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Neural crest motility on fibronectin is regulated by integrin activation

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Abstract

Cell migration is essential for proper development of numerous structures derived from embryonic neural crest cells (NCCs). Although recent work has shown that receptor recycling plays an important role in NCC motility on laminin, the molecular mechanisms regulating NCC motility on fibronectin remain unclear. One mechanism by which cells regulate motility is by modulating the affinity of integrin receptors. Here, we provide evidence that cranial and trunk NCCs rely on functional regulation of integrins to migrate efficiently on fibronectin (FN) *in vitro*. For NCCs cultured on fibronectin, velocity decreases after Mn^{2+} application (a treatment that activates all surface integrins) while velocity on laminin (LM) is not affected. The distribution of activated integrin $\beta 1$ receptors on the surface of NCCs is also substratum-dependent. Integrin activation affects cranial and trunk NCCs differently when cultured on different concentrations of FN substrata; only cranial NCCs slow in a FN concentration-dependent manner. Furthermore, Mn^{2+} treatment alters the distribution and number of activated integrin $\beta 1$ receptors on the surface of cranial and trunk NCCs in different ways. We provide a hypothesis whereby a combination of activated surface integrin levels and the degree to which those receptors are clustered determines NCC motility on fibronectin.

Keywords

Neural crest; integrin activation; cell movement; laminin; fibronectin; integrins; endocytosis; extracellular matrix

INTRODUCTION

The neural crest is a transient embryonic cell population which migrates long distances through the vertebrate embryo during development and gives rise to diverse derivatives. Neural crest cells (NCCs) arise on the dorsal side of the neural tube and, after an epithelial to mesenchymal transition, migrate along defined pathways to generate many cell types, including the neurons and glia of the peripheral and autonomic nervous systems, craniofacial connective tissue and bone, melanocytes, as well as the outflow tract of the heart (reviewed in [1-4]). The extensive migratory ability of the neural crest distinguishes this cell population from most embryonic and adult cell types which generally remain stationary. Unlike stationary cells, NCCs are able to adapt to changing extracellular environments and thereby migrate efficiently through diverse tissues over the course of many days. This behavior suggests that NCCs are able to maintain motility by continuously monitoring and responding to changes in the extracellular matrix

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(ECM), however, the molecular mechanisms responsible for such flexibility are only partially understood.

During development, the integrin family of cell adhesion receptors are required for proper cell migration, proliferation, survival and differentiation of many embryonic cell populations, including the neural crest. Integrins are heterodimeric transmembrane receptors that bind both ECM and cytoskeletal proteins, and thus provide a transmembrane link between extracellular signals and intracellular mechanical forces. Efficient cell motility requires an intermediate level of cell adhesion because cells that are too weakly attached cannot generate enough traction for movement, and cells that are too strongly adhered remain stationary. Thus, the ability to modulate cell adhesion *via* integrin function differentiates stationary cells, which remain restricted to certain tissues *in vivo*, from migratory cells, which can invade new environments. Hence, it is likely that regulation of cell adhesion is fundamental for proper migration of NCCs.

Previous work indicates that changes in integrin surface expression [5] and surface integrin distribution [6] can regulate cell adhesion and motility. In addition, ligand affinity is regulated by rapid and reversible conformation changes in the extracellular domains of integrin heterodimers. The process by which integrin-ligand affinity is regulated is called integrin activation, and refers to the change from an inactive (low-affinity) to an active (high-affinity) conformation [7,8]. It is thought that integrin receptors remain in a state of flux between the two conformations until intracellular signals, ligand- or divalent cation-binding holds the receptor in the active state. Furthermore, clustering of activated integrins into focal adhesions increases integrin avidity and cell adhesion, and subsequently slows cell migration [8,9].

The adhesion and migration of NCCs, both *in vivo* and *in vitro*, are primarily mediated by beta 1 integrins, whose ligands include two major ECM molecules, laminin and fibronectin [10-19]. Both laminin and fibronectin are abundantly expressed in neural crest migration pathways *in vivo* [20,21]. Previous analysis of NCC migration *in vitro* using a variety of monoclonal and polyclonal antibodies to integrin $\beta 1$ as substrata has shown that both the affinity of receptors for the substratum and the number of receptors bound to the substratum are critical in regulating NCC motility [22]. It has also been shown that two subpopulations of neural crest, cranial and trunk (based on their axial level of origin), differ in their requirement of divalent cations for attachment to laminin and fibronectin, suggesting that differences in integrin activation levels may underlie distinctions between embryonic cell populations [23]. However, the contribution of integrin activation to NCC motility has not been well studied.

Early investigation of NCC motility *in vitro* has shown that cranial and trunk NCCs migrate efficiently across a wide range of fibronectin concentrations [16]. Cranial NCCs regulate surface expression of integrin $\alpha 4$ (a fibronectin receptor) in response to the concentration of ligand present in the substratum, presumably to maintain the intermediate level of adhesion necessary for optimal motility. In contrast, trunk NCCs do not regulate surface fibronectin receptor levels, suggesting that this population of cells use another mechanism to compensate for changing fibronectin densities. Whether trunk NCCs accommodate different fibronectin levels either by regulation of integrin function or by compensatory alterations in downstream integrin signaling is unknown. Interestingly, overexpression of fibronectin receptors in cranial NCCs does not slow their motility on fibronectin, suggesting that, like trunk NCCs, other regulation mechanisms must control cranial NCC motility. Thus, we hypothesized that integrin functional regulation, *i.e.* the ratio of activated to inactivated receptors on the cell surface, may be an important mechanism for cranial and trunk NCC motility on fibronectin.

In the present study, we examined the role of integrin functional regulation in cranial and trunk NCC motility *in vitro*. We find that integrin functional regulation is substratum-dependent and plays a greater role in NCC motility on fibronectin than on laminin. Activated integrin $\beta 1$

distribution on the surface of NCCs cultured on fibronectin is distinct compared to the distribution on laminin, indicating that integrin clustering is also substratum-dependent. On varying concentrations of fibronectin, ligand availability regulates the speed of cranial but not trunk NCCs after activation of surface integrins with Mn^{2+} treatment. This treatment alters the distribution of activated integrin $\beta 1$ receptors on the cell surface, while only varying the level of activated surface receptors slightly. We provide a summary of our core findings whereby a combination of activated surface integrin levels and receptor clustering determines NCC motility on fibronectin *in vitro*, and we hypothesize that this mechanism may provide NCCs the flexibility to migrate through changing environments *in vivo*.

MATERIALS and METHODS

Substratum preparation and cell culture

Glass coverslips (Goldseal; Fisher) were acid washed, rinsed in dH_2O , and baked at $350^\circ C$ for 12 hours. 13mm holes were drilled in the bottom of 35mm tissue culture dishes (Fisher) and the prepared coverslips were affixed to the dish with Sylgard (Dow Corning). Bovine plasma fibronectin (FN; Gibco BRL) and natural mouse laminin (LM; Gibco) were diluted to low (1 $\mu g/ml$) and high (20 $\mu g/ml$) concentrations in phosphate buffered saline (PBS), and 300 μl of the protein solution was added to the coverslips, incubated for 3 hours at room temperature and then rinsed once with PBS. As previously published [16], absolute protein concentration on the coverslips was determined by measuring the amount of tritiated laminin and fibronectin bound to glass as described in [24]. Application of laminin at 1 $\mu g/ml$ (low laminin) and 20 $\mu g/ml$ (high laminin) resulted in a density of 45 ng/cm^2 and 430 ng/cm^2 bound laminin, respectively. Application of fibronectin at 1 $\mu g/ml$ (low fibronectin) and 20 $\mu g/ml$ (high fibronectin) resulted in 35 ng/cm^2 and 700 ng/cm^2 bound fibronectin, respectively.

White Leghorn chicken eggs (supplied by Utah State University) were incubated at $38^\circ C$ until the embryos reached [25] stages 8 (cranial cultures) and 12 (trunk cultures). All animal studies were approved by the University of Utah Institutional Review Board. Neural tubes from the mesencephalic level (cranial) or adjacent to the caudal-most 6-8 somites (trunk) were dissected away from the surrounding tissue with tungsten needles. Neural tubes were cultured at $37^\circ C$ with 5% CO_2 for 48 hours in 250 μl of Neurobasal Medium (Gibco) supplemented with 25 μM glutamic acid (Sigma), 500 μM L-glutamine (Sigma), 1x B-27 and N-2 (Gibco), 10 ng/ml NT3 (Chemicon), 100 ng/ml EGF (Upstate), 10 ng/ml FGF (Upstate), and 50 ng/ml NGF (R & D Systems). Neurobasal medium without the supplements and buffered with N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES; USBiological) was used for the timelapse assays.

Reagents and Antibodies

Antibodies against the following proteins were used in these experiments: activated integrin $\beta 1$ (TASC: MAB19294; Chemicon) and actin (AAN01; Cytoskeleton Inc., and Alexa Phalloidin; Molecular Probes.) Manganese chloride (Sigma) was applied at a final concentration of 0.25 mM. Secondary antibodies were from Molecular Probes.

Timelapse Analysis

Cranial and trunk neural crest were cultured for 48 hours. The neurobasal media was replaced with HEPES-buffered neurobasal media for filming. Independent cells (25-35/condition) at the outer edge of the explant were videographed at 10X for 30 minutes to establish the pre-treatment velocity. We chose to investigate the explant edge at this time point in order to maximize the number of solitary, migratory cells for our study. Phase images were acquired (Metamorph 5.0) every 150 seconds. Manganese chloride or H_2O alone (vehicle) was then added, and after 15 minutes the post-treatment velocity was determined by filming the cells

for an additional 30 minutes. Cell velocity was determined by tracking the position of the nucleus over time (Metamorph 5.0), and any cell that was not actively migrating just prior to treatment was not included in the analysis. Cumulative distribution graphs were generated by sorting each cell's velocity from highest to lowest speed, and determining the percentage of cells (in the same condition) that traveled slower than that cell. The speed of the cell was plotted against the percentage of cells traveling slower in order to show the velocity of each cell in relation to the whole population. Specific points in each graph are not directly comparable to corresponding points in other graphs as each point merely reflects a cell's speed in relation to the whole population.

Immunohistochemistry and Focal Adhesion Analysis

For the analysis of activated integrin $\beta 1$ distribution, cranial and trunk neural crest cultures were grown for 48 hours and incubated with Mn^{2+} or the vehicle control (H_2O) for 20 minutes at 37 °C. Cells were then rinsed and fixed with 2% paraformaldehyde (EMS) with 35% sucrose for 20 minutes at room temperature. This fixation technique does not permeabilize these cells. Anti-activated integrin $\beta 1$ antibody (TASC; 1:500) was diluted in blocking buffer (PBS- with 5% normal goat serum) and added to the cells for 1 hour at room temperature. Secondary antibodies (1:500; Molecular Probes) were also diluted in blocking buffer and added to the cells for 1 hour at room temperature. Images were acquired from all conditions on the same day at 60X magnification with a CCD camera using Spot acquisition software (Diagnostic Instruments, Inc.)

To quantify the number of focal adhesions and the focal adhesion area, cells were treated as detailed above. All data acquisition and analysis was done by an observer blinded to the experimental condition. Using criteria we have previously published [16,26], the entire area of a cell with a migratory morphology (leading lamella, rearward nucleus, and in some cases, a retraction fiber) was selected and measured (Metamorph 5.0). Two deconvolution steps, flatten background and no neighbors, were applied to each cell image to select focal adhesions. Focal adhesion area and number were then measured.

Immunoprecipitations

For the analysis of the ratio of active versus inactive integrin $\beta 1$ on the cell surface, cranial and trunk neural crest cultures were grown for 48 hours and incubated with 0.25 mM Mn^{2+} or the vehicle control (H_2O) for 20 minutes at 37 °C. Cell surface receptors were labeled with NHS-SS-biotin (Pierce) on ice and the cells (100,000) were then lysed on ice in a solution containing 0.5% triton x-100 and protease inhibitors. Protein concentration in each cell lysate was determined with a BCA protein assay (Pierce) and equalized across conditions. Activated integrin $\beta 1$ was immunoprecipitated with the TASC antibody using standard protocols [24], and immunoprecipitated proteins were size fractionated under nonreducing conditions on acrylamide gels and electrophoretically transferred to nitrocellulose membranes. Biotinylated proteins were detected by using strep-Avidin conjugated to HRP and a chemoluminescent reagent (Pierce) followed by exposure to film. Exposures of blots that were in the linear range were quantified using GelDoc and QuantityOne (BioRad).

RESULTS

Integrin activation slows NCC motility on fibronectin

To determine the role of integrin functional regulation in NCC motility on laminin and fibronectin, we examined the velocity of cranial and trunk NCCs before and after treatment with the divalent cation manganese (Mn^{2+}). Because Mn^{2+} holds integrins in an active conformation, we postulated that cells which require a balance of inactive and active surface integrins for efficient motility would be slowed after treatment. Using time-lapse

videomicroscopy, we established a pre-treatment velocity for each cell and graphed these data in a cumulative distribution plot (see Materials and methods for generation of these plots) to show the range of velocities in these heterogeneous cell populations. On high concentrations of fibronectin (FN20) and laminin (LM20) cranial NCCs traveled at the same velocity, whereas trunk NCCs were significantly faster on laminin compared to fibronectin (Fig. 1A). The average velocity of cranial NCCs pre-treatment was $179 \mu\text{m/hr} \pm 10$ (SEM) on FN20 compared to $186 \mu\text{m/hr} \pm 8$ on LM20, whereas the average velocity of trunk NCCs pre-treatment was $142 \mu\text{m/hr} \pm 5$ on FN20 compared to $173 \mu\text{m/hr} \pm 8$ on LM20. After following each cell for 30 minutes, Mn^{2+} was applied at a final concentration of 0.25 mM and allowed to act for 15 minutes. Each cell was then followed for another 30 minutes to determine a post-treatment velocity (Fig. 1B). The average velocity of cranial NCCs post-treatment was $106 \mu\text{m/hr} \pm 10$ on FN20 compared to $171 \mu\text{m/hr} \pm 9$ on LM20. The average velocity of trunk NCCs post-treatment was $89 \mu\text{m/hr} \pm 5$ on FN20 compared to $157 \mu\text{m/hr} \pm 6$ on LM20.

Cumulative distribution plots of the change in each cell's velocity after Mn^{2+} treatment show that nearly 90% of both cranial and trunk NCCs cultured on FN20 slowed when all surface integrins were activated (Fig. 1C); post-treatment velocities were significantly different from pre-treatment velocities ($P < 0.001$; paired t test). In contrast, there was no change in velocity for cranial NCCs cultured on LM20; pre- and post-treatment velocities were not significantly different from each other ($P > 0.05$; paired t test). Trunk NCCs cultured on LM20 and treated with Mn^{2+} slowed slightly; post-treatment velocities were significantly different from pre-treatment velocities ($P < 0.05$; paired t test). These results indicate that integrin functional regulation is substratum-dependent and plays a greater role in NCC motility on fibronectin compared to laminin.

Activated integrin $\beta 1$ distribution in NCCs is substratum-dependent

To determine whether the substratum-dependent slowing of NCCs was due to differential distribution of activated integrin receptors, we immunostained NCCs for activated integrin $\beta 1$ before and after Mn^{2+} treatment. Integrin $\beta 1$ heterodimerizes with a variety of integrin alpha subunits, some of which bind laminin, or fibronectin or both molecules. Clustering of activated integrins into large focal adhesions increases cell adhesion to the substratum and slows cell motility [11]. For cranial NCCs cultured on high concentrations of fibronectin, activated integrin $\beta 1$ was observed in clusters both before and after Mn^{2+} treatment (Fig. 2A). In contrast, there was much less clustering of activated integrin $\beta 1$ in cranial NCCs cultured on high laminin both before and after Mn^{2+} treatment. For trunk NCCs cultured on high fibronectin, activated integrin $\beta 1$ was observed in clusters both before and after Mn^{2+} treatment (Fig. 2B). Activated integrin $\beta 1$ also clustered in trunk NCCs cultured on high laminin, although perhaps to a lesser degree than on high fibronectin. These results indicate that both the motility of NCCs and their surface activated integrin $\beta 1$ distribution is substratum-dependent.

Integrin activation slows cranial NCC motility in a substratum-dependent manner

Activation of all surface integrins would be predicted to slow cells to a greater degree on high concentrations of fibronectin where more ligand is available to adhere cells to the substratum. To determine whether substratum density regulates the levels of surface integrin activation and thereby regulates NCCs motility, we examined the velocity of cranial and trunk NCCs cultured on low (FN1) and high (FN20) concentrations of fibronectin. Using the same experimental method as in Figure 1, we established pre-treatment velocities for cranial and trunk NCCs cultured on FN1 and FN20. On both fibronectin concentrations, cranial NCCs traveled at the same velocity, whereas trunk NCCs were slightly faster ($P < 0.05$; t test) on FN1 compared to FN20 (Fig. 3A). The average velocity of cranial NCCs pre-treatment was $179 \mu\text{m/hr} \pm 10$ on FN20 compared to $172 \mu\text{m/hr} \pm 8$ on FN1, whereas the average velocity of trunk NCCs pre-treatment was $142 \mu\text{m/hr} \pm 5$ on FN20 compared to $157 \mu\text{m/hr} \pm 6$ on FN1. After following

each cell for 30 minutes, Mn^{2+} was applied and allowed to act for 15 minutes. Each cell was then followed for another 30 minutes to determine a post-treatment velocity (Fig. 3B). Both populations of NCC on both concentrations of FN slowed in response to treatment. The average velocity of cranial NCCs post-treatment was $106 \mu\text{m/hr} \pm 10$ on FN20 compared to $128 \mu\text{m/hr} \pm 11$ on FN1. The average velocity of trunk NCCs post-treatment was $89 \mu\text{m/hr} \pm 5$ on FN20 compared to $115 \mu\text{m/hr} \pm 6$ on FN1.

Cumulative distribution plots of the change in each cell's velocity after Mn^{2+} treatment show that approximately 80% of both cranial and trunk NCCs cultured on FN1 or FN20 slowed when all surface integrins were activated (Fig. 3C); post-treatment velocities were significantly different from pre-treatment velocities ($P < 0.001$; paired t test). Cranial NCCs cultured on FN20 slowed to a greater degree than the same cell type cultured on FN1 ($P < 0.05$; t test), as would be predicted from high fibronectin concentrations mediating a greater increase in cell adhesion. In contrast, trunk NCCs slowed to the same degree on either FN1 or FN20 ($P > 0.05$; t test). These results indicate that amount of ligand available regulates the speed of cranial but not trunk NCCs cultured on fibronectin.

Surface ratios of inactive to active integrin $\beta 1$

To further investigate the differences between cranial and trunk NCCs cultured on low and high fibronectin, we examined the surface levels of active integrin $\beta 1$ before and after Mn^{2+} treatment. Although NCC motility on fibronectin is mediated by both $\beta 1$ and $\beta 3$ integrins, only integrin $\beta 1$ can be studied in its activated form with the available antibodies. An increased level of activated integrin $\beta 1$ on the cell surface after Mn^{2+} treatment would be predicted to slow cells possibly by increasing adhesion to the substratum, or by altering surface presentation of the receptor [27]. Cranial and trunk NCCs were cultured on low (FN1) and high (FN20) concentrations of fibronectin and surface activated integrin $\beta 1$ was immunoprecipitated with and without Mn^{2+} treatment (Fig. 4). There was no statistically significant difference between the amount of activated integrin $\beta 1$ on the surface without (red bars) or with (black bars) Mn^{2+} treatment for any condition. These results indicate that the majority of integrin $\beta 1$ on the surface of NCCs cultured on FN is already activated, and that the Mn^{2+} -induced slowing is not due to activation of a large pool of inactivated surface receptors. However, there was a trend toward an increased level of activated surface integrin $\beta 1$ after Mn^{2+} treatment for cranial NCCs on FN20 and trunk NCCs on FN1. Thus, these results suggest that the distribution of activated integrins on the cell surface, rather than the surface level, regulates NCC motility.

Clustering of activated integrin $\beta 1$ in cranial neural crest

In some cases, integrin activation can slow cells by increasing the number and/or strength of adhesions to the substratum [8]. However, manganese-induced changes in integrin activation can also be transient, with cells regulating total surface levels of activated protein [27]. Since Mn^{2+} treatment did not cause a significant increase in the number of activated surface integrin $\beta 1$ for any condition, we predicted that NCC motility on fibronectin is instead regulated by the distribution of activated integrins. Clustering of activated integrins into large focal adhesions increases cell adhesion to the substratum and slows cell motility [9]. To verify that activated integrin $\beta 1$ clusters were indeed focal adhesions, *i.e.* linked to the cytoskeleton, NCCs were cultured on fibronectin and stained for activated integrin $\beta 1$ and actin. Clusters of activated integrin $\beta 1$ colocalized with the tips of actin stress fibers (data not shown), consistent with previous analysis of clustered integrins in focal adhesions [28].

To quantify the change in distribution of activated integrin $\beta 1$ in cell surface adhesions, cranial NCCs were fixed but not permeabilized, and stained for activated integrin $\beta 1$ without and with Mn^{2+} treatment (Fig. 5A). The total area and focal adhesion area of each cell was measured to determine the percentage of total cell area that contained adhesions (Table 1). To determine

the degree of clustering, the percentage of adhesion area was divided by the total number of adhesions to generate a clustering index. Thus, an increase in the clustering index number indicates greater integrin avidity and increased cell adhesion. For cranial NCCs cultured on FN1, there was a significant increase ($P < 0.05$; t test) in clustering of activated surface integrin $\beta 1$ after Mn^{2+} treatment (Fig. 5A). In contrast, the clustering of activated surface integrin $\beta 1$ in cranial NCCs cultured on FN20 did not change after Mn^{2+} treatment (Table 1 and Fig. 5A). These results, in combination with the results presented in Figure 4, suggest that after Mn^{2+} treatment, cranial NCCs on FN1 slow due to increased integrin avidity, whereas cranial NCCs on FN20 slow due to increased integrin affinity.

Clustering of activated integrin $\beta 1$ in trunk neural crest

As in Figure 5A, trunk NCCs were fixed but not permeabilized, and stained for activated integrin $\beta 1$ without and with Mn^{2+} treatment (Fig. 5B). For trunk NCCs the effect of Mn^{2+} treatment on clustering was opposite that of cranial NCCs. On FN1, there was no increase in clustering of activated surface integrin $\beta 1$ after Mn^{2+} treatment, whereas on FN20 there was an increase in clustering of activated surface integrin $\beta 1$ after Mn^{2+} treatment (Table 1 and Fig. 5B). These results, in combination with the results presented in Figure 4, suggest that after Mn^{2+} treatment, trunk NCCs on FN1 slow due to increased integrin affinity, whereas trunk NCCs on FN20 slow due to increased avidity. Together, these results indicate that a combination of the levels of activated surface integrins and their distribution regulates NCC motility, and furthermore, that cranial and trunk NCCs differ in their response to substratum density.

DISCUSSION

In this work we have investigated the role of integrin activation in regulated cranial and trunk NCC motility on fibronectin *in vitro*. We show that integrin functional regulation is substratum-dependent and plays a greater role in NCC motility on fibronectin than on laminin, indicating that on fibronectin NCCs maintain a balance of integrin affinity and avidity to preserve motility. Activated integrin $\beta 1$ is distributed differently on the surface of NCCs cultured on fibronectin compared to laminin, indicating that integrin clustering is substratum-dependent. On varying concentrations of fibronectin, activation of surface integrins with Mn^{2+} slows cranial but not trunk NCCs in a substratum concentration-dependent manner. Although there is only a small change in the number of activated integrins on the surface after Mn^{2+} treatment, the distribution of activated integrins changes significantly, in particular on the cell surface of cranial NCCs cultured on low fibronectin and trunk NCCs cultured on high fibronectin. Our findings (summarized in Fig. 6) are consistent with the possibility that a combination of activated surface integrin levels (receptor affinity) and the degree to which those receptors are clustered (receptor avidity) determines NCC motility on fibronectin. Our results suggest that efficient motility on fibronectin is regulated by the appropriate matching of receptor affinity and avidity to ligand availability (Fig. 6). On low concentrations of fibronectin (FN1), there is very little ligand available so efficient motility may require a high receptor affinity, *i.e.* mostly active receptors on the surface to increase the probability of ligand binding events. Based on immunoprecipitation analysis, cranial NCCs have a greater number of activated receptors on their surface on FN1 compared to trunk NCCs on the same substratum which may underlie the difference in their rate of migration. Mn^{2+} treatment may slow cranial NCCs on FN1 by clustering activated receptors thereby increasing avidity to the substratum, and slows trunk NCCs on the same substratum by activating the inactive pool of surface receptors thereby increasing affinity. In contrast, on high concentrations of fibronectin (20-fold higher density), ligand is more readily available and fewer receptors need to be activated to achieve the same probability of ligand binding. On high fibronectin (FN20), greater numbers of activated surface receptors would increase cell adhesion and concomitantly decrease motility, which presumably

is why trunk NCCs travel slower than cranial NCCs on FN20. Mn^{2+} treatment may slow cranial NCCs on high fibronectin by activating the inactive pool of surface receptors thereby increasing affinity to the substratum, and slow trunk NCCs on the same substratum by clustering activated receptors thereby increasing cell avidity.

Integrin activation and motility

The first observation that functional regulation of integrins played a role in the motility of developmentally important cells showed that developing retinal neurons which had lost responsiveness to laminin regained the ability to bind laminin after integrin activation [29]. These authors postulated that the observed developmental change in retinal neurons must be due to mechanisms which lead to the reversible inactivation of receptor populations on the cell surface. Furthermore, in a different cell population, integrin activation had little effect on neurite outgrowth of dorsal root ganglion neurons [30], suggesting that intrinsic integrin activation states differ among distinct cell types. Our finding that integrin activation regulates NCC motility on fibronectin is consistent with the notion that some cells fine tune integrin activation to promote efficient motility.

The fact that integrin activation regulates NCC motility to a greater degree on fibronectin compared to laminin indicates that substratum-specific mechanisms regulate neural crest migration. This finding is consistent with previous studies which have shown that the effect of integrin activation on lymphoblast motility is dependent not only on the substratum, but also on the ligand concentration [31]. It is also possible that changes in the activation state of non $\beta 1$ containing integrins, particularly $\alpha V\beta 3$ or $\alpha V\beta 5$, may play an important role in regulating neural crest motility on fibronectin. This possibility is consistent with the data shown in Figure 1, that manganese has greater effects on a fibronectin, a ligand for αV -containing integrins [17,38], compared to laminin, a substratum that interacts primarily with $\beta 1$ -containing integrins [11,14,22,23]. However, the lack of reagents suitable for monitoring the activation state of αV -containing integrins in this system limits our ability to investigate this possibility.

Alternatively, immunoprecipitation might not fully reveal all of the activated $\beta 1$ -containing integrins, leading to an underestimate of the level of activation receptors by manganese. However, following manganese treatment there is a statistically significant difference between the surface levels of activated integrin $\beta 1$ on cranial and trunk NCCs cultured on low fibronectin concentrations (Fig. 4), suggesting that differences in $\beta 1$ activation state make different contributions to neural crest motility on fibronectin depending on the axial origin of the cells (Fig. 6)

Interestingly, the effect of integrin activation on NCC motility was dependent on the substratum concentration, but only for cranial cells. Early analyses of cranial and trunk NCC motility *in vitro*, established that cranial and trunk NCCs differ in their mechanisms of adhesion to fibronectin [23], however these authors only looked at one concentration of fibronectin and did not investigate whether these differences translated to differences in motility. It is tempting to speculate that differences between the way these two NC subpopulations modulate their adhesion to the substratum contribute to differences in their migration *in vivo*, especially since it is likely that both cranial and trunk NCCs encounter large variations in fibronectin density during migration.

Cytoskeletal organization and motility

Substratum-induced changes in cytoskeletal organization have been implicated in cell migration [32]. Integrin receptors can be found on the surface of cells either associated with stable, focal adhesions or migratory, focal contacts. Thus, the difference between stationary and migratory cells typically reflects a balance of integrin receptor usage and the functional

regulation of those receptors. Integrins cluster into focal adhesions which are rich in structural proteins including vinculin, talin, paxillin, zyxin, and α -actinin, as well as tyrosine kinases such as focal adhesion kinase (FAK) and Src [33-35]. One of the earliest analyses into the spatial distribution of cytoskeletal components in migrating NCCs *in situ* compared three different neural crest subpopulations, cranial, vagal and trunk, and found that trunk and vagal NCCs express increasing amounts of vinculin as they migrate [36]. Moreover, this was not the case for cranial NCCs and thus it was postulated that particular adhesive behaviors of these cells may be related to their distinct differentiation. Our data shows that regulation of both receptor affinity and avidity control NCC motility, however, cranial and trunk NCCs regulate these processes in response to substratum concentration in opposite ways. These data suggest that cell-type specific regulation of adhesive structures in combination with ligand availability determines NCC motility on fibronectin.

Clearly, the regulation of adhesion in migrating NCCs must be a dynamic and multifaceted process. Tyrosine kinases play important roles in controlling substratum adhesion [37] and promoting cell motility [38]. Both the size and distribution of focal adhesions are acutely sensitive to the actions of tyrosine kinases and phosphatases, and inhibitors of these co-regulators often have cell-specific effects [39,40]. Previous studies have investigated the role of phosphotyrosine signaling in regulating a balance of cell-cell adhesion and contact inhibition of movement, versus cell-substratum interactions during trunk NCC migration [41]. It is possible that the activation state of tyrosine kinases and phosphatases differs between cranial and trunk NCCs, and that these differences correspond to the distinct way these cell types cluster activated integrins.

Neural crest motility and cell fate

In the developing embryo, different populations of neural crest (cranial, vagal, trunk, and sacral) follow different migratory pathways and give rise to distinct derivatives [42]. Generally, NCCs can assume position-appropriate fates when grafted into ectopic environments. However, some NCC subpopulations will not migrate or differentiate appropriately when grafted into a heterotopic environment. For example, trunk NCCs cannot form all mesectodermal derivatives when transplanted into cranial levels [43,44]. Interestingly, trunk NCCs *can* contribute to the trigeminal ganglia and corneal stroma, indicating that although they are able to migrate in a new environment, only certain pathways are available to them [45]. Because target-derived differentiation cues lie far away from the origin of neural crest migration and these signals may be short-lived, the ability to migrate efficiently in a specific embryonic environment may determine (or limit) the final destinations and cell fates available to particular NCCs.

There is evidence that neural crest phenotypes are specified in broad domains along the anterior/posterior (A/P) axis, and that gene expression patterns respect these broad domains [46-48]. For example, the matrix metalloprotease ADAM13 [49] and the hyaluronic acid receptor CD44 [50] are known to be expressed in cranial, but not trunk NCCs at early stages. Furthermore, our lab has previously shown that cranial and trunk NCCs differ in their migratory ability and integrin regulation when cultured on high concentrations of laminin [16]. Our finding that trunk NCCs are faster on low concentrations of fibronectin compared to high, while cranial NCCs travel at the same rate on both concentrations of fibronectin, suggests that the ability to migrate efficiently in a specific embryonic environment may determine the final destinations available to neural crest populations. This suggests that differences in NCC migratory ability may influence where NCCs go and thereby what derivatives they are able to produce.

Although the roles of integrin activation and receptor clustering have been well studied in transformed cell lines and adult primary cell populations, very little is known about how these mechanisms regulate developmental cell motility. During migration, NCCs encounter distinct

environments and must dynamically regulate their adhesion in order to maintain an efficient rate of motility during migration to their target tissues. Our results suggest that both substratum-specific and cell type-specific differences between cranial and trunk NCCs regulate their cytoskeletal organization and subsequently, their migratory behavior.

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Figure 1.

Integrin activation regulates NCC motility on fibronectin, but not on laminin. (A) Cumulative distribution plots of cranial and trunk NCC velocity before Mn^{2+} treatment are shown where each point represents the pre-treatment velocity of a single cell. Cumulative percent on the y-axis refers to the percent of the population traveling that speed or faster. The average velocity of cranial NCCs pre-treatment is $179 \mu\text{m/hr} \pm 10$ (SEM) ($n = 23$) on FN20 (filled circles) compared to $186 \mu\text{m/hr} \pm 8$ ($n = 19$) on LM20 (open circles). The two pre-treatment conditions are not statistically different ($P > 0.05$; t test). The average velocity of trunk NCCs pre-treatment is $142 \mu\text{m/hr} \pm 5$ (SEM) ($n = 24$) on FN20 (filled triangles) compared to $173 \mu\text{m/hr} \pm 8$ ($n = 26$) on LM20 (open triangles). The difference between the two pre-treatment conditions is statistically significant ($P < 0.01$; t test). (B) Cumulative distribution plots of cranial and trunk NCC velocity after Mn^{2+} treatment. The average velocity of cranial NCCs post-treatment is $106 \mu\text{m/hr} \pm 10$ on FN20 compared to $171 \mu\text{m/hr} \pm 9$ on LM20. The average velocity of trunk

NCCs post-treatment is $89 \mu\text{m/hr} \pm 5$ on FN20 compared to $157 \mu\text{m/hr} \pm 6$ on LM20. The differences between the post-treatment conditions for both cranial and trunk NCCs are statistically significant ($P < 0.001$; t test). (C) Cumulative distribution plots of the change in cell velocity after Mn^{2+} treatment where each point represents the change in velocity of a single cell following Mn^{2+} treatment. Cranial NCCs cultured on FN20 and treated with Mn^{2+} slow an average of $73 \mu\text{m/hr} \pm 11$ (post-treatment velocities are statistically different from pre-treatment velocities; $P < 0.001$; paired t test). In contrast, cranial NCCs cultured on LM20 and treated with Mn^{2+} slow an average $15 \mu\text{m/hr} \pm 10$ (pre-and post-treatment velocities are not significantly different; $P > 0.05$, paired t test). Trunk NCCs cultured on FN20 and treated with Mn^{2+} slow an average of $52 \mu\text{m/hr} \pm 8$ (post-treatment velocities are statistically different from pre-treatment velocities; $P < 0.001$; paired t test). Trunk NCCs cultured on LM20 and treated with Mn^{2+} slow an average $16 \mu\text{m/hr} \pm 7$ (post-treatment velocities are significantly different from pre-treatment velocities; $P < 0.05$, paired t test). Specific points in each graph are not directly comparable to corresponding points in other graphs as each point merely reflects a cell's velocity, or change in velocity, in relation to the whole population.

Figure 2.

Activated integrin $\beta 1$ distribution in NCCs is substratum-dependent. Cranial and trunk NCCs were cultured on high concentrations of fibronectin or laminin and immunostained for activated integrin $\beta 1$ before (–) and after (+) Mn^{2+} treatment. On high concentrations of fibronectin, activated integrin $\beta 1$ clustered both before and after Mn^{2+} treatment (Fig. 2A). In contrast, there was much less clustering of activated integrin $\beta 1$ in cranial NCCs cultured on high laminin both before and after Mn^{2+} treatment. For trunk NCCs cultured on high fibronectin, activated integrin $\beta 1$ clustered both before and after Mn^{2+} treatment (Fig. 2B). Activated integrin $\beta 1$ also clustered in trunk NCCs cultured on high laminin, although perhaps to a lesser degree than on high fibronectin.

Figure 3.

Integrin activation regulates cranial NCC motility in a substratum concentration-dependent manner, but not trunk NCC motility. (A) Cumulative distribution plots of cranial and trunk NCC velocity before Mn^{2+} treatment are shown where each point represents the pre-treatment velocity of a single cell. Cumulative percent on the y-axis refers to the percent of the population traveling that speed or faster. The average velocity of cranial NCCs pre-treatment is $179 \mu\text{m/hr} \pm 10$ (SEM) ($n = 23$) on FN20 (filled circles) compared to $172 \mu\text{m/hr} \pm 8$ ($n = 24$) on FN1 (open circles). The two pre-treatment conditions are not statistically different ($P > 0.05$; t test). The average velocity of trunk NCCs pre-treatment is $142 \mu\text{m/hr} \pm 5$ (SEM) ($n = 24$) on FN20 (filled triangles) compared to $157 \mu\text{m/hr} \pm 6$ ($n = 26$) on FN1 (open triangles). The difference between the two pre-treatment conditions is statistically significant ($P < 0.05$; t test). (B) Cumulative distribution plots of cranial and trunk NCC velocity after Mn^{2+} treatment. The average velocity of cranial NCCs post-treatment is $106 \mu\text{m/hr} \pm 10$ on FN20 compared to 128

$\mu\text{m/hr} \pm 11$ on FN1. The two post-treatment conditions are not statistically different ($P > 0.05$; t test). The average velocity of trunk NCCs post-treatment is $89 \mu\text{m/hr} \pm 5$ on FN20 compared to $115 \mu\text{m/hr} \pm 8$ on FN1. The difference between the two post-treatment conditions is statistically significant ($P < 0.01$; t test). (C) Cumulative distribution plots of the change in cell velocity after Mn^{2+} treatment where each point represents the change in velocity of a single cell following Mn^{2+} treatment. Cranial NCCs treated with Mn^{2+} show an average of $73 \mu\text{m/hr} \pm 11$ on FN20 versus $44 \mu\text{m/hr} \pm 11$ on FN1 (post-treatment velocities on both substrata are statistically different from pre-treatment velocities; $P < 0.001$; paired t test). The difference between the changes in velocity for cranial NCCs on FN20 and FN1 is statistically significant ($P < 0.05$; t test). Trunk NCCs treated with Mn^{2+} show an average of $52 \mu\text{m/hr} \pm 8$ on FN20 versus $42 \mu\text{m/hr} \pm 8$ on FN1 (post-treatment velocities on both substrata were statistically different from pre-treatment velocities; $P < 0.001$; paired t test). The change in velocity for trunk NCCs on FN20 or FN1 is not statistically significant ($P > 0.05$; t test). Specific points in each graph are not directly comparable to corresponding points in other graphs as each point merely reflects a cell's velocity, or change in velocity, in relation to the whole population.

Figure 4.

Surface levels of active and inactive integrin $\beta 1$ in cranial and trunk NCCs cultured on low (FN1) and high (FN20) concentrations of fibronectin. (A) Cell surface labeled integrins were immunoprecipitated and analyzed on western blot. Average band intensities of activated integrin $\beta 1$ were determined for cranial and trunk NCCs cultured on FN1 or FN20 without Mn^{2+} treatment (red). Average band intensities of activated integrin $\beta 1$ with Mn^{2+} treatment are shown in black. The levels of activated integrin $\beta 1$ on the surface of cranial NCCs cultured on FN1 is significantly different from that of trunk NCCs cultured on the same substratum ($P < 0.05$; t test). (B) All eight conditions were simultaneously analyzed in a total of seven experiments. Representative gel from one experiment.

Figure 5.

Mn^{2+} -induced clustering of activated integrin $\beta 1$ differs between NCCs cultured on low and high fibronectin. (A) Cranial NCCs were cultured on low (FN1) high (FN20) concentrations of fibronectin and stained for activated integrin $\beta 1$ without ($-Mn^{2+}$) and with ($+Mn^{2+}$) treatment with Mn^{2+} . For cranial NCCs, the clustering index (percent of focal adhesion area/focal adhesion number) significantly increases after Mn^{2+} treatment on FN1 ($P < 0.01$; t test) but not on FN20 ($P > 0.05$; t test). (B) Trunk NCCs were cultured on low (FN1) high (FN20) concentrations of fibronectin and stained for activated integrin $\beta 1$ without ($-Mn^{2+}$) and with ($+Mn^{2+}$) treatment with Mn^{2+} . For trunk NCCs, the clustering index increases after Mn^{2+} treatment on FN20 ($P < 0.05$; t test), but not on FN1 ($P > 0.05$; t test).

Cranial

Little inactivation
 Mn^{2+} induces clustering
 Slows due to increased avidity

Some inactivation
 Mn^{2+} induces activation
 Slows due to increased affinity

Trunk

Some inactivation
 Mn^{2+} induces activation
 Slows due to increased affinity

No inactivation
 Mn^{2+} induces clustering
 Slows due to increased avidity

| activated $\beta 1$ integrin L inactivated $\beta 1$ integrin

Figure 6.

Diagram summarizing results of neural crest motility on fibronectin. NCC motility on fibronectin is mediated by a combination of activated surface integrin $\beta 1$ levels and integrin $\beta 1$ clustering. On low fibronectin concentrations (FN1), cranial NCCs have few inactivated $\beta 1$ receptors on their surface. Mn^{2+} treatment induces integrin $\beta 1$ clustering in cranial NCCs cultured on FN1, and may slow their motility due to increased avidity to the substratum. On high fibronectin concentrations (FN20), cranial NCCs have some inactivated $\beta 1$ receptors on their surface. Mn^{2+} treatment activates some inactive $\beta 1$ integrins and may slow cell motility due to increased affinity for the substratum. The effects of Mn^{2+} treatment on non- $\beta 1$ -containing integrins has not been determined, but $\alpha V\beta 3$ and $\alpha V\beta 5$ are known to play important roles in neural crest motility. In contrast to cranial NCCs, on low fibronectin (FN1), trunk NCCs have some inactivated $\beta 1$ receptors on their surface. Mn^{2+} treatment activates some inactive $\beta 1$ integrins and may slow cell motility due to increased affinity for the substratum. For trunk NCCs on high fibronectin (FN20), all surface $\beta 1$ integrins are activated. Mn^{2+}

treatment induces integrin $\beta 1$ clustering in trunk NCCs cultured on FN20, and may slow their motility due to increased avidity to the substratum.

Table 1

Integrin clustering.

Condition (# of cells)	Cell area (μm^2)	Focal adhesion area	% Focal adhesion area	Focal adhesion count	Clustering index
CF1– (<i>n</i> = 23)	2424 ± 175	255 ± 23	10.6 ± 0.7	244 ± 20	4.8 ± 0.4
CF1+ (<i>n</i> = 35)	2035 ± 99	251 ± 18	12.5 ± 0.7	212 ± 12	6.1 ± 0.3
CF20– (<i>n</i> = 30)	2742 ± 135	270 ± 20	9.8 ± 0.6	216 ± 15	5.0 ± 0.3
CF20+ (<i>n</i> = 21)	2787 ± 188	278 ± 22	10.1 ± 0.6	247 ± 21	4.6 ± 0.4
TF1– (<i>n</i> = 33)	2153 ± 120	249 ± 18	11.4 ± 0.4	235 ± 16	5.3 ± 0.3
TF1+ (<i>n</i> = 29)	2192 ± 115	250 ± 15	11.6 ± 0.5	237 ± 14	5.1 ± 0.3
TF20– (<i>n</i> = 33)	2312 ± 130	258 ± 12	11.7 ± 0.5	224 ± 11	5.5 ± 0.3
TF20+ (<i>n</i> = 45)	2152 ± 108	255 ± 13	12.2 ± 0.5	210 ± 10	6.2 ± 0.3