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Control of neural crest cell behavior and migration

Insights from live imaging

Matthew R. Clay and Mary C. Halloran*

Departments of Zoology and Anatomy, and Cell and Molecular Biology Program; University of Wisconsin-Madison; Madison, WI USA

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Neural crest cells (NCCs) are a remarkable, dynamic group of cells that travel long distances in the embryo to reach their target sites. They are responsible for the formation of craniofacial bones and cartilage, neurons and glia in the peripheral nervous system and pigment cells. Live imaging of NCCs as they traverse the embryo has been critical to increasing our knowledge of their biology. NCCs exhibit multiple behaviors and communicate with each other and their environment along each step of their journey. Imaging combined with molecular manipulations has led to insights into the mechanisms controlling these behaviors. In this Review, we highlight studies that have used live imaging to provide novel insight into NCC migration and discuss how continued use of such techniques can advance our understanding of NCC biology.

Neural crest cells (NCCs) are a pluripotent population of cells that migrate from the dorsal neuroepithelium and give rise to multiple cell types including neurons and glia of the peripheral nervous system, pigment cells and craniofacial bone and cartilage.[1] An important hallmark of NCCs is their remarkable ability to migrate over long distances and along specific pathways through the embryo. NCC migration begins with an epithelial to mesenchymal transition (EMT), in which NCCs lose adhesions with their neighbors and segregate from the neuroepithelium.[2,3] Following EMT, NCCs acquire a polarized morphology and initiate directed migration away from the neural tube. While migrating along their pathways to their target tissues, NCCs are guided by extensive communication with one another and by other cues from the extracellular environment. Each of these aspects of NCC migration requires precise regulation of cell motile behaviors, although the mechanisms controlling them are still not well understood. A critical step toward understanding the molecular control of NCC motility is characterization of NCC behaviors as they migrate in their native environment. In the past 15 years, multiple studies have analyzed specific behaviors associated with NCCs along the various stages of their journey and have begun to identify molecules controlling these behaviors. In this review we will focus specifically on these studies that employ live imaging and will highlight the strength of live imaging to reveal mechanisms regulating NCC motility and migration pathways.

*Correspondence to: Mary C. Halloran; Email: mchalloran@wisc.edu
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Epithelial to Mesenchymal Transition

The onset of directed NCC migration is preceded by EMT, which is a dramatic, multistep process wherein cells lose epithelial adhesions, acquire motility and segregate from the neuroepithelium.[2,3] Only some cells in the neuroepithelium become NCCs and undergo EMT, while others remain in the neuroepithelium and become part of the central nervous system. Thus, NCCs must disassemble adhesions while other neighboring cells maintain them. Precise regulation of these dynamic processes is therefore essential for proper development of both the neural tube and NCC derivatives, yet how they are coordinated and regulated in vivo remains poorly understood.

Two recent studies have used live imaging to characterize NCC behaviors before and during EMT while cells are in their native environment. These studies of either zebrafish cranial NCCs in vivo[4] or of chick trunk NCCs in a semi-intact slice preparation[5] have defined specific cell behaviors underlying EMT and have provided novel insight into mechanisms of EMT. Ahlstrom and Erickson[5] used long-term imaging in slices to examine the behavior of chick trunk NCCs within the neuroepithelium before EMT. Neuroepithelial cells and premigratory NCCs span the width of the pseudo-stratified neuroepithelium with adherens junction attachments at the apical surface (**Fig. 1A**). There have been several proposed hypotheses of how NCCs break their cell attachments within the neuroepithelium to allow EMT to occur. One hypothesis is that apical adhesions must be downregulated or disassembled and that this loss of adhesion is the driving force in NCC EMT.[6-9] Alternatively, NCCs may be able to generate enough motile force to break away from adhesions without the need to downregulate them.[10,11] Ahlstrom and Erickson[5] found that premigratory NCCs usually lose their apical attachments and components of adherens junctions before retraction of the apical tail and translocation out of the neural tube (**Fig. 1A and cell 1a**). This is not always the case however, as occasionally

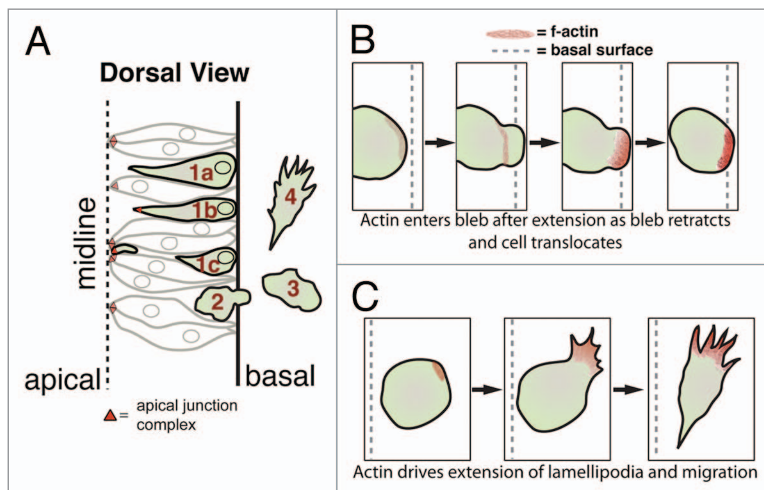

Figure 1. Schematic depiction of neural crest EMT and associated behaviors. (A) NCCs are depicted in green while neuroepithelial cells are gray. EMT includes a series of behaviors. 1(a–c) Premigratory NCCs detach from the apical surface of the neuroepithelium by three mechanisms: 1(a) downregulation of the apical junction complex (red triangle) precedes NCC tail retraction; 1(b) translocation of apical junction complex along with apical NCC tail as it retracts; 1(c) rupture of apical NCC tail during retraction leaving behind apical junction complex. (2) After tail retraction NCCs bleb at the basal surface extending protrusions and translocating out of the neuroepithelium. (3) Following delamination, NCCs can continue to bleb adjacent to the neural tube. (4) NCCs polarize and transition to directed migration. (B) Blebs protrude away from areas of underlying actin. Actin fills blebs as they retract and concentrates as the cell body translocates into the area of the bleb. (C) Lamellipodia/filopodia are formed by actin that persists within these structures throughout their lifetime. These protrusions result in a polarized cell morphology and drive directed migration.

junctional components were still present after detachment and migrated along with the retracting tail (**Fig. 1A and cell 1b**). Rarely, a NCC retracted its apical tail while leaving behind adherens junction components, suggesting that the cell generated enough force to shear its tail while adherens junctions were intact at the separated apical tip (**Fig. 1A and cell 1c**). These data suggest that downregulation of adherens junction components usually occurs prior to segregation from the neuroepithelium, but is not necessarily a prerequisite. It has also been proposed that generation of a NCC via asymmetric cell division may bypass the need to disassemble adherens junctions.[11,12] Following a cell division in a plane parallel to the lumen, a daughter cell that has no connection to the apical surface would be produced and thus could readily delaminate from the neural tube. While Ahlstrom and Erickson[5] found that the basal-most daughter of this type of division most often delaminated, on occasion both of the daughters underwent EMT. Also, after a perpendicular mitosis, where both daughters could maintain apical connections, at least one of the daughters frequently underwent EMT during the imaging period. From these observations, it appears that the plane of division does not affect the ability to undergo EMT. Consistent with this idea, a recent study that tracked labeled NCCs over extended time periods in chick embryos showed that all progeny of labeled cells eventually underwent EMT, suggesting that asymmetric divisions giving rise to one NCC daughter and one neuroepithelial daughter do not occur in the chick trunk.[13] On the whole, this work suggests that there is not a single method NCCs must use to detach from the neuroepithelium. Multiple mechanisms, including disassembly of adherens junctions and cell traction force, may work independently or together to break apical adhesions and allow NCC EMT.

To define specific behaviors of NCCs as they move out of the neuroepithelium, we imaged cranial NCCs in the zebrafish hindbrain in vivo.[4] We used higher temporal resolution imaging (imaging at 10–30 second intervals as compared to 7 min intervals used by Ahlstrom and Erickson[5]) to visualize the dynamics of transient cell protrusions and a biosensor to visualize the F-actin cytoskeleton,[14] while NCCs delaminate from the neural tube. This analysis revealed that cranial NCCs display a stereotyped series of behaviors characterized by distinct types of cell protrusions. Just before migrating out of the hindbrain, NCCs take on a rounded morphology at the basal edge of the neuroepithelium, presumably resulting from loss of apical and cell-cell contacts and extend membrane bleb protrusions at the basal surface of the neural tube (**Fig. 1A and cell 2**). Blebbing is followed by translocation of the cell soma in the direction of blebbing and then by extension of filopodial and lamellipodial protrusions as the cells migrate out of the neuroepithelium. Interestingly, membrane blebbing has been identified as a potential protrusive force driving cell migration in some cell types during development and is associated with amoeboid type cell migration.[15-18] Moreover, studies of carcinoma cells show they can alternate between bleb-associated and filopodial/lamellipodial-based migration depending on their extracellular environment.[19] Our imaging data suggest NCCs use blebbing motility to exit the neuroepithelium and then transition to lamellipodial/filopodial-based motility to initiate directed migration away from the neuroepithelium.

To gain insight into how the cytoskeleton controls these behaviors, we imaged F-actin distribution. We found that filopodial and lamellipodial protrusions contain F-actin from their initial formation (**Fig. 1C**), as expected since these protrusions are known to be actin-based. In contrast, bleb protrusions show a different F-actin pattern. Cells exhibiting blebbing display an intense F-actin signal at the site of the protrusion; however, during bleb extension the membrane separates from the underlying actin. Only after full bleb extension does F-actin signal appear beneath the bleb membrane and it intensifies during bleb retraction or while the cell moves in the direction of the bleb[4] (**Fig. 1B**). A similar pattern of F-actin accumulation has been described in blebs of other cell types.[15,20-22] Thus, the two distinct types of protrusions NCCs exhibit as they undergo EMT have unique F-actin dynamics. These results from imaging transient protrusions and F-actin accumulation form a foundation for future studies to analyze the molecular control of cytoskeletal dynamics during EMT.

Together, these studies demonstrate the diversity of cellular behaviors that take place during NCC EMT. Given this diversity, there are likely several molecular mechanisms that work in

parallel to regulate detachment and delamination of NCCs from the neuroepithelium. Cell blebbing and/or lamellipodial/filopodial attachment to the extracellular matrix outside the neuroepithelium may provide the forces allowing the NCC to pull away from cell attachments while at the same time molecular components of adherens junctions are downregulated. Also, these two processes may be partially redundant means to exit the neuroepithelium and one may compensate for potential inefficiency of the other. In order to fully understand the complex EMT process, it will be critical to build on these behavioral characterizations by investigating the molecules that control specific changes in cell behavior.

We have begun to investigate molecular mechanisms controlling cell motility during EMT by combining live imaging with molecular manipulations.[4] We found that zebrafish cranial NCC blebbing is regulated by Myosin II and Rho-kinase (ROCK), a downstream effector of the small GTPase Rho. Inhibition of Myosin II or ROCK causes a decrease in the number of NCC blebs indicating that actomyosin contraction is important for blebbing.[4] Interestingly, direct observation of NCC EMT following Myosin II or ROCK inhibition also showed a decrease in the number of NCCs delaminating from the neural tube. These data suggest that not only is NCC blebbing regulated by Myosin II and Rho/ROCK activity, but it may also promote NCC EMT by generating a protrusive force similar to that used by migrating zebrafish primordial germ cells.[15,18] Our results are consistent with earlier studies that, while not using live imaging, did demonstrate a role for Rho in NCC EMT. Inhibition of Rho using C3 transferase treatment of chick trunk neural tube explants caused a decrease in the number of cells that delaminated from the explant.[23] In addition, overexpression of RhoB along with Sox9 in chick embryos leads to an increase in delaminating trunk neural crest cells.[24] On the contrary, a more recent study concluded that Rho/ROCK activity maintains the epithelial state in chick trunk NCCs and that Rho/ROCK inhibition promotes EMT.[25] These authors treated explants with cell-permeable C3 or a ROCK inhibitor and found an increase in delaminating NCCs from chick trunk explants. Similarly, introduction of C3 or DN-Rho into embryos led to a premature delamination of NCCs. Finally, another study showed that dominant negative RhoA had no effect on delamination of chick cranial NCCs.[26] These differing reports of Rho function in EMT may reflect the fact that precise spatiotemporal control of Rho activity determines its effect. The sub-cellular localization of regulators of Rho activity and its downstream effectors are tightly controlled and the activity of Rho and its regulators are strongly affected by signals from the extracellular environment.[27] Thus outcomes of experimental manipulation will be highly context dependent. It is also possible that there are different molecular requirements for Rho signaling in EMT between different species or in cranial versus trunk NCCs, which would not be surprising given the differences in surrounding tissues. Continued use of live imaging based studies will help to elucidate these details. In particular, in vivo live imaging of NCC behavior and manipulation of Rho signaling while cells are in their natural environment can avoid some of the potential confounding variables introduced by in vitro culture conditions. Furthermore, recent technology has permitted imaging of the subcellular localization of active Rho signaling in vivo[18,28] (and Matthew R. Clay and Mary C. Halloran unpublished results), which will in the future provide important insight into the precise spatiotemporal location of active Rho in NCCs.

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Initiation of Directed Migration Away from the Neural Tube

After exit from the neuroepithelium, NCCs must initiate directed migration away from the neural tube. Our live imaging studies showed that zebrafish cranial NCCs transition between bleb-associated migration and lamellipodial/filopodial based migration during this step, and that there is some variability in the time required for NCCs to make this transition.[4] Many cells extend lamellipodia and filopodia immediately after blebs, while they are crawling out of the neuroepithelium and then quickly initiate directed migration. Less often, NCCs remain adjacent to the neural tube after delamination and display undirected blebbing without migrating for 30–60 min (**Fig. 1A and cell 3**), before finally acquiring polarized morphology and initiating migration (**Fig. 1A and cell 4**). In addition, many cranial NCCs pause and undergo cell division after exit from the neuroepithelium and before migrating.[4] Live imaging of chick cranial NCCs showed that they also exhibit variability in this behavior, with some cells migrating immediately away from the neural tube and others remaining stationary for a couple hours before migration.[29] Thus it appears that the signals capable of initiating directed NCC migration are present as cells undergo EMT, but in many cases cells require time to read these signals and extend polarized protrusions. One possibility is that the time required for NCCs to initiate directed migration is related to whether the cell divides after exit from the neural tube. Longer term imaging and tracking of individual cells after division could help resolve this question.

Recent studies using a combination of live imaging with molecular manipulation have begun to reveal the mechanisms that induce polarized cell morphology and cause NCCs to initiate migration in the correct direction. There is strong evidence that non-canonical Wnt-planar cell polarity (PCP) signaling functions in this process for cranial NCC migration in Xenopus and zebrafish and that two parallel pathways involving Wnt11 and Syndecan4 (Syn4) converge on PCP signaling.[28,30] Wnt11 is expressed in a band of cells just lateral to cranial NCCs at the delamination stage. Using a combination of approaches, including overexpressing Wnt11, expressing a dominant negative Wnt11 or expressing mutant forms of Disheveled (Dsh) that interfere with non-canonical Wnt signaling, De Calisto et al.[30] showed that non-canonical Wnt signaling is required for NCCs to migrate away from the neural tube. Live imaging of cultured NCCs showed that blocking Wnt11 resulted in fewer lamellipodial protrusions, suggesting that non-canonical Wnt signaling helps form or stabilize polarized protrusions and is required for generating locomotion. Syn4, a proteogylcan expressed by cranial NCCs, functions in parallel to Wnt11.[28] Syn4 can be activated

by fibronectin, which is present in the extracellular matrix that NCCs encounter upon exit from the neuroepithelium.[31] Matthews et al.[28] used live imaging in zebrafish embryos to show that Syn4 knockdown reduced the directionality or persistence of migration and caused NCCs to extend protrusions in all directions instead of in a polarized manner. This study also showed that Syn4 knockdown caused increased levels of active Rac and that inhibition of non-canonical Dsh caused a decrease in active RhoA. The authors suggest a mechanism in which PCP signaling is specifically activated at the trailing edge of the cell where it inhibits Rac and activates Rho, thereby inhibiting protrusions at the back of the cell and promoting leading edge protrusions and thus directed migration.[28,32] The relative contributions of and interactions between the Wnt11 and Syn4 pathways are not yet fully understood, although both appear required. Interestingly, only a subpopulation of NCCs express the Wnt11 receptor Fz7.[30] These cells are the lateral-most cranial NCCs, which are likely the first to delaminate. It is possible that later delaminating NCCs are not directly competent to respond to Wnt11, but initiate migration by cell contact with and guidance by the first NCCs. Alternatively later cells may rely more heavily on the Syn4 pathway. The variability in expression of receptors for PCP signaling may also account for the variable time it takes for NCCs to initiate migration, observed in the live imaging studies described above.

Overall, these studies show a clear role for non-canonical Wnt, Syn4 and PCP signaling. However, a critical question that remains unanswered is how PCP signaling is activated only at the back of the cell. One possibility is that a signal from the neuroepithelium controls this polarity. Live imaging in chick trunk slices showed that delaminated NCCs occasionally extend processes back into the neuroepithelium, suggesting they are still sensing information from neuroepithelial cells.[5] Alternatively, NCCs may retain some sub-cellular polarity information from their former apical-basal polarity within the neuroepithelium. Indeed, imaging of centrosome localization showed that the centrosome remains at the apical side of delaminating NCCs even after apical detachment.[5] Future studies that image subcellular localization of labeled polarity molecules during the entire process of EMT through initial migration would help to elucidate what drives the acquisition of polarized migration.

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Guidance of Migration Pathways

After initiating polarized, directed motility, NCCs must maintain persistent migration and correctly navigate pathways to their final targets. Live imaging of the motile behaviors of NCCs migrating along these pathways has provided insight into the mechanisms that control migration. In particular, such studies have revealed the importance of three general types of guidance mechanism: cell-cell communication between NCCs, repulsion from inappropriate tissues surrounding NCC pathways and attractive signals from targets that draw NCCs to their proper targets. Although many studies have investigated potential molecules controlling NCC migration pathways, we focus here specifically on studies that use live imaging of cell behavior to reveal aspects of migratory mechanisms.

Neural crest cell-cell communication. A prevalent behavior of migrating NCCs seen in most live imaging studies is that they make extensive contacts with one another as they migrate. Although the nature and proposed function of the contacts varies among specific NCC populations, it appears that cell-cell communication is important for guidance of all NCCs. The first study to image NCCs migrating along their pathways in situ used chick trunk explants to characterize migratory behaviors.[33] Trunk NCCs migrate along two pathways: a ventromedial pathway through the medial part of the somite sclerotome containing NCCs that will form the dorsal root ganglia (DRG) and sympathetic ganglia (SG) and a dorsolateral pathway over the somites containing NCCs that will form pigment cells. NCCs in the ventromedial pathway are restricted to the rostral part of each somite and do not migrate through the caudal somite[34] (**Fig. 2**). Live imaging demonstrated that NCCs migrating through the rostral somite often maintain close contact with one another.[33] More recently, a modified trunk explant was developed that has allowed higher resolution imaging of the behaviors of these cells as they migrate along their pathways and as they form the DRG and SG.[35] This study showed that trunk NCCs migrate in chain-like arrays through the rostral somite, with continuous filopodial contacts between them. Occasionally, individual cells venture into the caudal somite, but usually maintain filopodial contact with the chain and return to it after sampling the caudal somite tissue. In the rare cases that a cell broke contact with the chain, the isolated cell lost directionality and did not rejoin the chain or populate a DRG or SG, demonstrating the importance of the connections for proper migration. Interestingly, live imaging of the NCC-derived cells that form the enteric nervous system has shown that these cells also migrate as connected chains or strands as they populate the gut.[36,37]

Importantly, the explant preparation developed by Kasenmeier-Kulesa et al.[35] allowed the first detailed imaging of trunk NCCs further along their pathway, as they form the SG. It was previously thought that the segregated pattern of trunk NCC streams through the rostral somite was the basis for subsequent segmental arrangement of the SG.[38] However, live imaging demonstrated a strikingly different picture.[35] After migrating through the somite, NCCs intermingle extensively along the rostral-caudal axis before resegregating and coalescing into separate ganglia (**Fig. 2**). The authors imaged cell behaviors of NCCs in the inter-ganglionic regions during this re-segregation process. When a cell contacted another NCC in the SG anlagen, the filopodia thickened and widened to increase contact and then moved into that SG anlagen. In contrast, contact with another NCC in the interganglionic space resulted in retraction. Thus, live imaging suggests that both attractive and repulsive cell-cell interactions are central to the SG segregation process. A more recent study extended this work by combining live imaging with molecular manipulation.[39] The authors showed that both EphB-ephrinB signaling and N-cadherin homophilic adhesion are required for NCCs to segregate into SGs. N-cadherin mediates the attractive cell-cell contacts between NCCs in this process, while ephrinB1 in the interganglionic tissue repels NCCs from this region. It remains to be determined whether additional

molecules exist that mediate repulsive contacts between NCCs in interganglionic regions, however it is possible that the cell-cell repulsion observed in the interganglionic region could simply be the result of lower relative levels of N-cadherin on those NCCs.

Similar mechanisms appear to guide cranial NCC pathways. Cranial NCCs migrate from the hindbrain in three streams and cells within streams often form chain-like arrays in which they migrate collectively while maintaining filopodial contact[29,40] (**Fig. 3**). Live imaging of labeled subsets of cranial NCCs showed that they maintain spatial order throughout migration, with the first delaminating NCCs remaining at the leading front.[41] Interestingly, high resolution imaging revealed that cranial NCCs at a distance from one another within a stream are sometimes connected by very long filopodia (up to 100 μm),[40] suggesting a means of communication between distant NCCs. Cell tracking showed that cranial NCCs within chains have a higher directionality than those migrating individually.[29] Moreover, cranial NCCs were often found to change their direction to follow another cell after filopodial contact with it. These observations suggest that the filopodial chain connections contribute to the ability of the cells to stay on course. Interestingly, a precise balance of Rho activity has been shown to be important for maintaining positive cell contacts in NCC chains. Expression of either constitutively active or dominant negative Rho resulted in fewer NCC chain arrays.[26] Imaging of the cranial NCC streams in ovo showed that occasionally they can intermingle and contact NCCs in an adjacent stream; however, they usually then migrate back to their original stream, suggesting they receive an inhibitory signal from NCCs in the neighboring stream.[42] Interestingly, ablation of portions of the dorsal neural tube and the associated NCC stream results in rerouting of NCCs from adjacent streams into the ablated area.[43] This rerouting occurs in locations where cells normally intermingle and "sample" NCCs in an adjacent stream, further suggesting that the sampling is normally important for directing NCCs to stay in their correct stream rather than entering a neighboring stream. Additionally, the ablations caused a disruption of the chain arrays in the remaining NCC streams, suggesting that inhibitory signals from neighboring streams may help to drive the formation of chains.

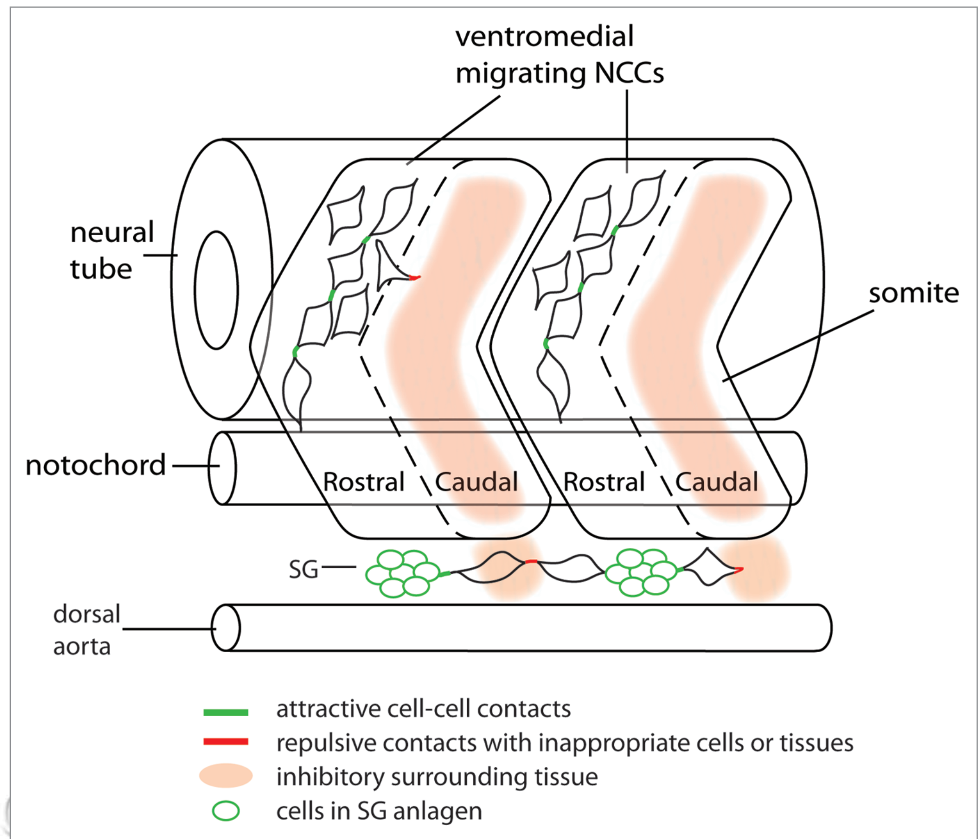

Figure 2. Schematic of mechanisms guiding trunk NCC migration. Ventromedial trunk NCCs migrate through the rostral somite in chain arrays mediated by positive contacts between NCCs (green) and are inhibited by the caudal somite (red shading). Near the dorsal aorta, NCCs intersperse before resegregating into SG (green cells) using a combination of positive cell-cell contacts in the SG anlagen (green contacts) and inhibition from interganglionic regions (red).

Although cell-cell contact is frequently involved in promoting chain formation and migration, a recent report has explored in detail the mechanisms and results of contact inhibition between cranial NCCs.[44] These authors used live imaging of Xenopus and zebrafish cranial NCCs both in vitro and in vivo to show that these NCCs display repulsion upon contact with other NCCs, and that this contact inhibition is mediated by the PCP signaling pathway. Imaging of NCCs in PCP pathway mutants or of NCCs expressing the PCP-specific Dsh mutant showed loss of contact inhibition. In addition, this study showed that Rho is activated at cell-cell contacts, consistent with the previous studies linking PCP signaling with Rho activation during acquisition of NCC polarity,[28,30] and suggesting that local activation of Rho leads to local inhibition of cell protrusions. The authors hypothesize that persistent directional migration of the cranial NCC streams can be driven by contact inhibition that prevents protrusions from forming on the sides and back edge of the cells, and preferentially allows protrusion along the leading front. Furthermore, they suggest that contact inhibition could sustain persistent migration without attraction from external guidance cues. Some of the observations from earlier imaging studies lend support to this idea. For example, chick cranial NCCs at the leading front of migrating streams show more persistent directionality than those within the stream,

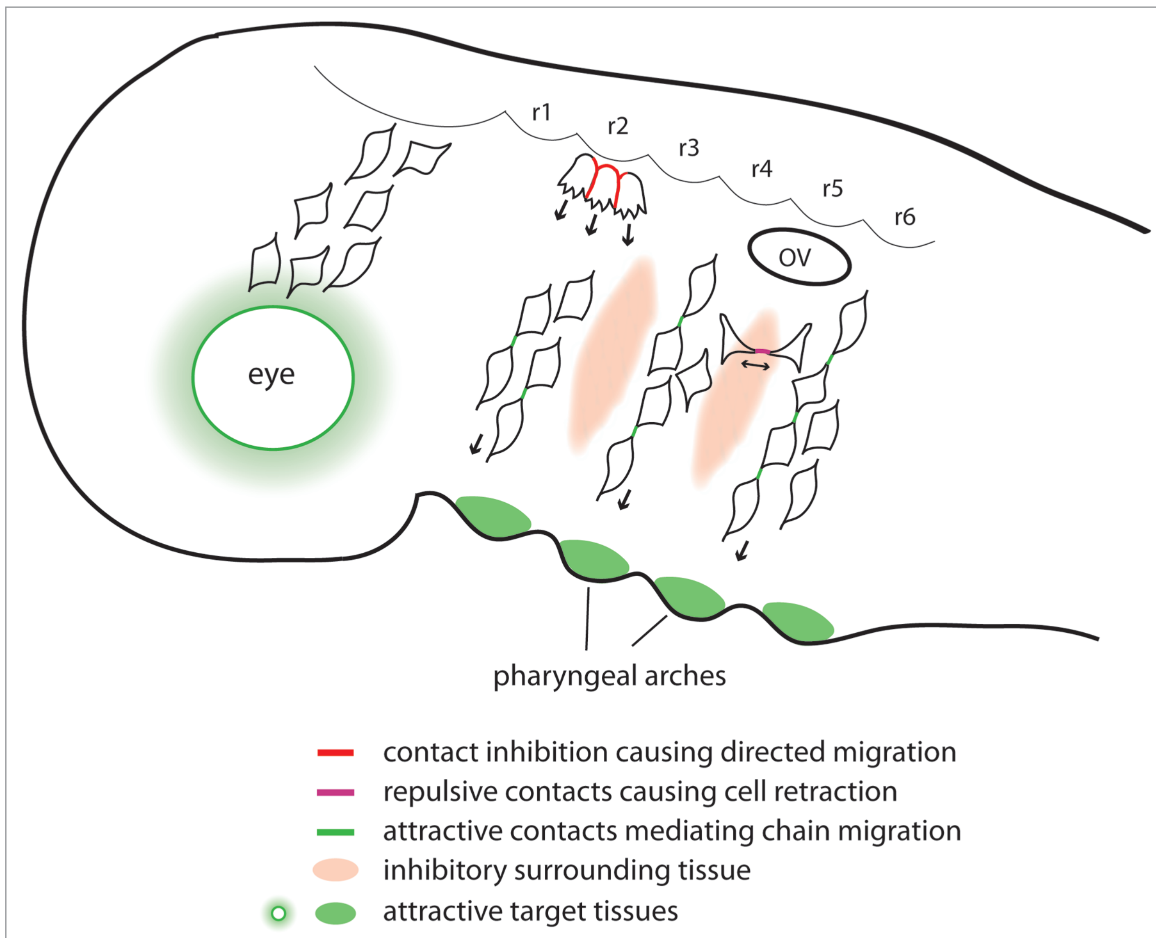

Figure 3. Schematic of mechanisms guiding cranial NCC migration. Migration is driven by contact inhibition between NCCs that initiates directed migration (red contacts), repulsive contacts between NCCs in neighboring streams resulting in retraction (purple contacts) attractive cell-cell contacts between NCCs forming chains (green contacts), inhibition from interstream regions (red shading) and attraction from pharyngeal arch and eye targets (green). OV = otic vesicle.

which could be because these cells only have contacts (and contact inhibition) with other NCCs at their trailing edges and thus can more readily form protrusions into the NCC-free space at their leading edges.[29] In addition, trunk NCCs at the center of a forming DRG anlagen display less protrusive activity than those at the periphery.[35] However, other observations contradict the contact inhibition hypothesis and suggest positive or attractive results of cell-cell contact. For example, NCCs can change direction to follow another cell after filopodial contact and chain contacts between NCCs can prevent them from straying too far out of the migratory chain.[29,35] Furthermore, live imaging of zebrafish trunk NCCs in vivo showed that these cells do not retract protrusions or display repulsive behaviors upon contact with other NCCs[45] suggesting that contact inhibition between NCCs is not an important mechanism regulating trunk NCC directed migration. Finally, several studies have shown that both trunk and cranial NCCs can have tortuous pathways and often move backward,[29,33] and that NCCs can extend protrusions in a backward direction, toward NCCs behind them.[29,35,40]

There are several possible reasons for the different behaviors seen upon NCC contact. First, it is possible that both contact-mediated attraction and inhibition are occurring at once, i.e., cell-cell contact may lead to adhesion, causing cells to co-migrate while at the same time leading to local inhibition of protrusions at the contact point. Second, different groups may have observed different subpopulations of NCCs with different behaviors. Imaging of chick cranial NCCs showed that each of the three streams displays somewhat different patterns of cell contacts and migration.[29] Even within an individual stream, cells destined to populate the cranial ganglia likely behave differently than cells continuing on to populate the pharyngeal arches; however, previous studies have not distinguished between these cell types. Future longer term imaging and cell tracking to follow the ultimate fate of individual cells will help answer these questions. On the whole, these live imaging studies have shown that cell-cell communication between NCCs is a widespread phenomenon and is essential for accurate guidance along migratory pathways. Moreover, both attractive and inhibitory cell-cell contacts likely play important roles.

Inhibitory barriers to incorrect pathways. In addition to cell-cell communication, NCC migratory pathways are also influenced by signals from other surrounding tissues in their environment, and in many cases these signals appear to be

inhibitory barriers to migration into incorrect tissues. Imaging cell behaviors can often distinguish between attractive versus inhibitory guidance signals, especially when combined with manipulation of potential molecular cues. Live imaging of trunk NCCs migrating through the rostral somite in chick trunk explants showed that these cells are actively inhibited by the caudal somite[33] (**Fig. 2**). Peanut agglutinin (PNA) binding molecules are specifically localized to the caudal somite and treatment of trunk explants with PNA caused NCCs to migrate into both the rostral and caudal somite,[33] suggesting that PNA treatment occludes an inhibitory signal in the caudal somite. A subsequent study used a combination of molecular manipulation and imaging in trunk explants and cultured NCCs to show that inhibition by the caudal somite is mediated by ephrinB1-EphB signaling.[46] After suppression of ephrinB1-EphB binding, NCCs invade the caudal somite. In addition, these authors used live imaging of NCCs in vitro to show that NCCs collapsed and retracted their protrusions in the presence of ephrinB1. A similar function for EphB-ephrinB signaling was also shown to be important for rodent trunk NCCs.[47] Interestingly, differences in the details of NCC behaviors after PNA treatment versus ephrinB1 treatment suggests that these two manipulations are not the same and thus that more than one molecule contributes to the inhibitory nature of the caudal somite.[46] Indeed, recent studies of mouse mutants, while not using live imaging, have demonstrated that inhibitory Semaphorin-Neuropilin signaling is also involved in restricting NCCs to the rostral somite in mice.[48,49] As NCCs migrate further along the ventromedial pathway they encounter another inhibitory barrier whose presence was revealed by live imaging, although its molecular identity remains unknown. These cells can either populate the more dorsal DRG or migrate further ventral to the region of the dorsal aorta and form the SG.[35] Earlier in their migration, NCCs can move between the DRG and SG anlagen; however, after a particular stage, they appear to encounter an inhibitory barrier between these two structures that prevents migration of cells between the DRG and SG.[35]

Another pathway choice required by trunk NCCs is the decision to migrate along the dorsolateral versus ventromedial pathways. Live imaging of zebrafish trunk NCCs in vivo shows that ventromedial NCCs choose their pathway because inhibition by the lateral somites prevents migration lateral to the somites.[45] NCC contact with lateral somites causes retraction or collapse of protrusions. In chick embryos this choice has been shown to be mediated by ephrinB-EphB signaling.[50] The earlier migrating ventromedial NCCs are inhibited by ephrinB, while the later migrating melanocytes that take the dorsolateral pathway, are attracted to ephrinB, which promotes their migration laterally. While this study did not use live imaging of NCC behavior, it did show that early migrating NCCs plated on ephrin-B1 showed markedly different morphology, cytoskeletal arrangement and adhesions compared to later migrating melanocytes, consistent with the hypothesis that trunk NCCs undergo a switch in responsiveness to ephrinB that allows migration along the previously inhibitory dorsolateral pathway.

The three streams of cranial NCCs that migrate from the hindbrain are separated by NCC-free zones adjacent to hindbrain rhombomeres 3 and 5 (**Fig. 3**). Live imaging studies in chick embryo explants and in ovo have provided a couple lines of evidence that these interstream regions contain signals that are inhibitory to NCC migration.[29,42] First, NCCs just emerging from r3 and r5 were found to collapse filopodia and change direction to migrate either anteriorly or posteriorly and join the NCC streams migrating from r2 or r4, suggesting the tissue adjacent to hindbrain r3 and r5 is inhibitory. Second, during migration along the streams, cells at the edge of the stream that extend filopodia toward the interstream region retract them and continue migration laterally in the stream. These observations from live imaging have been supported by numerous other studies that have begun to identify inhibitory molecules present in interstream regions. Two predominant repulsive signaling pathways involved are Eph-ephrin signals[51-53] and semaphorin-neuropilin signaling.[54-57] The fact that multiple molecules appear to be involved suggests the interstream regions are not simple barriers to migration, but rather more complex mechanisms may be regulating the sorting of cranial NCCs to different targets. A future challenge will be to determine effects of combinatorial molecular manipulation on the dynamic behaviors of specific subsets of cranial NCCs.

Attraction by targets. Although it has been hypothesized that NCCs may be capable of tracking to their target sites in the absence of any external attractants,[44] and that inhibitory cues from surrounding tissues could be the prominent means to shape migration pathways, recent studies have shown that external attractants also contribute to NCC migration and invasion of target tissues. Live imaging of midbrain-derived NCCs in zebrafish reveals the existence of an attractive signal from the eye for NCCs that populate the orbit.[58] These NCCs show high directionality in their migration toward the eye. This directionality and the distance migrated are both significantly reduced in embryos lacking eyes. It is possible that eye morphogenesis somehow modifies the extracellular matrix around the eye and that this is necessary to create an environment permissive to NCC migration. It is also possible that the eye secretes an attractive cue or combination of cues necessary for NCC guidance (**Fig. 3**). Another live imaging study in zebrafish showed that Pdgf signaling is required for the proper migration of cranial NCCs around the eye and into the oral ectoderm.[59] Loss of Pdgf signaling results in failure of NCCs to migrate around the eye to the optic stalk. This study showed that the microRNA Mirn140 mediates the response of NCCs to Pdgf ligand (Pdgfaa) by controlling the level of receptor expressed (Pdgfra). While it is clear that migration of NCCs around the eye is dependent on these signals, it is not known whether Pdgf, which is expressed in the optic stalk and the midbrain rudiment, is the main attractant cue produced by the eye.

Indeed, live imaging in zebrafish has also shown that the chemokine Sdf1a, which is expressed in the pharyngeal arch endoderm and the optic stalk, signals via the Cxcr4a receptor to guide NCCs to the arches and around the eye.[60] Following Cxcr4a knockdown, NCCs initiate migration from the neural tube correctly, but migrate to ectopic locations over the eye and yolk and fail to condense in the pharyngeal arches. These results suggest that Sdf1a also acts as an attractant that helps NCCs hone to their targets.

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In chick, another chemoattractant has recently been identified that is specifically required for invasion of cranial NCCs into branchial arch targets. Neuropilin-1 (Nrp-1) acts as a co-receptor to transduce signals from semaphorin or VEGF ligands based on the other co-receptors in its complex.[61] NCCs in the r4 migratory stream express Nrp-1 and live imaging after Nrp-1 knockdown in NCCs in ovo showed that they migrate toward BA2, but fail to completely invade this tissue.[62] These cells exhibit normal formation of filopodia and contact with other NCCs during migration, but once they reach the entrance to BA2 their filopodia collapse, migration ceases and invasion never occurs. These results suggest that without Nrp-1 NCCs are incapable of responding to a cue to invade BA2. In an extension of this work, McLennan et al.[63] found that r4 NCCs also express VEGFR2, which complexes with Nrp-1 to transduce VEGF signals. Interestingly, VEGF expression becomes strong in BA2 at stages that correlate with invasion of the r4 NCCs. Live imaging of explanted chick neural tubes showed that r4 NCCs tracked to BA2 tissue and VEGF soaked beads. In addition, r4 NCCs deviated from their normal routes and migrated into normally crest-free zones when VEGF soaked beads or VEGF expressing cells were implanted into crest-free zones. Moreover, VEGF depletion resulted in phenotype similar to Nrp-1 knockdown where r4 NCCs migrated toward BA2 but failed to invade it. Together these data suggest that VEGF signaling is mediated by an Nrp-1/VEGFR co-receptor complex and is a necessary attractive cue for r4 NCC invasion into BA2. Although VEGF is expressed in the overlying ectoderm as NCCs are migrating, loss of Nrp-1 or soluble VEGF does not disrupt the majority of the r4 migration pathway. This suggests that while invasion of BA2 is accomplished via an attractive signal from VEGF, migration to BA2 is either mediated by a different attractive signal, or does not require an attractive cue. Chick r4 NCCs also express several plexins,[63] which typically mediate responses to repulsive semaphorins. Thus the initial migration may be controlled by the onset of polarized cell migration coupled with repulsive signals and positive cues from cell-cell contacts as discussed above, while chemoattractive cues are necessary once NCCs reach their final destination.

In Xenopus, a similar effect on cranial NCC invasion of the pharyngeal arches results from the loss of Cadherin 11, a molecule typically associated with cell-cell adhesion rather than attractive cues.[64] Live imaging of Cadherin-11 depleted NCCs shows they migrate approximately halfway along their pathway and then lose directionality and fail to invade the pharyngeal arches. Moreover, imaging of explanted wild-type NCCs revealed that Cadherin-11 localizes to filopodia and to cell-cell contacts. Cadherin-11 knockdown causes NCCs to switch their protrusive activity from filopodial/lamellipodial protrusions to membrane blebs. Given the localization of Cadherin-11 to filopodia and the switch in membrane protrusions following its depletion, it is reasonable to suggest that Cadherin-11 may be necessary for the formation of filopodial protrusions. Interestingly, expression of Trio (a guanine nucleotide exchange factor (GEF) and activator of Rho GTPase signaling) or various Rho GTPases rescues the Cadherin-11 knockdown migration defect and restores the formation of filopodial protrusions. Rescue of these phenotypes was also achieved by expressing a version of Cadherin-11 lacking its extracellular adhesive domain. These data suggest that Cadherin-11 plays a role upstream of the Rho GTPases and independent of its role in cell adhesion. Because the Cadherin-11 loss of function phenotype is remarkably similar to the loss of VEGF signaling in chick, it is possible that Cadherin-11 mediates an attractive cue to invade the pharyngeal arches. What is perhaps most interesting is the fact that Cadherin-11's role in promoting NCC migration is independent of its role in cell adhesion. This is consistent with previous reports that overexpression of full-length Cadherin-11 or just its extracellular and transmembrane domains inhibits cranial NCC migration, suggesting Cadherin-11 mediated adhesion is inhibitory to NCC migration.[65,66] Furthermore, the protease ADAM13, which is also necessary for normal cranial NCC migration,[67] can cleave the extracellular cell adhesion domain of Cadherin-11.[66] ADAM13 overexpression can rescue the loss of NCC migration caused by Cadherin-11 overexpression. Therefore a potential scenario exists where ADAM13 cleavage of Cadherin-11 may release Cadherin-11 from its role in cell-cell adhesion and create a molecule that can respond to attractive cues, activate RhoGTPase signaling, generate filopodia and stimulate invasion into the branchial arches. Similarly, signaling by an N-cadherin proteolytic cleavage product has been implicated in NCC EMT,[9] thus cleavage of cadherins may represent a more widespread, adhesion-independent role for cadherins in NCC migration. It is also possible that the role of Cadherin-11 may be a general one in generating cell motility rather than as a receptor for attractive cues. Future work will be required to determine whether Cadherin-11 acts to mediate signals from attractive ligands.

Conclusion

In conclusion, imaging the behavior of NCCs in living tissue has given extensive insight into mechanisms controlling NCC migration and provides a foundation for future studies that investigate molecular mechanisms. These studies to date show that the regulation of cell adhesions and onset of motility in the neuroepithelium, as well as the targeted migration along specific pathways through the embryo are governed by complex interacting signals. The challenge for the future will be to determine the function of these signals in regulating specific cell motile events. This effort will benefit from application of higher technology imaging methodology such as biosensors to image molecular activity within NCCs as they migrate in vivo and transgenic technology to label particular subsets of NCCs and watch their behavior relative to others. The multiple embryonic preparations developed thus far for NCC live imaging, from chick embryo explants to intact zebrafish embryos, set the stage for a more complete understanding of the mechanisms governing the migration of this important cell type.

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