V, ventral

Formation of iterated, discrete sympathetic ganglia is not the direct result of patterned crest cell migration through the somites

After making the lengthy dorsoventral migration and arriving at the site of the incipient SG, neural crest cells fail to maintain segregated streams, suggesting that the inhibitory factors that restricted the neural crest to the anterior portion of the somite may no longer influence the neural crest once the cells traverse the ventral border of the sclerotome. Instead, we show here that individual SG arise as a consequence of extensive neural crest cell reorganization, coalescence and finally condensation into discrete ganglia. Furthermore, this segregation process is mediated by dynamic intercellular contacts and reiterated extension and retraction of multiple filopodia, a behavior that may also be exhibited by neighboring streams of migrating cranial neural crest cells and by gut neural crest cell streams (Kulesa and Fraser, 2000; Young et al., 2004a; Teddy and Kulesa, 2004).

Eph/ephrin interactions have been implicated in restricting the trunk neural crest cells to the anterior portions of the somite (Krull et al., 1997; Wang and Anderson, 1997). Once they have migrated through the ventral border of the sclerotome, the fact that neural crest cells are then free to spread rostrally and caudally suggests that these inhibitory molecular mechanisms are no longer operative. Instead, in the absence of inhibitory guidance cues, intercellular interactions among crest cells may dominate. In the cranial region lateral to the neural tube, it has been shown that neighboring neural crest cell streams interact extensively (Kulesa and Fraser, 2000). Thus, if the neural crest cells relied on intrinsic destination cues from the neural tube, this alteration in behavior from one of strict axial segregation to one of extensive intermixing would not be expected. Instead, our data suggest that the local environment near the site of the incipient SG plays an important role in influencing the behavior of neural crest cells.

The segregation of neural crest cells into specific areas along the dorsal aorta to form the SG suggests a local cell sorting mechanism. Neural crest cells that fill into the areas between the forming SG ultimately coalesce with one of the incipient SG. The mechanism driving the compaction of cells into specific SG may be repulsive and/or attractive. A repulsive molecule(s) may become expressed in the (nonpermissive) region between the developing SG that causes the neural crest cells to move away from the interganglionic space and toward the SG sites. By contrast, or in addition, a cell adhesive molecule may be expressed by differentiating sympathetic precursors that induces the neighboring neural crest cells to adhere and coalesce with the developing SG. Our data clearly indicate that increased intercellular contact with another SG is accompanied by decreased physical contact with a cell in the interganglionic space. Sorting of neural crest cells into distinct subpopulations also takes place in the cranial region of the developing vertebrate nervous system. In the hindbrain, Eph/ephrin signals at the rhombomere boundary sites act to sort individual cells into particular rhombomeric segments (Xu et al., 1999). Specifically, some gene expression boundaries appear to correlate with rhombomere boundaries, suggesting a mechanism that relatively precisely marks a border between two cell populations (Xu et al., 1999). The expression of several cell adhesion molecules correlates with the onset of cell coalescence into discrete SG: both N-cadherin and NCAM are expressed in the nascent SG (Duband et al., 1985; Akiyata and Bronner-Fraser, 1992). However, the operative molecular mechanisms mediating the segregation of neural crest cells into discrete ganglia remain to be elucidated.

Our finding, based on imaging neural crest cells in their native environment, of the dynamic behavior of neural crest cells en route to their destination sites adds complexity to the

idea that the metameric organization of neural-crest-derived structures depends on the alternation of rostrocaudal properties within the somite. Several classes of guidance molecules have spatiotemporal patterns in the somites consistent with a role in neural crest and/or axon guidance; including semaphorins, neuregulins and BMPs. However, these studies cannot explain our finding of neural crest cell dispersion adjacent to the dorsal aorta followed by crest cell re-segregation into discrete ganglia. What are the molecular mechanisms mediating these distinct behaviors? In-vitro studies have shown that a class of semaphorins, namely Sema3A, induces the collapse of sympathetic and DRG growth cones and of migrating neural crest cells in vitro (Adams et al., 1997; Eickholt et al., 1999; Vastrik et al., 1999; Bron et al., 2004). RNA interference of the sema3 receptor, neuropilin-1, in chick neural crest cells causes the premature (i.e. dorsal) arrest of neural crest cells destined to form SG (Bron et al., 2004). The phenotype of mice with targeted deletions of either Sema3A or its receptor neuropilin 1 is complex (Kawasaki et al., 2002). Mutant mice neural crest cells migrate normally through the sclerotome, reach the dorsal aorta and turn on MASH1. However, they fail to arrest and aggregate at the dorsal aorta and hence to give rise to mature SG. Disruptions in expression of Neuregulins and/or their ErbB family of tyrosine kinase receptors results in severe hypoplasia of the primary sympathetic ganglion chain. Mice with targeted deletions in either the ligand or its receptors, exhibit a lack of neural crest precursor cells in the anlage of the primary sympathetic ganglion chain (Britsch et al., 1998). Although very informative, studies addressing whether these molecules regulate the dispersion and re-segregation of neural crest cells once they reach the dorsal aorta will be required. The question remains, Why set up a metameric migration pattern when in the end you redistribute and refine at the target location (Young et al., 2004b)?

In summary, the time-lapse analysis has revealed particularly intriguing, unexpected aspects of DRG and SG formation. The trunk neural crest cell behaviors in chains mimic behaviors reported in cranial and gut neural crest cells. Surprisingly, these tightly segregated cell streams disperse uniformly once at their target site near the dorsal aorta, yet within hours these neural crest cells sort and reassemble themselves into discrete SG. What remains is a careful dissection of the molecular mechanisms that mediate the cellular phenomena orchestrating the formation of two of the major neural crest derivatives, the DRG and SG.

This work is dedicated to the memory of Dr Robert A. Oakley. We thank Dr Cathy Krull (University of Michigan) for the pCAX and pMES vectors and Dr Rusty Lansford (Caltech) for the pLZRS.gap43.GFP. Grant support to F.L. was from the National Institutes of Health, NS35714, and to J.C.K. from NIH-NCRR, P20-RR-16455.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/2/235/DC1

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