Regional differences in neural crest morphogenesis

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Neural crest cells are pluripotent cells that emerge from the neural epithelium, migrate extensively and differentiate into numerous derivatives, including neurons, glial cells, pigment cells and connective tissue. Major questions concerning their morphogenesis include: (1) what establishes the pathways of migration? And (2), what controls the final destination and differentiation of various neural crest subpopulations? These questions will be addressed in this Review. Neural crest cells from the trunk level have been explored most extensively. Studies show that melanoblasts are specified shortly after they depart from the neural tube and this specification directs their migration into the dorsolateral pathway. We also consider other reports that present strong evidence for ventrally migrating neural crest cells being similarly fate restricted. Cranial neural crest cells have been less analyzed in this regard but the preponderance of evidence indicates that either the cranial neural crest cells are not fate-restricted or are extremely plastic in their developmental capability and that specification does not control pathfinding. Thus, the guidance mechanisms that control cranial neural crest migration and their behavior vary significantly from the trunk.

The vagal neural crest arises at the axial level between the cranial and trunk neural crest and represents a transitional cell population between the head and trunk neural crest. We summarize new data to support this claim. In particular, we show that: (1) the vagal-level neural crest cells exhibit modest developmental bias; (2) there are differences in the migratory behavior between the anterior and the posterior vagal neural crest cells reminiscent of the cranial and the trunk neural crest, respectively and (3) the vagal neural crest cells take the dorsolateral pathway to the pharyngeal arches and the heart, but take the ventral pathway to the peripheral nervous system and the gut. However, these pathways are not rigidly specified because of prior fate restriction. Understanding the molecular, cellular and behavioral differences between these three populations of neural crest cells will be of enormous assistance when trying to understand the evolution of the neck.

Introduction

Neural crest cells are mesenchymal cells that emigrate from the dorsal neural tube and are remarkable both for their extensive migration and the multiple derivatives to which they give rise. For these reasons, the neural crest has been a popular model system with which to investigate morphogenesis. The question that will be addressed in this review is what controls the positioning of these different phenotypes of the neural crest in the appropriate places during embryogenesis? Because the situation is somewhat simpler at the trunk level, this review will focus initially on patterning of the trunk neural crest before addressing the more complex patterns at the head and neck. Most of the experimental studies have used the chicken as a model system, so we will primarily consider the avian neural crest, although recent genetic studies in mouse and zebrafish will be referenced when appropriate. We have not included Xenopus, lamprey and humans for lack of space and because there are good recent reviews.

One model for patterning neural crest derivatives proposes that neural crest cells are multipotent and migrate haphazardly into the available paths where they differentiate according to the cues that they encounter (reviewed in ref. 1 and 2). This model is supported by experimental evidence from heterotopic grafting experiments, as well as back-transplantation of crest-derived structures into the early migratory crest pathways, which show that, as a population, neural crest cells migrate and differentiate according to their new environment and not their origin.

Recent studies have revealed that an alternative mechanism directs the migration of chicken melanoblasts (precursors to the epidermal melanocytes) into the dorsolateral path: (1) melanoblasts are fate-restricted at the time that they depart from the neural tube,2,3 (2) neural crest cells that are specified primarily as melanocytes enter the dorsolateral path2,4,5 and (3) melanoblasts are the only neural crest cells that can exploit the dorsolateral path under experimental conditions.6-8 Thus, another model for positioning of neural crest derivatives, based on the study of melanocytes, is that their final distribution is directed by cell-autonomous migratory properties unique to each subpopulation.

After reviewing the trunk neural crest we will compare and contrast their migratory behavior with those at the cranial and “vagal” levels. The latter represents the merger or overlap of the brain and spinal cord in the region of the neck. We will then survey the molecular mechanisms by which neural crest cells are directed to their final destination at each of these axial levels.
and then migrates in a medial-to-lateral trajectory, initially using the basal lamina of the myotome as a substratum for migration.

It is during the invasion through the somites that the first important patterning event occurs. Neural crest cells only penetrate the anterior half of the somite while scrupulously avoiding the posterior region. This restriction establishes, at a very early stage, the segmental order of the adult peripheral nervous system. As neural crest cells migrate through the somite, they begin to coalesce to form the ganglia of the peripheral nervous system: some cells aggregate adjacent to the dorsal neural tube and form the sensory (or dorsal root; DRG) ganglia. Other neural crest cells migrate ventrally and stop at the dorsal aorta to form the primary sympathetic chain. At a slightly later stage in development these latter neural crest cells migrate dorsally to form the secondary sympathetic chain, and at somite-level 18–24 some of these cells migrate ventrally and laterally and coalesce adjacent to the kidney to give rise to the adrenal medulla. Other ventrally migrating cells spread along the ventral root motor fibers and differentiate into glial cells. Finally, at the sacral level of the trunk (posterior to somite 28), neural crest cells migrate ventrally to the dorsal aorta and into the mesenchyme dorsal to the gut, from which point they will eventually invade the post-umbilical gut and differentiate into the neurons and glial cells of the enteric nervous system.

Correct patterning of the ventrally migrating neural crest cells requires: (1) molecular mechanisms to control the pathway of migration through the somite and (2) cues to control the localization and differentiation of the many different lineages (sensory, sympathoadrenal, glial, enteric) that develop along the ventral path. These two patterning events will be considered separately, although mechanisms that control them are not likely to be mutually exclusive.

Migration around and through the somite. As neural crest cells migrate through the somites their distribution becomes asymmetrically patterned along the anterior/posterior axis. Experimental studies have revealed that the failure of neural crest cells and ventral root motor fibers to invade the posterior somite is controlled by environmental cues in the somite. If a strip of segmental plate or a cluster of the last-formed somites is excised and then rotated 180°, neural crest cells and the ventral root fibers still invade what had been previously specified as anterior somite. Direct observations of migrating neural crest cells labeled with DiI reveal that they retrait their lamellipodia and reorient their migration when...
they contact the posterior somite. Together, these data suggest that the posterior half of the somite contains barrier molecules that are inhibitory to neural crest cell migration.

A number of candidate molecules have been localized to the posterior sclerotome and could possibly inhibit neural crest migration. Some of these are extracellular matrix (ECM) molecules: chondroitin-6-sulfate proteoglycan (CSPG), peanut agglutinin (PNA)-binding molecules, collagen type IX, versican, and F-spondin. T-cadherin, a cell-cell adhesion molecule, is expressed on posterior sclerotome cells. Additionally, ligands that inhibit neuronal migration have also been identified in the posterior sclerotome: ephrin-B1, a ligand for the Eph family of receptor tyrosine kinases, and Semaphorin 3A (SEMA3A) and Semaphorin 3F (SEMA3F), two of the many members of the collapsin/semaphorin family. Although most of these molecules can inhibit neural crest cell migration in vitro (reviewed in ref. 44 and 45), several of them are probably not involved in controlling segmental migration in vivo. Collagen IX inhibits neural crest migration, but it is not expressed until after they begin to invade the somite. T-cadherin mediates homophilic cell-cell adhesion and is also expressed on motor neurons, but is not expressed by the neural crest, so it is not likely to affect neural crest cell migration by a homophilic mechanism. And SEMA3A forms a segmental pattern only well after neural crest cells have invaded the somite so that its timing eliminates it as a likely inhibitor of posterior migration.

In order to confirm if any of these posteriorly-expressed molecules inhibits neural crest migration, their function needs to be perturbed in the embryo. Such an analysis has been facilitated by the development of an in vitro explant assay in which a segment of a chick trunk is excised, the neural crest cells are labeled with DiI and their behavior is observed directly as development proceeds. Additionally, these explants can be exposed to perturbing agents in the bathing medium, obviating the need for injecting them and assuring that the agents will permeate the tissue thoroughly. Using this assay, two different molecules appear to have a role in the segmental migration of the neural crest.

PNA-binding molecules, which have not been identified, were the first for which a barrier function was suggested. When trunk pieces are treated with PNA, neural crest cells now enter the posterior sclerotome, presumably because the inhibitory epitopes to which PNA binds are camouflaged. However, migration into the posterior sclerotome does not mimic anterior migration, suggesting that PNA-binding molecules alone are not responsible for this pattern. For example, neural crest cells are still delayed compared to entry in the anterior half and their direction and rate of migration are different from the cohorts in the anterior half.

Other ligands that play a role in inhibiting neural crest migration are those that bind to the Eph receptors. This family of receptors and their ligands have been implicated in many axonal guidance events. One member of the Eph family of receptor tyrosine kinases, EphB3, is expressed on early migratory chick neural crest cells, whereas one of its complementary ligands, ephrin-B1, is expressed in the posterior sclerotome. A similar distribution of Eph-family receptors and ligands has been discovered in the mouse. Furthermore, when neural crest cells are confronted in vitro with a substratum derivatized with stripes of ephrin-B1, neural crest cells avoid the ephrin-B1 stripes and migrate preferentially on the control lanes. Using the explant assay, Krull and colleagues treated trunk segments with monomeric, soluble ephrin-B1, which binds to the receptor on the neural crest and presumably competes with extracellular ephrin-B1. In treated explants, neural crest cells invade both the anterior and posterior somite halves. These data persuasively argue that ephrins inhibit neural crest cell invasion of the posterior somite.

At least one ECM molecule appears to influence anterior/posterior segmentation. F-spondin is expressed in the posterior sclerotome, the perinotochordal matrix in the anterior somite and the dermamyotome—all regions that are refractory to early neural crest migration. When function-blocking antibodies are injected into the space between the somite and neural tube, the neural crest cells invade the perinotochordal mesenchyme, the posterior sclerotome and even invade the dermamyotome, suggesting that F-spondin is yet another inhibitory signal that patterns neural crest migration through the somite.

Recently another inhibitor of migration has been added to the mix and is likely the primary control of segmental migration. The collapsins/semaphorins are secreted molecules that were first identified by their ability to collapse the growth cones of migrating neurons. SEMA3F and its receptor, neuropilin 2 (Nrp2), appear to be critical in the segmental migration of the neural crest. SEMA3F is expressed in the posterior-half somite and Nrp2 on a subpopulation of the neural crest, suggesting that SEMA3F might act to repel neural crest cells from the posterior-half somite. When either gene is knocked down in the mouse, neural crest cells invade the posterior half of the somite as massively as they do the anterior half, showing that this semaphorin plays a critical role in blocking crest migration. Curiously at later stages in development, despite the uniform invasion of the somite, there is still some segmental distribution of the sensory ganglia. The reason for this appears to be that both the SEMA3A and SEMA3F are required for appropriate patterning.

Eickholt and co-workers reported that SEMA3A is expressed in the posterior half of the somite, although this distribution is not seen until well after segmental migration is underway. Further, early migrating neural crest cells express Nrp1, which is the receptor for SEMA3A. When SEMA3A is immobilized in stripes alternating with fibronectin, explanted neural crest cells avoid the collapsin stripe. Knockdown of Nrp1 in the chicken embryo using siRNA does not result in disruption of segmental migration. (Kasemeier-Kulesa and Erickson, unpublished data). However, when SEMA3A or its receptor Nrp1 is mutated or knocked down in mouse embryos, neural crest cells that normally migrate through the anterior half of the somite now migrate in the intersomitic space, resulting in the formation of ectopic sympathetic ganglia. When double knockouts of the Nrp1 and Nrp2 receptors are produced the segmental organization of the dorsal root ganglia is lost. Thus, the two signaling systems work together. SEMA3F/Nrp2 controls anterior migration in the somite and prevents migration in the posterior somite. SEMA3A/Nrp1 seems to be involved in two steps in patterning. First, it provides a somite
boundary to keep neural crest cells out of the intersomitic space (or perhaps drives neural crest cells from the intersomitic space into the anterior somite) and consequently plays a role in segmentation of the sympathetic nervous system, whereas later this signaling system reinforces segmentation of the dorsal root ganglia.28

In summary, mutations of the semaphorins and their receptors suggest that they play the predominant role in segmental migration. However, there are other molecules that can prevent neural crest migration into the posterior somite, implying that there is considerable redundancy in establishing segmental migration. It might be informative to knockout several of these genes together or perturb several of these molecules at the same time to observe a more dramatic phenotype. Given this redundancy, it is perplexing that in the explant assay, there is a robust effect when either the EphB3 receptor or PNA-binding molecules are perturbed individually. This observation suggests that perhaps each of these signaling systems has a unique role to play in segmental migration through the somite or that different subpopulations of the crest are differentially sensitive to the many cues. Alternatively, it raises the disturbing and confounding possibility that antibody perturbation experiments yield fundamentally different results from genetic loss-of-function studies.

The data suggest that neural crest cell migration is inhibited through the posterior somite and so they migrate by default through the anterior somite. It is important to ask what substratum in the anterior somite is permissive for migration. The anterior somite (as well as the posterior) contains a number of ECM molecules on which neural crest cells can migrate, including fibronectin, laminin, collagen and vitronectin (reviewed in ref. 44). Which of these is used as the predominant migratory substratum is still not known. Numerous studies have attempted to perturb cell-matrix interactions by injecting function-blocking antibodies into the lumen of the somite and these have had little effect. Whether this is because the antibody is expelled from the somite or whether there is functional redundancy is not known. However, using the chick explant system, α4 integrin function was perturbed with function-blocking antibodies or a peptide that competes with fibronectin binding to the α4 receptor and neural crest cell migration was substantially inhibited.29 Because the α4β1 integrin largely mediates adhesion to fibronectin, it is reasonable to conclude that the neural crest cells at the trunk level use fibronectin as their primary migratory substratum (although thrombospondin-1, whose distribution is strictly correlated with neural crest migratory pathways, also binds to α4). There is still much to clarify, however, because neural crest cells are known to express other integrins, including those that mediate attachment to laminin30 and vitronectin.31 Moreover, not all neural crest cells express α4.32-34 Any analysis is bound to be complicated, because integrins can functionally compensate for each other.35

One additional extracellular matrix molecule that appears to stimulate migration and is present only in the anterior half of the somite is thrombospondin-1. It is distributed in the basal lamina of the neural tube along which neural crest cells first migrate and is associated with the basal surface of the myotome, on which the neural crest cells spread once they enter the somite.36 Moreover, thrombospondin-1 promotes adhesion and migration in vitro to approximately the same extent as fibronectin.

A combination of cues, stimulatory, inhibitory and permissive, determines the paths that neural crest cells follow ventrally. But the proper pathway of migration is not sufficient to generate the peripheral nervous system. Other factors must control when neural crest cells should stop migrating and how they must differentiate, which we will consider now.

Control of lineage segregation and migratory behavior in the ventral path. We introduced this review with the notion that there are two possible ways that the correct neural crest cell type will become localized and differentiate at the appropriate site: (1) either the neural crest cells are pluripotent and differentiate according to the environmental cues along their path of migration or (2) they are already specified before they disperse on the ventral path and arrive at the appropriate destination owing to cell-autonomous migratory properties that direct them. The melanoblasts are an example of a neural crest lineage that is specified prior to entering its path of migration. The question is whether there is any evidence that the ventrally migrating neural crest cells are also specified at the time they segregate from the neural tube and if so, do these lineages have unique migratory and path-finding capabilities?

When are neural crest lineages specified? The preponderance of evidence has supported the view that, as a population, the neural crest is pluripotent. The classic heterotopic transplant studies of Le Douarin and Teillet show clearly that neural crest cells from one axial level will migrate and differentiate according to another axial level if they are transplanted there.65 Subsequent studies in which neural crest-derived structures, such as autonomic ganglia, were back-transplanted to the early migratory pathway also reveal that neural crest cells that had already migrated to one spot are capable of remigrating and giving rise to a variety of phenotypes.66-68 These studies all focused on populations of neural crest cells and not individual cell capabilities, but at the very least they demonstrate that the neural crest population, as a whole, is extremely plastic.

The developmental potential of individual neural crest cells has been tested by employing the limit-dilution cloning strategies first developed by Sieber-Blum and Cohen.69 By taking neural crest cells that had emigrated from neural tubes in culture and subcloning them, they and other investigators since have established that some neural crest cells are capable of differentiating into a range of phenotypes. For example, neural crest cells derived from the thoracic axial level can give rise to clones that contain neurons, glial cells and pigment cells.69 Clones derived from cranial neural folds are even more diverse in their developmental repertoire.70-73 Similarly, in vivo labeling of single dorsal neural epithelial cells and early migratory neural crest cells with rhodamine dextran as a lineage marker has revealed that the resulting clones give rise to neural crest cells that are found in a variety of structures, although how they are actually differentiating has not been rigorously tested owing to the eventual dilution of the injected marker and the lack of availability of cell-type-specific markers.73,74 These studies give the clear impression...
that at least some neural crest cells are multipotent. Moreover, even differentiated neural crest cells have been observed to switch their phenotype when placed in a different environment.\textsuperscript{75,76} Consequently, the prevailing belief is that the patterning of the ventrally distributed neural crest is dependent upon environmental cues that control neural crest cell differentiation.

It is now clear that the neural crest is a much more heterogeneous population of cells than the above studies predicted.\textsuperscript{77,79} First, numerous markers for many of the crest lineages have been developed, and these have revealed a level of heterogeneity in the early migratory crest that was not initially suspected. However, these markers cannot be used to infer developmental potential or specification.\textsuperscript{77} Second, when data from the cloning studies cited above are examined, a large percentage of clones is discovered to be comprised of only one cell type, suggesting that at the time a particular neural crest cell was isolated, it was already fate-restricted. But it is a study by Henion and Weston\textsuperscript{3} that unequivocally demonstrates that a large number of neural crest cells are already fate-restricted at the time they leave the neural tube and, together with some other studies in zebrafish,\textsuperscript{80} suggests that we should reevaluate the mechanisms that control patterning of the ventral migrating crest.

Henion and Weston addressed the state of specification of trunk neural crest cells by culturing quail neural tubes, labeling a single neural crest cell with lyseinated rhodamine dextran as it emerged from the neural tube, and then assessing the phenotypes of the cells in the resulting clone, using lineage-specific markers for neurons, glial cells and melanocytes.\textsuperscript{3} There are several advantages of this approach over traditional cloning techniques: (1) limit-dilution cloning studies generally use 24 h outgrowths, which have already changed a great deal in their developmental potential and (2) the neural crest cells in the Henion and Weston study remain as an interacting population and so could influence each other’s development as they might in the embryo. Further, they assessed the phenotypes that arise from clones whose founder cells were labeled at different times after their emergence from the neural tube: at 6 h, between 13–16 h and at 30–36 h. Their results were surprising. When the phenotypic capabilities were assessed after only 6 h of migration, 44.5% of the clones gave rise to only one cell type (i.e., neuron, glial or melanocyte), showing that at the initiation of migration almost half the neural crest cells were already lineage restricted. By 13–16 h after the initiation of migration, 72.2% gave rise to only one cell type. The rest of the clones at 6 h were partially restricted, containing either neurons and glial cells or glial cells and melanocytes, showing that the neuronal and melanocyte lineages are already segregated at the time they detach from the neural tube. Almost no pigment cells differentiate from cells migrating during the initial 6 h. In a complementary study, Reedy and colleagues showed that when quail neural tubes are serially replated, there are no pigment cells that emerge during the first 6 h, and only during later stages of migration do pigment cells progressively appear.\textsuperscript{8} Recent in vivo lineage studies from the Lefcourt lab show unequivocally that there are specified progenitors for the neurons and glial cells of the DRG and these are temporally and spatially segregated from other premigratory neural crest cells (reviewed in ref. 81–83).

The unsuspected level of heterogeneity in developmental potential of the early migratory neural crest implies that the patterns of neural crest cell migration may be, in part, regulated by cell autonomous migratory properties that accompany lineage restriction. In the following discussion, we will review the evidence that this model accounts for the migration, localization, proliferation and maintenance of sensory neurons, sympathetic neurons and enteric neurons.

**Sensory neuron lineage.** Sensory neurons differentiate from the neural crest cells that coalesce adjacent to the dorsal neural tube and form the segmental dorsal root or sensory ganglion.\textsuperscript{23,84} The dorsal positioning of the DRG is almost certainly due to some cue from the dorsal neural tube. If the neural tube is transplanted into nonspecific mesoderm, such as the limb bud or lateral plate mesoderm,\textsuperscript{85,86} or the neural tube is rotated around its dorsoventral axis,\textsuperscript{86,87} the ganglion always develops adjacent to the dorsal neural tube and independent of any mesodermal influence. Conversely, the segmentation of the DRG is controlled by the somite, as we have described above. If somites are constructed that are comprised of two anterior halves, a large unsegmented ganglion develops.\textsuperscript{87,88} If a somite is comprised of two posterior halves, a very small unsegmented ganglion forms dorsal to the somite itself.\textsuperscript{88} However, an important question remains: are sensory neuron precursors determined early, and are they uniquely able to respond to these cues to form a ganglion?

**When does the sensory lineage arise?** In vitro clonal analysis reveals that at least some neural crest cells are able to give rise to both sensory neurons and catecholaminergic neurons.\textsuperscript{89} However, this same study suggests that there are also cells that become fate-restricted early in the culture period. Clonal analysis of migratory neural crest cells in vivo also shows that fully 25% of all clones analyzed contribute only to the DRG.\textsuperscript{89} Back-transplantations of sympathetic ganglia or enteric neurons fail to give rise to any sensory neurons, again pointing to an early segregation of this lineage.\textsuperscript{88,91} These disparate results suggest that there may be multiple lineages that produce sensory neurons.

A number of studies support the idea that the sensory ganglion is comprised of a variety of lineages that are spatially, temporally and molecularly distinct (reviewed in ref. 83 and 92). First, the avian ganglion consists of neurons in the dorsomedial and ventrolateral quadrants that are morphologically dissimilar and undergo apoptosis at different times. Second, using the SSEA marker for sensory neurons, two sources of sensory neurons have been identified in outgrowth cultures: (1) cells that differentiate early in the center of the outgrowth in association with remnants of the neural tube and (2) cells that differentiate later that are
on the periphery of the outgrowth.97 Third, analysis of cells that emigrate from the neural tube show that components of the sensory ganglion migrate in three temporal waves: an early- and a late-migrating population of neural crest cells, as determined by lineage analysis of virally infected neural epithelium in chick.95 EGFP-labeled premigratory neural crest cells91 or neurogenin-expressing cells in mice,94 and a third wave from the boundary cap cells,95,96 which also give rise to pigment cells. And, as discussed in the next paragraph, several studies reveal that there are markers that identify at least a portion of the sensory lineage, and these markers are already expressed in premigratory neural crest cells.

One marker expressed by chick premigratory crest and later in some cells that settle in the DRG, as determined by in situ hybridization, is the trkC receptor for the neurotrophin, NT-3.97 However, this is questionable since detailed studies examining the expression of trkA, B and C by immunocytochemistry reveal that these markers are displayed only after migration begins.98 Other markers of a subpopulation of sensory neurons are two members of the basic helix-loop-helix transcription factor, neurogenin.94,99 Ngn2 expression coincides with the first wave (St. 15–18)83 of DRG precursor migration and neurogenesis whereas ngn1 is expressed by the second wave (St. 19–22). The neurogenins are important in ganglion development, because in neurogenin-null mice, the sensory lineage does not develop and ectopic expression of chick neurogenins in other tissues, including non-neuronal tissue such as the dermamyotome, results in the expression of sensory neuron-specific markers.99

It seems reasonable to conclude that although some neural crest cells retain multipotency and can give rise to a variety of neurons, one or several subpopulations of the crest are already restricted to the sensory lineage at the time they initiate migration.

Do these lineages selectively migrate to the site of the DRG? The expression of these sensory lineage markers early in migration suggests that there are one or several distinct sensory lineages that may already be specified. If this is so, is there any evidence that they selectively migrate to the site of the developing DRG? In a provocative experiment, Perez and colleagues overexpressed the neurogenins in the premigratory crest using RCAS viruses, and found that most of the crest cells expressing neurogenin were localized in the DRG.99 Possibly, neurogenin expression promotes the migration of these cells to the DRG. Similarly, the lineage analysis of the second wave of neurogenesis in the avian DRG by George et al.81 shows clearly that one subpopulation derived from the more lateral dorsal neural tube migrates ipsilaterally and localizes to the core of the developing DRG, whereas the medi ally derived cells migrate both ipsilaterally and contralaterally and form a perimeter around the DRG. Not only do these different subpopulations give rise to different types of neurons, showing that they are lineage specified at the time of migration, but also this specification results in different migratory behavior.

There does appear to be at least one receptor/ligand pair that promotes chemotaxis of sensory neuronal precursors to the correct site. Belmadani and colleagues observed in the mouse that the receptor CXCR4 is distributed on neural crest cells found in the developing DRG and its ligand SDF-1 is expressed along their migratory route.100 In an in vitro chemotaxis assay, this lineage of the neural crest will chemotact to a source of SDF-1 and when CXCR4 is knocked out in the mouse, many neural crest cells remain dorsal to the site of the DRG, which develops abnormally. The role of CXCR4/SDF-1 in the development of the chick DRG is not known, although the expression of SDF-1 at the time of coalescence of the DRG is in the ectoderm and not the neural tube or somite, although later it is expressed around the dorsal aorta.101 Therefore it may not have the same function in chick DRG development as the mouse.

Sympathoadrenal lineage. Neural crest cells give rise to at least three catecholamine-containing cells types in the peripheral nervous system: the adrenergic neurons of the sympathetic ganglia, the chromaffin cells (or pheochromocytes) of the adrenal gland, and a third cell type called small intensively fluorescent cells, which are found in the sympathetic ganglia, the adrenal medulla and also in the carotid body and in small paraganglia in the gut.102 All of these catecholaminergic cells are thought to arise from a common progenitor cell, which initially localizes in the primary sympathetic chain by the dorsal aorta.26,103 From this site, the cells then disperse dorsally to form the definitive sympathetic ganglia and also ventrally to the developing kidney, where they contribute to the pheochromocytes of the adrenal gland. Experimental evidence is consistent with the notion that this lineage first differentiates into neurons,26 and the neuronal traits are subsequently lost when they migrate to the site of the adrenal gland. The loss of neuronal traits is likely due to exposure to glucocorticoids in the developing adrenal gland.25 However, there is some recent evidence to suggest that there may also be a separate chromaffin sublineage88 so that the relationship of the chromaffin and sympathetic lineages is now in question (reviewed in ref. 105). Experimental studies have identified environmental signals from the ventral neural tube, notochord and somite that are required for sympathoblast differentiation.106,107 These include exposure to norepinephrine (NE) produced by the notochord,108 and BMPs produced at the dorsal aorta that provide instructive signals to control neuronal differentiation109-113 (reviewed in ref. 105).

There is no direct evidence for a fate-restricted sympathoadrenal precursor cell in the early migratory neural crest.77 Rather, evidence from studies using chick and rat model systems suggest that the sympathoadrenal lineage is derived from multipotent, partially restricted progenitors that can also produce some sensory neurons and glial cells. For example, limit-dilution clonal studies by Sieber-Blum produced single clones that contain both adrenergic and sensory neurons.89 Moreover, markers for adrenergic neurons do not appear until well after migration has begun. The earliest-known markers for these neurons include the uptake system for NE,114 MASH1 and Phox2 genes,115-117 which appear just prior to or after contact with the dorsal aorta. Some evidence has been interpreted to support an early segregation of the sensory and autonomic lineages in the early migratory neural crest. For example, when six-day sympathetic ganglia were back-grafted into the early migratory pathway, these neural crest cells re-migrated and gave rise to glial cells and sympathetic neurons, but never to sensory neurons.68 However, such studies could just as easily be interpreted to mean that lineage restrictions occur
relatively late and after migration is complete. The most parsimonious conclusion to be drawn from the current data is that cells of the sympathoadrenal lineage are derived from precursors that are multipotent as they are migrating, and that their specification and differentiation is controlled largely by environmental signals.

Although the sympathoadrenal lineage may not be fate-restricted at the time the neural crest cells initiate migration, there may still be cell autonomous migratory cues that direct some neural crest cells ventrally. Britsch and colleagues have shown that in knockout mice for the EGF-like growth factor neuregulin-1 or for its receptors ErbB2 or ErbB3, that neural crest cells fail to arrive at the site of sympathetic ganglion formation adjacent to the dorsal aorta, and these mice never develop an adrenal medulla or secondary ganglia. The absence of ventral neural crest cells is not believed to be due to apoptosis, as revealed by TUNEL assay, although apoptosis may have been missed since it occurs so quickly. The accumulation of excess neural crest cells in the region of the sensory ganglion suggests that the phenotype is due to the failure of neural crest cells to move ventrally below the neural tube. Moreover the distribution of neuregulin-1 is compatible with a role in directing the neural crest either by chemotactic or haptotactic cues; initially it is present in the sclerotome of the somite, and eventually it is focused in a ventral stripe adjacent to the dorsal aorta. Finally, if this receptor-ligand system is guiding the crest, then the ErbB2/ErbB3 receptor dimer should be present on early migrating neural crest cells, and it is. Similar results have been reported in the zebrafish. If neuregulins pattern neural crest migration, then neural crest cells are predicted to accumulate at sites of ectopically expressed neuregulin. Also, the ErbB2 receptor may be a marker for a sympathetic lineage that segregated early from the sensory lineage. Finally, it will be interesting to see if a similar expression pattern exists in the chick embryo, in which experimental manipulation would be possible to show directly if neuregulin-1 is a positive guidance cue that directs a subpopulation of neural crest cells ventrally.

Schwarz and colleagues have recently shown that various subpopulations of ventrally migrating crest in the mouse express different combinations of neuropilin receptors. The first neural crest cells to migrate ventrally do so in the intersomitic space along the intersomitic blood vessels and are Nrpl-negative. These cells are the first to reach the dorsal aorta and are likely to be the founders of the sympathetic ganglia. The later crest cells to reach the developing sympathetic chain are primarily Nrpl-positive, whereas the founders of the DRG are Nrp2-positive. These observations suggest that the ventrally migrating neural crest cells are molecularly distinct and the combination of neuropilin receptors plays a role in their final distribution, as described above.

It is unclear what directs neural crest cells to the dorsal aorta, where they stop and spread longitudinally, although neuregulin is likely to play a role. An interesting question is what regulates segmentation of the sympathetic ganglia once they reach the aorta. Kasemeier-Kulla and colleagues observed using time-lapse recording in slice cultures that neural crest cells migrate longitudinally along the dorsal aorta but eventually cluster to form segmental ganglia. The molecular basis for this is at least two-fold: these crest cells bear the EphB2 receptor whereas ephrin-B1 is expressed in the interganglionic mesenchyme, which apparently repels neural crest cells. Secondly, N-cadherin is expressed by these sympathetic precursors and allows them to coalesce. Perturbing either of these signaling systems inhibits gangliogenesis.

**Dorsolateral migration.** By stages 19–20, beginning at the wing-bud level, dorsolateral migration is initiated (Fig. 1) as ventral migration gradually ceases. The reason for the onset of neural crest migration into the dorsolateral path was originally suggested to be a gradual loss of inhibitory molecules, namely chondroitin sulfate proteoglycan and PNA-binding substances, which appeared to retreat from the dorsolateral path in advance of neural crest invasion, potentially rendering the dorsolateral path permissive for migration. Some experimental evidence seemed to support this view. If the dermamyotome, which presumably produced the inhibitory cue, was ablated prior to the onset of neural crest migration, precocious invasion of the dorsolateral path was observed. However, when melanoblasts are grafted adjacent to the neural tube in younger embryos (stage 13/14), they immediately migrate dorsolaterally, and well in advance of the time that dorsolateral migration usually takes place. In contrast, non-melanogenic neural crest cells grafted into the early pathway only migrate ventrally. In fact, melanoblasts are the only neural crest subpopulation tested that is capable of migrating into the dorsolateral path. Moreover, there appears to be minimal change in the environment to facilitate dorsolateral migration, because non-melanogenic crest grafted into the embryo at stage 19, which is when dorsolateral migration begins, still only migrate ventrally. These observations suggested that melanoblasts acquire distinctive properties as they are specified that permit them to migrate dorsolaterally. Other studies, which exploited markers for melanoblasts (serum from Smyth line chickens, MEBL-1, MITF), revealed that neural crest cells are specified as pigment cells prior to entering the dorsolateral path, and almost all of the neural crest cells in the dorsolateral path at all stages express melanoblast-specific markers. Taken together, these data show that in order to migrate into the dorsolateral path, a neural crest cell must already be differentiating into a pigment cell.

What molecular properties allow melanoblasts to access the dorsolateral path? There is now significant information about the mechanisms that direct or permit melanoblast migration in the dorsolateral path. All of these result from molecular changes that occur prior to the onset of dorsolateral migration and are cell autonomous changes in the neural crest cells themselves, rather than in the environment.

It appears that melanoblasts develop unique sensitivity to positive guidance cues from the dermamyotome or ectoderm, allowing them to overcome inhibitory cues in the dorsolateral path. In the mouse, melanoblasts depend upon Steel factor produced by the dermamyotome for their initial dispersal onto the dorsolateral path, and they express the receptor for Steel factor, c-kit, prior to embarking on the dorsolateral path. In the avian embryo, however, c-kit is first expressed by melanoblasts long...
after they have migrated dorsolaterally (stage 25) and Steel factor is not produced by the dermamyotome, but rather by the ectoderm and only after stage 25. Moreover siRNA knockdown of c-kit in chick neural crest cells has no effect on melanoblast migration. Thus, acquiring responsiveness to Steel factor owing to the expression of c-kit does not control timing of dorsolateral migration in the chicken.

The first identified factor that stimulates migration of melanoblasts in the chick is ephrin-B1. The ephrins were initially found to trigger the collapse response of neurons and as discussed already, ephrin-B1 inhibits migration of neural crest cells through the posterior somite. Ephrin-B1 is also expressed in the dorsolateral pathway and was predicted to inhibit neural and glial precursors from taking this path. Inhibiting ephrin signaling, either by using Fc-fusion proteins or by knocking down Eph receptors using siRNA, results in the inappropriate invasion of neural and glial precursors into the dorsolateral space. How then do melanoblasts overcome this inhibitory cue? The surprising answer is that ephrins stimulate the migration of melanoblasts. This difference in behavior is receptor specific. EphB3 triggers the collapse response and EphB2 regulates the migration response. As melanoblasts differentiate they upregulate the expression of EphB2. Whether this stimulatory behavior is the result of increasing adhesiveness to the fibronectin substratum or whether it acts as a chemotactic factor is not known, although in vitro assays indicate that ephrins can act in either capacity.

A more recently described chemotactic molecule for melanoblasts is endothelin-3 (ET3). ET3 is expressed by the ectoderm and dermamyotome in early chicken embryos at the time that neural and glial precursors migrate ventrally. The ventrally migrating neural crest cells express the endothelin receptor EDNRB. Later, as melanoblasts are specified in the migration staging area, they downregulate EDNRB and upregulate EDNRB2. This switch in receptors suggested that EDNRB2/ET3 signaling might positively regulate dorsolateral migration. shRNA knockdown of EDNRB2 inhibits melanoblast migration, whereas misexpression of EDNRB2 in neural and glial precursors allows their invasion of the dorsolateral path. Remarkably if EphB2 is knocked down but EDNRB2 is overexpressed, dorsolateral migration is rescued. These latter results show that the increase in total expression level of these receptors, and not the specific receptor itself, allows for overcoming negative cues in the dorsolateral path. This suggests that precise regulation of receptor expression is not necessary; rather, several receptors loosely regulated can accomplish the task of allowing melanoblasts to invade this hostile pathway.

Undoubtedly these two receptors are not the whole story. It has been reported that the Robo1 receptor is expressed on neural and glial precursors and its ligand Slit is expressed in the dorsolateral space. Dominant-negative inhibition of Robo1 function allows neural crest cells to invade the dorsolateral path precociously. There appears to be some downregulation of Robo1 and 2 in late-migrating neural crest, suggesting that melanoblasts are refractory to the inhibitory Slit cue by losing the Robo receptors. These data point to the fact that control of pathfinding is likely regulated by a complex regulatory network in which inhibitory signaling receptors are removed and stimulatory receptors are expressed.

The Cranial Neural Crest

Migration of the neural crest cells at the cranial level. At the cranial level, neural crest cells migrate predominantly in the dorsolateral pathway (Fig. 2) (i.e., between the ectoderm and the underlying mesoderm), and they differentiate into the various components of the head and neck. The mesectodermal origin of the facial skeleton and cartilage is restricted to the cranial neural crest as far posterior as the level of somite 5. Although initial grafting experiments of Noden suggested that the cranial neural crest is prespecified, it is now clear that this cell population is extremely plastic in its migratory behavior and fate, as described in the following sections.

Patterns of migration in the dorsolateral pathway. The migratory behavior of the cranial neural crest was described by using orthotopic quail transplantation into chick embryo hosts. This study showed that there are very few neural crest cells originating from the anterior-most region at the prosencephalon, whereas there is an abundance of cells from the diencephalon and rostral mesencephalon that begin to migrate rostrally by stage 10. These cells then migrate ventrally toward and around the optic stalks and the anterior portion of the head (prosencephalon). The cells from the posterior mesencephalon migrate laterally under the ectoderm by stage 12 and colonize the first branchial arch (BA1) to give rise to parts of the mandible. Finally, the neural crest cells from the metencephalon and myelencephalon, which can be subdivided into seven segments known as rhombomeres, migrate laterally and ventrally to form three streams of cells that invade the branchial arches. A more detailed distribution of the cranial neural crest (Fig. 3) from each rhombomere was determined by quail neural tube transplantations into the chick and Dil labeling. Cranial neural crest cells from the posterior mesencephalon and r1-r2 populate the branchial arch 1 (BA1), with a small contribution from r3, which remains predominantly crest-free. Neural crest cells from the level of r4 contribute extensively to BA2, with a minute contribution from r3 and r5. Finally, the third branchial arch (BA3) is populated by neural crest cells from the level of r6 and r7, with a small contribution from r5. The many specific components that differentiate from each of the branchial arches are detailed elsewhere.

When neural crest cells emigrate from the midbrain they move as an unsegmented sheet beneath the ectoderm. However, at the hindbrain neural crest cells form separate streams that subsequently invade the branchial arches. Specifically neural crest cells from r1 and r2 form a stream that fills BA1. Neural crest cells from r4 invade BA2 and neural crest cells from r6, 7 invade BA3. Relatively few neural crest cells emigrate from r3 and r5 and those that do migrate anteriorly and posteriorly to join the
Adjacent to r3 or r5, they fail to migrate laterally, suggesting that the crest-free space is imposed by the environment and is not a cell-autonomous effect. Time-lapse images show that if cells from r3 or r5 very rarely wander into the crest-free space adjacent to r3 and r5, they round up, collapse their filopodia and lamellipodia or reverse their migration and join an adjacent neural crest stream. Several studies have shown that the receptors Nrp1 and Nrp2 and their ligands SEMA3A and SEMA3F play a role in the segmental migration of the cranial neural crest and in gangliogenesis. Moreover, when neuropilin/semaphorin signaling is perturbed using neuropilin-Fc fusion proteins there are defects in stream formation and invasion of the arches, although the specificity of the probes is suspect. However, when Nrp2 is specifically inhibited either in mouse mutants or using siRNA knockdowns in

Figure 2. Cranial neural crest cells migrate in the dorsolateral pathway. Beginning at stage 10, cranial neural crest cells migrate dorsolaterally, between the ectoderm and the underlying paraxial mesoderm (grey shading). Representative cross-sections at stages 10 (St 10), 12 (St 12) and 13 (St 13). Once the neural crest cells reach the first branchial arch (labeled BA1 in the St 14 section), they migrate on both sides of the muscles (orange shading) within the forming jaw. The thick bar indicates the level of the representative cross sections. BA1, branchial arch 1; BA2, branchial arch 2; BA3, branchial arch 3; BA4, branchial arch 4; DA, dorsal aorta; CV, cardinal vein; S1, somite 1; S2, somite 2; S3, somite 3; Ph, pharynx; r1-7, rhombomeres 1–7.
chick, neural crest cells from the first and second branchial arch streams cross between each other, suggesting that SEMA3F is at least partially responsible for the crest-free region. Moreover, the trigeminal ganglion is not properly condensed. Nrp1 signaling is more complex. When Nrp1 is knocked down in chick using Nrp1 siRNA, stream formation is not affected. However, the neural crest cells fail to invade BA2 fully. Confusing this analysis is the fact that Nrp1 is a receptor for SEMA3A and also for vascular endothelial cell growth factor (VEGF). When VEGF is reduced instead of Nrp1, the phenotype is the same as Nrp1 knockdown, suggesting that when Nrp1 signaling is perturbed, it is the VEGF response that is inhibited. Therefore the potential role of SEMA3A in stream formation is unclear. The most parsimonious model is that Nrp2/SEMA3F signaling creates the r3 and r5 crest-free spaces, whereas VEGF is a chemoattractant that positively directs neural crest cells into BA2 (its role in other arches has not been reported).

It is unlikely that the semaphorins and VEGF act alone. For example, the ErbB4 tyrosine kinase receptor and its ligand neuregulin have been implicated in the non-cell autonomous control of cranial neural crest cell migration by regulating the expression of repulsive cues at the r2/r3 and r3/r4 boundaries and therefore resulting in the r3 crest-free region. Golding and colleagues report that, in the mouse, the expression of ErbB4 is localized to the r3 neuroepithelium whereas neuregulin is expressed in r2 and r4 neuroepithelium. When wild-type mouse neural crest cells from the level of r4 are isotopically transplanted in ErbB4 knockout mouse, they invade the normally crest-free region adjacent to r3. Therefore, the authors suggest that the ErbB4-neuregulin signaling results in the production of a putative repulsive cue that repels the migration of r2- and r4-derived neural crest cells from the region adjacent to r3. The putative repulsive cues remain to be characterized.

Eph/ephrin signaling has also been implicated in the migration of the cranial neural crest. In Xenopus, even though the cranial neural crest cells do not initially form distinct streams, the cells within them nevertheless do not mix. This is as a result of Eph/ephrin signaling. Their role in chick and mouse is not so clear-cut. In the mouse, ephrin-B2 is expressed in the ectoderm of the branchial arches and the neural epithelium. When ephrin-B2 is knocked down in mutant mouse lines, neural crest cells fail to migrate into BA2 but the cranial crest streams are still segregated. On the other hand ephrin-B1 is expressed on cranial neural crest cells, and in null mutant mice it acts cell autonomously to result in neural crest wandering between streams migrating into BA3 and BA4. It is not known what Eph receptors are involved in this signaling or where they are expressed (on the crest, in the adjacent ectoderm or mesoderm). Moreover, the situation is complicated because these mutations result in defects in different branchial arches. No experimental perturbation of Eph receptors or ephrins has been reported in the chick but in situ hybridization suggests that complementary expression of EphA and B receptors on the cranial crest and ephrin-B1 ligand and EphB2 by non-neural cells in the crest-free spaces may also play a role of segregating streams and directional migration into the branchial arches.

We are very far from a detailed understanding of what directs cranial neural crest cells from the neural tube, what keeps them in streams and what directs them into the branchial arches. While a few signaling systems have been identified, there are others likely to be discovered in the future. For example, the chemokine Sdf1 and its receptors Cxcr4 and Cxcr7 have recently been identified in zebrafish to direct cranial neural crest cells into the arches and to play a role in the condensation of the ventral cartilages.

**Control of lineage segregation of the cranial neural crest cells.** We previously discussed that melanoblasts are an example of a neural crest lineage that is specified prior to entering its path of migration. Moreover, as detailed above, there is significant evidence that the ventrally migrating neural crest cells are also specified at the time they segregate from the neural tube, and have some unique migratory and pathfinding capabilities (reviewed in refs. 81–83).

To address the developmental potential of the cranial neural crest cells, previous studies have used the limit-dilution cloning techniques on isolated quail neural crest cells. The results of these studies show that the cranial neural crest is a developmentally heterogeneous cell population composed of multipotent, partially fate-restricted and specified cells. However, these clonal analyses used neural crest cells derived from 24 h cultures, and therefore do not address whether they become fate-restricted over time or whether the majority of the cells truly remain multipotent and differentiate according to the environmental cues they encounter. As far as we are aware there are no studies similar to Henion and Weston’s that examine lineage
restriction using lineage-marking techniques, nor cell-marking experiments in vivo similar to George et al. Thus, lineage restriction at the time of cranial crest emigration has not been tested directly.

Although any given neural crest cell appears to have considerable flexibility to differentiate into a neuron, a glial cell, ectomesenchyme or a pigment cell, there is evidence for lineage restriction along the anterior/posterior axis. Moreover the expression pattern of the Hox genes confers a specific axial identity (reviewed in refs. 163 and 164). The neural tube rostral to r3 is Hox-negative, whereas the posterior region that spans r3 to r8 is Hox-positive, thus creating a differential expression pattern along the anterior/posterior axis. Transplantation of quail neural tubes into chick embryo hosts has shown that the neural crest cells that originate from the Hox-negative region contribute to the frontonasal and facial skeletal, whereas the neural crest cells that arise from the Hox-positive region give rise to more limited skeletal derivatives such as the hyoid cartilage. Mouse experiments in vivo similar to George et al. Thus, lineage restriction using lineage-marking techniques, nor cell-marking experiments in vivo similar to George et al. Thus, lineage restriction at the time of cranial crest emigration has not been tested directly.

Further evidence for axial level specification of the neural crest came from a transplantation study carried out by Noden, who showed that r1-r2 neural crest cells (presumptive BA1) transplanted into the r4 region (presumptive BA2 neural crest cells) results in the migration of the transplanted cells into BA2 where the lower jaw is duplicated, although some BA2 structures are also formed. This initially suggested that the cranial neural crest was predominantly prespecified, and that the transplanted cells migrate according to their new environment but are patterned according to their origin. However, the work of Couly and colleagues showed that Noden’s results could only be duplicated when the neural tube was included in the graft, and that the neural fold was insufficient to duplicate the lower jaw. Subsequently, Trainor proposed that the isthmus, and its expression of FGFr8, is responsible for the conserved skeletogenic (BA1-derivatives) differentiation of the transplanted presumptive-BA1 neural crest cells. Briefly, when the presumptive-BA1 neural crest cells are transplanted to the level of r4 without the isthmus, the transplanted cells migrate into BA2 where they differentiate into BA2-derivatives. This study also shows that FGFr8 represses Hoxa2, which is proposed to be the reason for the lack of Hox expression in the neural tube and the neural crest cells that originate from regions anterior to r2; it is also the basis for the skeletogenic differentiation of the neural crest cells that originate from that axial level. This is further supported by the fact that the ectopic expression of Hoxa2 in all BA1 tissue, including the ectoderm, leads to the differentiation of BA1 into BA2 structures. Thus, specification appears to depend upon signaling from the isthmus.

Whether heterotopic grafting results in a change in neural crest patterning or not depends on the number of cells transplanted. If a large cohort is grafted, they differentiate according to their origin, whereas if only a few cells are transplanted they are extremely plastic and develop according to their new environment. Similarly if small numbers of cranial neural crest cells are diverted experimentally, they will differentiate according to their new pathway. We conclude that the cranial neural crest is very plastic in its developmental potential.

**Does specification dictate pathway choice?** Given this plasticity, it is not surprising that there appears to be little evidence for the idea that prior specification dictates pathway choice. The only study that addresses directly this idea is the work of Baker and colleagues. They showed that the early (stage 9) mesencephalic neural crest cells contribute to both dorsal (i.e., melanocytes, dorsal dermis and neurons of the ciliary ganglion) and ventral structures (i.e., Schwann cells and cartilage, bone and dermis of the jaw), whereas the late migrating neural crest cells (stage 11) give rise to more dorsal structures and much less cartilage and bone of the jaw. When heterochronic transplants of early and late migrating crest cells were performed, the transplanted cells behave like the endogenous neural crest cells. Especially interesting was the observation that when mesencephalic neural crest cells were ablated and then late neural crest cells were grafted into a late embryo, the late-migrating neural crest cells are able to invade the branchial arches, presumably because their migration is not interfered with by early migrating crest. This is the soundest evidence yet that prior specification of early vs. late crest does not dictate migratory behavior. However, given the plasticity of the cranial neural crest lineage and the importance of the environment in directing their differentiation, these results are not surprising. Potentially there could still be cell autonomous cues that dictate migration into the arches vs. dorsal aggregation to form cranial ganglia, but these might never be detected in light of the extreme plasticity noted in the above studies.

Although it is clear that the pre- and early-migrating cranial neural crest cells are plastic, Keown and colleagues showed that this plasticity is gradually lost as they reach their final destinations. For instance, when neural crest cells taken from the arches are transplanted into younger embryo hosts (stage 7–11), their ability to contribute to the trigeminal ganglia and to migrate to BA1 decreases. Moreover, when neural crest cells taken from the trigeminal ganglia (stage 14–19 embryos) are transplanted into younger embryo hosts (stage 7–11), these cells are able to reach the host’s BA1, but they are unable to differentiate into ectomesenchyme. Finally, when stage-14 neural crest cells from the dorsolateral pathway or isolated from regions close to the branchial arches are transplanted into younger embryo hosts (stage 7–11), the former transplanted neural crest cells can migrate further and in greater number into BA1 than the latter. Therefore, the initial plasticity described by Baker and colleagues is progressively lost as neural crest cells age and as they reach more ventrolateral positions.

**The Neural Crest from the “Neck”:**

**A Hybrid Between the Cranial and the Trunk**

Migration of neural crest cells at the cardiac/vagal level. The border between rhombomere 8 and the spinal cord is considered to be at the intersomitic space between somite 4 and 5. This
is also the position of the occipitocervical junction. The vagal neural crest from somite level 1–7 appears to be a molecular and behavioral interface between the cranial and trunk neural crest and reflects an evolutionary transition from the brain to the spinal cord. The surrounding embryonic structures, namely the somites and the pharyngeal arches, are similarly superimposed in this region to suggest an overlapping transitional zone (the posterior arch 6 is at the same axial level as the somite 4/5 boundary). It is therefore a region of intense interest because it represents the area of merger between the head and trunk but whose evolution is unknown and is the topic of considerable speculation. Because of the important role that neural crest cells have played in the evolution of the vertebrate head and neck, we consider the neural crest cells from this region separately.

Neural crest cells from the post-otic region to the trunk level have been divided into different categories depending on the cell types to which they contribute. The “vagal” neural crest was initially identified by Le Douarin and her colleagues as derived from the region between somites 1–7 that gives rise to the enteric neurons (ENS) of the gut (reviewed in refs. 30, 65, 78, 79, 132 and 135) as well as a variety of other cell types, including neurons and glial cells of the peripheral nervous system, ectomesenchyme derivatives such as the cartilage of the neck and smooth muscle cells of the heart and the walls of the aortic arch arteries. Recent studies from Burns and colleagues have significantly refined our understanding of how the gut is populated. The “cardiac” neural crest was identified by ablation and chick-quail chimera transplantations. The ablation of neural crest cells from the level of somites 1–3 results in significant heart defects, which include outflow tract septation defects, persistent truncus arteriosus and double right outlet right ventricle. Isotopic migration from r6-r8 has been substantiated using time-lapse imaging. These authors introduced the concept of the circumparyngeal ridge, then populate the pharyngeal arches 3–6 and the outflow tract. They also migrate along the circumparyngeal ridge to the gut. By stage 13, neural crest cells from the level of somites 1–4 cease their migration in the dorsolateral pathway and begin to migrate in the ventral pathway, while those from the level of somites 5–7 continue their migration in the ventral pathway (Fig. 4). By stage 23, the ventrally-migrating cells from the level of somites 1–3 contribute to the sensory and sympathetic ganglia and the anterior foregut. The ventrally-migrating cells from the level of somites 4–6 migrate to the sensory and sympathetic ganglia and the foregut as far posteriorly as the stomach (Kuo and Erickson, submitted) (Fig. 5). The neural crest cells from the level of somite 7 only populate the sensory and sympathetic ganglia. Finally, by stage 21, the last wave of migrating cells, the melanoblasts, take the dorsolateral pathway to populate the ectoderm (Fig. 4). Therefore, the vagal neural crest cells from the level of somites 1–4 behave like the cranial crest (by first migrating dorsally), whereas the neural crest cells from the level of somites 5–7 behave like the trunk crest (by initially migrating in the ventral pathway). This highlights the transitional nature of the vagal neural crest, which shares characteristics found both at the cranial and trunk levels.

Refinement of the migratory pathways at the cardiac/vagal level.

We were interested in the behavior of neural crest cells once they arrive at the circumparyngeal ridge (Fig. 4). The ridge is a large mass of neural crest cells beneath the ectoderm and neural crest cells get there by migrating in the dorsolateral pathway. These cells are also contiguous with the medial mesoderm that surrounds the gut (see Fig. 5 for the anatomy of this region). A question that then concerned us is whether the cells that initially migrated in the dorsolateral space stayed dorsally and moved into the branchial arches or whether they could cross over into the ventral pathway and migrate into the gut. Conversely, did neural crest cells that initiated migration in the ventral pathway only invade the gut or could they cross over into the arches and populate the heart? Our specific concern was to understand whether pathway choice at the initiation of migration determined whether neural crest cells would migrate to the heart or the gut. To address
this question we differentially labeled the dorsal vs. ventral waves of neural crest cells by electroporation of GFP. We observed that ventrally migrating neural crest cells always continued into the gut and never crossed over to the branchial arches and the heart. Conversely, most dorsally migrating neural crest cells migrated to the arches and heart, with the exception of some cells from the level of somites 1–4 that traveled along the circumpharyngeal ridge to populate the foregut. These data suggest that early pathway choice is correlated with the final destination (heart vs. gut).

Mouse and chick neural crest cells behave differently at the trunk level (mouse neural crest cells invade the dorsolateral and ventral pathways simultaneously whereas in the chick dorsolateral migration is delayed by 24 h) and this seems to be the case at the vagal level. Chan and colleagues labeled mouse neural crest cells with DiI or gold-conjugated WGA and observed that at the vagal level, cell migration occurred simultaneously in both the ventral and dorsolateral pathway (as opposed to migrating initially in the dorsolateral pathway). Interestingly in the splotch mutant mouse, which shows severe defects in heart development, neural crest cells were not found in the dorsolateral pathway. This suggests that even in the mouse, those crest cells destined for the heart primarily take the dorsal pathway.

Molecular control of the vagal neural crest cell migration. The regulation of the migration of the vagal neural crest has not been investigated in as much detail as the cranial and the trunk neural crest but a few studies are instructive. In the chick, the semaphorins play a role directing the migration of the cardiac neural crest to the heart. Specifically, in situ hybridization shows that the Plexin-A2, Plexin-D1 and Nrp1 receptors are expressed by the cardiac neural crest cells, whereas SEMA3C ligand is expressed in the outflow tract of the heart and SEMA6A and 6B are expressed in the dorsal neural tube and in the pharyngeal mesenchyme. The authors show that there is a strong binding affinity between PlexinA2/Nrp1 and SEMA6A/6B, while PlexinD1/Nrp1 has a high affinity to SEMA3C. In vitro migration assays demonstrate that SEMA6A/6B repels the cardiac crest, but SEMA3C attracts them. These results suggest that SEMA6A/6B drives the cardiac crest cells away from the neural tube and SEMA3C in the outflow tract (the target) attracts them. The authors further knocked down PlexinA2, PlexinD1 and Nrp1 and observed that the downregulation of PlexinA2 leads to the accumulation of...
Moreover, the PlexinA2 mutant mouse has an outflow tract separation defect associated with a decrease of neural crest cells at the septation site. Together these data show that semaphorins play a critical role in directing cardiac neural crest cells to the heart.

Figure 5. For figure legend, see page 581.

neural crest cells in the dorsal region close to the neural tube with a significant absence from the outflow tract, whereas the downregulation of PlexinD1 and Nrp-1 results in an increase of neural crest cells in the region between the neural tube and the outflow tract and a significant absence in the outflow tract. Moreover, the PlexinA2 mutant mouse has an outflow tract separation defect associated with a decrease of neural crest cells at the septation site. Together these data show that semaphorins play a critical role in directing cardiac neural crest cells to the heart.
There are many reports regarding the migration of the enteric neural crest cells in the gut, but these are generally focused on migration along the length of the gut rather than attraction to the gut in the first place. RET/GDNF signaling is the most studied and an important signaling pathway that regulates the migration and the differentiation of the enteric neural crest since its absence leads to enteric nervous system disorders, including Hirschsprung’s disease (reviewed in ref. 195–201). Moreover, GDNF has been demonstrated to be a chemoattractant for enteric neural crest cells.198 Finally, experimentally increasing the expression levels of RET in sacral neural crest cells makes them more migratory, suggesting that GDNF might function to speed up vagal neural crest cells and allow them to reach the gut. However in most mouse mutations of Ret or GDNF, neural crest cells reach the gut but do not migrate posteriorly, so that attraction to the gut initially is more likely regulated by other cues.

The interactions between the secreted Slit ligands and the Robo receptors have been proposed to play a role in restricting the entry of trunk neural crest cells into the gut.200 Slit1, 2 and 3 are expressed in the gut mesenchyme, while the Robo receptors, Robo1 and 2, are expressed by the trunk neural crest and not the vagal neural crest. Furthermore, when Slit2-expressing cells are grafted in the ventral pathway in the trunk, they block the migration of trunk neural crest cells once they come to the vicinity of the graft. A later report by Jia and colleagues also shows that the Slit ligands act as a repulsive cue to confine the trunk neural crest cells to the ventral pathway.201 Therefore the presence of Slits in the gut mesenchyme acts to prevent trunk neural crest cells from entering the gut, whereas this block does not occur at the vagal level.

Control of lineage segregation of vagal neural crest cells. Trunk neural tube replating experiments showed that there is a developmental bias of neural crest cells depending on the time they emigrated from the neural tube.202 Similar experiments using vagal neural tubes reveal that the early-migrating crest from the level of somites 1–3 or 4–7 from stage 10–13 embryos primarily differentiate into smooth muscle cells (α-smooth-muscle-actin-positive immunoreactivity) whereas the late-migrating cell population (stage 16+) exhibits a dramatic shift toward melanogenic differentiation (Kuo and Erickson, submitted). Previous limit-dilution clonal analysis of the vagal crest demonstrate that there are two lineage-committed cells: smooth muscle cells and pigment cells.78,204,205 We predict that the early-migrating and late-migrating neural crest cells that differentiate into smooth muscle cells and pigment cells, respectively, are fate restricted. Accordingly we asked whether this developmental bias regulates pathways of migration and neural crest targets at the vagal level.

Does prespecification dictate migratory pathway choice at the vagal level? The results of the cell culture and the fate mapping studies suggest a correlation between the early specification of the vagal neural crest cells and the pathways they take to reach the heart and the gut. The presumptive cardiac neural crest populates the heart by migrating in the dorsolateral pathway, whereas the presumptive enteric neural crest cells reach the gut by migrating into the ventral pathway. To test whether the early migratory pathways taken by the vagal crest are controlled by their developmental bias heterotopic transplantations of quail neural tube into chick embryo hosts were performed (Kuo and Erickson, unpublished). When early-dorsolaterally-migrating neural crest cells from the level of somites 1–3 were transplanted into stage-matched embryos at the level of somites 5–7 or in older embryos at either the level of somites 1–3 or 5–7, the transplanted cells invade the ventral pathway, mimicking the behavior of the endogenous neural crest cells. Conversely, when we transplanted early-ventrally-migrating neural crest cells from the level of somites 5–7 into stage-matched embryos at the level of somites 1–3, the transplanted cells maintained their migration in the ventral pathway, rather than migrating dorsolaterally. The results show that the neural crest cells from the level of somite 1–3 are plastic in their migratory behavior and independent of their developmental bias, whereas the neural crest cells from the level of somite 5–7 are more restricted in their migratory behavior. The plasticity of the anterior vagal crest resembles that of the cranial crest,173 and the restriction of the more posterior vagal crest resembles that of the trunk crest.206,207 These observations further reinforce the transitional nature of the vagal axial level.

In the same study, we observed that 48 h after the neural crest cells from the level of somites 1–3 are transplanted into the level of somites 5–7, they migrate ventrally and posteriorly into the foregut more extensively than they normally do, to reach the stomach. However, these cells do not migrate anteriorly into the pharyngeal arches and the heart. Conversely, neural crest cells from the level of somites 5–7 transplanted to the level of somites 1–3 migrate ventrally but nevertheless populate the heart. Additionally they migrate into the gut and invade posteriorly up to the stomach (as they normally do) 48 h later. Since the transplanted cells from the level somites 5–7 can still reach the branchial arch mesenchyme and the outflow tract, even after...
initially migrating in the ventral pathway, this suggests that the initial pathway taken by the vagal neural crest does not restrict those cells to certain structures (i.e., heart or gut). Additionally, neural crest cells from the level of somites 5–7, which are biased toward a cardiac fate (see previous section), can still find their way to the outflow tract, albeit by an irregular pathway. These data suggest that cardiac neural crest migration is regulated by environmental cues, which involve, but are not limited to, the Semaphorins, present in the heart. It is not known why neural crest cells derived from somite-levels 5–7 are more migratory along the gut. Potentially they express higher level of RET, whose ligand GDNF is present in the gut.

Future Directions

The cardiac/vagal neural crest represents a hybrid population located in a transition zone between the head and the trunk. Aside from its intermediate position, these cells also share migratory and differentiation properties found at both axial levels. In addition, the vagal population exhibits some developmental bias, as evidenced by cell culture and experimental perturbations, but nevertheless displays considerable behavioral plasticity. Understanding the molecular, cellular and behavioral differences between these three populations of neural crest cells will be of enormous assistance when trying to understand the evolution of the neck.

Although most evidence shows that the cranial neural crest as a population is extremely plastic in its behavior, especially when cells are challenged in small numbers, studies similar to Henion and Weston or George et al. to assess lineage restriction have not been done with cranial neural crest cells and it still remains possible that some subpopulations of the cranial crest are specified early and irreversibly. We have raised more questions about the vagal neural crest than we have answered. If the first wave of migratory neural crest cells is biased toward a smooth muscle (cardiac) fate, why do the cells from somite-levels 1–3 take a different pathway than those from somite-level 5–7? How do the neural crest cells from somite-level 5–7 that are biased toward a smooth muscle fate differentiate in the embryo, since they do not migrate to the heart or branchial arches normally? Why do they not reach the outflow tract normally since they seem to be attracted in some fashion to the heart? What keeps the ventrally and dorsolaterally migrating populations of neural crest cells separate since they do not mix in the circumpharyngeal ridge? Is there a developmental difference in the enteric neural crest cells that come from the ventral pathway vs. those that arrive in the pharynx from BA6? What are the mechanisms that regulate pathway determination at the vagal level? A combination of newly developed techniques for the chick model system, including cell labeling and in vivo time-lapse imaging, combined with genetic analysis using mouse and zebrafish, will allow us to answer these questions.

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