Modulation of mouse neural crest cell motility by N-cadherin and connexin 43 gap junctions

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Connexin 43 (Cx43α1) gap junction has been shown to have an essential role in mediating functional coupling of neural crest cells and in modulating neural crest cell migration. Here, we showed that N-cadherin and wnt1 are required for efficient dye coupling but not for the expression of Cx43α1 gap junctions in neural crest cells. Cell motility was found to be altered in the N-cadherin–deficient neural crest cells, but the alterations were different from that elicited by Cx43α1 deficiency. In contrast, wnt1-deficient neural crest cells showed no discernible change in cell motility. These observations suggest that dye coupling may not be a good measure of gap junction communication relevant to motility. Alternatively, Cx43α1 may serve a novel function in motility. We observed that p120 catenin (p120ctn), an Armadillo protein known to modulate cell motility, is colocalized not only with N-cadherin but also with Cx43α1. Moreover, the subcellular distribution of p120ctn was altered with N-cadherin or Cx43α1 deficiency. Based on these findings, we propose a model in which Cx43α1 and N-cadherin may modulate neural crest cell motility by engaging in a dynamic cross-talk with the cell’s locomotory apparatus through p120ctn signaling.

Introduction

The deployment of migratory cells plays an important role in embryonic development and disease. In vertebrates, a remarkable migratory cell population known as the neural crest cells plays an essential role in many aspects of development. These cells are derived from the dorsal neuroepithelium by an epithelial–mesenchymal cell transformation. They migrate long distances along distinct pathways to many different regions of the embryo and contribute to the differentiation and development of a diverse array of tissues. Various developmental defects can arise when neural crest cells do not migrate along their stereotypical pathways, or for other reasons, failed to reach their intended target sites. For example, perturbation in the deployment of the cardiac neural crest cells can result in persistent truncus arteriosus and various aortic arch anomalies (Kirby and Waldo, 1995). Thus, elucidating the mechanisms regulating neural crest cell migration is of great interest not only given its importance in vertebrate development, but also such studies have significant clinical relevance.

Recent studies have indicated an essential role for gap junctions in modulating the migratory behavior of neural crest cells. Gap junctions contain membrane channels that allow the movement of ions, second messengers, and small metabolites between cells (for review see Bruzzone et al., 1996). They are encoded by a multigene family known as the connexins. The gap junction protein, connexin 43 or α1, connexin (Cx43α1),* is expressed abundantly in migrating neural crest cells, and these cells are also functionally coupled via gap junction channels (Lo et al., 1999). These observations are consistent with the fact that neural crest cells migrate not as individual cells but as cohorts of cells that are organized in sheets or streams (Bancroft and Bellairs, 1976; Davis and Trinkaus, 1981). When the Cx43α1 gene is deleted in the Cx43α1 knockout mice, conotruncal heart malformations and outflow obstruction were elicited (Reaume et al., 1995). Using transgenic mouse models, we showed that the requirement for Cx43α1 in conotruncal heart development involves the cardiac neural crest cells (Ewart et al., 1997; Huang et al., 1998a; Sullivan et al., 1998). Cardiac neural crest cells, a neural crest subpopulation derived from the postotic hindbrain neural fold, play an essential role in outflow tract septation and tissue remodeling in the
conotruncal region of the heart. Transgenic mice exhibiting an elevation or inhibition of Cx43α1 function targeted to the cardiac neural crest cells show outflow tract obstruction and conotruncal heart malformations (Ewert et al., 1997; Huang et al., 1998b; Sullivan et al., 1998). These heart malformations are likely due to alterations in the abundance of neural crest cells reaching the heart. Thus an elevation of gap junction communication was associated with an enhanced rate of neural crest cell migration and an increase in the abundance of neural crest cells in the outflow tract (Huang et al., 1998a). In contrast, a reduction or inhibition of gap junction communication was associated with a reduced rate of neural crest cell migration, and concomitantly, a decrease in the abundance of crest cells in the outflow tract (Huang et al., 1998a). Based on these observations, we proposed previously that gap junctions may mediate the cell-to-cell movement of second messengers and other cell-signaling molecules involved in regulating cell locomotion and in this manner help coordinate the deployment of neural crest cells to the heart (Huang et al., 1998a; Lo and Wessels, 1998).

The present study was initiated to examine the role of cadherin-based adherens junction in modulating gap junction communication and the migration of mouse cardiac neural crest cells. Studies in tissue culture cells have shown previously that gap junction formation is dependent on cadherin-mediated cell–cell adhesion (Keane et al., 1988; Mege et al., 1988; Matsuzaki et al., 1990; Musil et al., 1990; Meyer et al., 1992; Frenzel and Johnson, 1996). In chick embryos, N-cadherin is expressed in the neural tube and emerging neural crest cells (Hatta and Takeichi, 1986). A specific requirement for N-cadherin in neural crest cell migration was shown by the finding that the inhibition of N-cadherin with function-blocking antibody perturbed the deployment of neural crest cells (Bronner-Fraser et al., 1992). In such antibody-treated embryos, neural crest cells were found inside the lumen of the neural tube or in the extraembryonic space dorsal to the neural tube but not in the expected dorsolateral neural crest migratory pathway. In addition, studies using adenoviral vectors to ectopically express various N-cadherin constructs have shown that overexpression of N-cadherin can disrupt the normal deployment of neural crest cells (Nakagawa and Takeichi, 1998). In the present study, we sought to elucidate the role of N-cadherin in the deployment of mouse cardiac neural crest cells and whether this involves the modulation of Cx43α1-mediated gap junction communication.

Our studies show that N-cadherin has an essential role in mouse neural cell migration. Mouse neural crest cells express N-cadherin in regions of cell–cell contact found between extended cell processes. N-cadherin is often colocalized or closely juxtaposed with Cx43α1 gap junctions. Interestingly, in N-cadherin–deficient neural crest cells, although Cx43α1 gap junction contacts remained abundant, gap junction communication was reduced to levels like that of the Cx43α1-deficient neural crest cells. These results suggest that N-cadherin and Cx43α1 gap junctions may have separable roles in neural crest cell locomotion. As β-catenin expression was reduced in the N-cadherin but not Cx43α1-deficient neural crest cells, we further examined the possible involvement of wnt signaling in neural crest cell motility. Neural crest cells from the wnt1 knockout mouse embryos exhibited normal cell motility, even though gap junction communication was reduced to levels like that of Cx43α1-deficient neural crest cells. As with the N-cadherin–deficient neural crest cells, Cx43α1 gap junction contacts remained abundant at the cell surface in wnt1-deficient neural crest cells. These observations suggest that N-cadherin and wnt1 may regulate gating of Cx43α1 gap junction channels in neural crest cells. As cadherin is known to bind p120 catenin (p120ctn), an Armadillo protein also involved in modulating cell motility (Anastasiadis and Reynolds, 2000; Grosheva et al., 2000; Noren et al., 2000), we examined p120ctn expression in neural crest cells. p120ctn was observed to be expressed abundantly in neural crest cells. It was found to be colocalized with N-cadherin and Cx43α1. Significantly, the distribution of p120ctn was altered in the Cx43α1- and N-cadherin–deficient neural crest cells. Based on these findings, we propose that neural crest cell motility may be modulated by the dynamic interactions of N-cadherin and Cx43α1 with the cell’s locomotory apparatus through p120ctn signaling.

Results

Cx43α1 gap junctions in N-cadherin–deficient neural crest cells

Our studies focused on the cardiac neural crest cells, a subpopulation of neural crest cells that emerge from the postotic hindbrain neuroepithelium at E8.5 (Fukishii and Morriss-Kay, 1992). This is before the time when N-cadherin–deficient embryos die from cell adhesion defects in the developing myocardium (Radice et al., 1997). To determine if N-cadherin is expressed in the cardiac neural crest cells, postotic hindbrain neural tube explant cultures were generated, and immunofluorescence microscopy was used to examine the newly emerged neural crest cells. Such studies showed the distribution of N-cadherin diffusely over the cell surface and also clustered in punctate spots along extended cell processes, many of which were thin projections barely visible by phase-contrast microscopy (Fig. 1 A). This pattern is similar to that reported for N-cadherin expression in neural crest cells in chick neural tube explant cultures (Monier-Gavelle and Duband, 1995). Double immunostaining with Cx43α1 and N-cadherin antibodies revealed in some regions the close juxtapositioning or colocalization of N-cadherin with Cx43α1, particularly along cell processes (Fig. 1 A, arrows). Examination of neural crest cells from the N-cadherin–deficient embryos surprisingly showed no detectable change in the abundance of Cx43α1 gap junction plaques at the cell surface (Fig. 2 A). The specificity of the Cx43α1 antibody was demonstrated by a parallel analysis of Cx43α1-deficient neural crest cells, which showed no significant immunostaining (Fig. 2 C). We also examined the expression of N-cadherin in the Cx43α1-deficient neural crest cells and showed no obvious change in the distribution of N-cadherin, which remained abundant at regions of cell–cell contact (data not shown).
Gap junction communication in neural crest cells requires N-cadherin function

To determine whether gap junction communication may be functionally altered by the loss of N-cadherin, we quantitated the level of dye coupling. This entailed carrying out microelectrode impalements into neural crest cells and iontophoretically injecting carboxyfluorescein, a fluorescent tracer that can pass through gap junction channels. After 2 min of dye injection, the electrode was withdrawn and the number of dye-filled cells counted to determine the extent of gap junction communication. Such studies carried out in neural crest outgrowth cultures showed that gap junction communication is reduced greatly in the N-cadherin–deficient neural crest cells to levels similar to that observed in the Cx43α1-deficient neural crest cells (Table I; Huang et al., 1998a). To determine whether the changes in dye coupling detected in vitro reflected changes in gap junction communication in vivo, we examined dye coupling in presumptive neural crest cells in the living embryo. For these studies, intracellular impalements were carried out into the dorsolateral region of the hindbrain neural fold, a region where cardiac neural crest cells are situated. Such studies confirmed that gap junction communication in the N-cadherin–deficient neural crest cells is reduced in vivo as in vitro (Table I). These observations in conjunction with the findings above would suggest that N-cadherin may have a role in regulating gating of the Cx43α1 gap junction channel.

N-cadherin deficiency reduces cell migration and elevates cell proliferation

Given our previous observations showing a reduction in the rate of neural crest cell migration with the loss or reduction of Cx43α1-mediated gap junction communication, we expected that the loss of N-cadherin would reduce the rate of neural crest cell migration. To monitor the overall rate of neural crest cell migration, we first examined the migration index, a method for estimating migration rate based on a measurement of the neural crest outgrowth area in neural tube explant cultures (Huang et al., 1998a). In the 24-h outgrowth cultures, the migration index was reduced significantly in the heterozygous and homozygous N-cadherin knockout mouse embryos. However, by 48 h no significant migration differences were detected (Table II). Analysis of BrdU incorporation suggests that this disparity could be due to alterations in the rate of cell proliferation. Thus, cell proliferation was significantly increased in the heterozygous and homozygous N-cadherin knockout explants (Table III).

Table 1. Dye coupling in neural crest cells of N-cadherin

| N-cadherin genotype | Number of dye-filled cells* |
|---------------------|-----------------------------|
|                     | In vitro | In vivo |
| ++                  | 3.14 ± 1.58 (28) | 4.90 ± 1.07 (20) |
| +/-                 | 1.98 ± 0.95 (42)* | 3.92 ± 1.10 (50)* |
| --                  | 1.46 ± 0.60 (22)* | 2.31 ± 0.58 (19)* |

*Number of impalements carried out indicated in parentheses.
*P = 0.0001 when compared with ++/+. 
which over 48 h could inflate the migration index (Table II). It is interesting to note that these results contrast with that of the Cx43\(\alpha_1\)/H9251-deficient neural crest cells, which do not show any change in the rate of cell proliferation (Huang et al., 1998a).

**Neural crest cell motility differentially affected by N-cadherin versus Cx43\(\alpha_1\) deficiency**

To determine precisely how cell motility is affected by the loss of N-cadherin and how it may compare with cell motility perturbation elicited by Cx43\(\alpha_1\) deficiency, we used time-lapse videomicroscopy and motion analysis to quantify various cell motility parameters in individual neural crest cells. For this study, images of neural tube explant cultures were captured every 10 min over a 20-h interval. Examination of the resulting time-lapse movies revealed no discernible difference in the timing of neural crest cell emergence in explants derived from the N-cadherin– or Cx43\(\alpha_1\)-deficient mouse embryos compared with wild-type littermates. Tracking and analyzing the migratory paths of individual neural crest cells provided quantitative information on three cell motility parameters: speed, directionality (ratio of net over total distance traveled), and persistence of cell movement (direction change divided by speed). Surprisingly, this analysis showed that the speed of neural crest cell locomotion was elevated in the N-cadherin–deficient neural crest cells, but this was accompanied by a decrease in the directionality of cell movement (Table IV). This reduction in directionality is sufficiently large enough that it can be discerned visually by the actual migratory paths of the individual neural crest cells (Fig. 3). In addition, the persistence of cell movement was increased in the N-cadherin–deficient neural crest cells (Table IV; data not shown). A parallel analysis of Cx43\(\alpha_1\)-deficient neural crest cells revealed a similar reduction in the di-
deficient neural crest cells, we observed a marked reduction in crest perturbations (Brault et al., 2001). In the N-cadherin–resulted in craniofacial defects indicative of cranial neural
distribution (Dufour et al., 1999), we examined changes in cadherin expression can affect crest cell motility (Olson et al., 1991; Olson and Moon, 1992) and suggest that the altered cell motility of the N-cadherin–deficient neural crest cells is not simply due to the perturbation of Cx43α1-mediated gap junction communication.

Wnt1 is essential for coupling but not neural crest cell motility
Since changes in cadherin expression can affect β-catenin distribution (Dufour et al., 1999), we examined β-catenin expression in neural crest cells by immunofluorescence microscopy. These studies are of particular interest, given a recent report showing that wnt1-Cre–mediated deletion of β-catenin in the dorsal neuroepithelium and neural crest cells resulted in craniofacial defects indicative of cranial neural crest perturbations (Brault et al., 2001). In the N-cadherin–deficient neural crest cells, we observed a marked reduction in cell surface expression of β-catenin at regions of cell–cell contact in extended cell processes (Fig. 2 B compare with Fig. 1 B). As Cx43α1 also has been shown to bind β-catenin (Ozawa et al., 1989; Ai et al., 2000), we carried out β-catenin and Cx43α1 double immunolocalization studies. In wild-type neural crest cells, β-catenin was expressed abundantly and exhibited extensive colocalization with Cx43α1 (Fig. 1 B). In the absence of Cx43α1, β-catenin continued to be expressed abundantly at regions of cell–cell contact, similar to that seen in wild-type neural crest cells (Fig. 2 D; data not shown). Due to technical constraints, we were unable to determine whether there were any changes in the level of β-catenin expression in the cytoplasm or nucleus. These results suggest the possibility that N-cadherin deficiency could alter β-catenin–mediated wnt signaling in neural crest cells. Previous studies have indicated a role for wnt signaling in neural crest cell proliferation and differentiation, but whether there is also a role in cell migration is not known (Ikeya et al., 1997; Dunn et al., 2000). To examine this question, we analyzed the motility of neural crest cells from the wnt1 knockout mouse embryos. Using time-lapse videomicroscopy and motion analysis, we found no change in any cell motility parameter with the loss of wnt1 function (Table VI). We also used lithium treatment to mimic wnt signaling in neural tube explant cultures and at 10 mM concentration (the highest compatible with viability) found no significant effect on neural crest cell motility (data not shown). These results indicate that wnt1 does not have an essential role in neural crest cell motility.

Since previous studies by others have indicated that wnt1 signaling potentially can modulate gap junction communication (Olson et al., 1991; Olson and Moon, 1992) and Cx43α1 transcription (van der Heyden et al., 1998; Ai et al., 2000), we also examined Cx43α1 gap junction expression known.

### Table II. Neural crest migration in neural tube explant cultures from N-cadherin knockout mouse embryos

| N-cadherin genotype | 24 h | 48 h |
|---------------------|------|------|
| +/-                 | 0.589 ± 0.018 (53) | 2.042 ± 0.084 (29) |
| +/-                 | 0.463 ± 0.016 (99) | 1.837 ± 0.055 (56) |
| +/-                 | 0.414 ± 0.021 (39) | 1.947 ± 0.073 (25) |

### Table III. Neural crest cell proliferation in explant cultures of N-cadherin knockout mouse embryos

| N-cadherin genotype | BrdU incorporation |
|---------------------|--------------------|
|                     | 24 h | 28 h |
| +/-                 | 9.5 ± 2.3 (22) | 10.9 ± 1.8 (21) |
| +/-                 | 10.1 ± 2.7 (43) | 12.2 ± 3.5 (37) |
| +/-                 | 13.5 ± 2.5 (12) | 15.1 ± 2.7 (18) |

### Table IV. Analysis of N-cadherin-deficient neural crest cell locomotion by time-lapse videomicroscopy

| N-cadherin genotype | Speed | Directionality | Persistence |
|---------------------|-------|---------------|-------------|
|                     | μm/h  | μm/min-deg    |             |
| +/- (52/5)          | 33.4 ± 2.49 | 0.413 ± 0.019 | 0.127 ± 0.006 |
| +/- (21/2)          | 42.8 ± 2.74 | 0.435 ± 0.034 | 0.172 ± 0.014 |
| +/- (29/2)          | 44.4 ± 2.40 | 0.347 ± 0.024 | 0.186 ± 0.017 |

Notes:

- The number of explants analyzed is indicated in parentheses.
- No statistically significant differences were found by ANOVA.
- \(P = 0.0001\) when compared with +/-.
and dye coupling in the wnt1 knockout mouse embryos. Analysis of the wnt1-deficient neural crest cells by immunohistochemistry revealed an abundance of Cx43α1 gap junctions (Fig. 4 A). N-cadherin expression also was not detectably altered (Fig. 4 B) nor was the cell surface expression of β-catenin (data not shown). Nevertheless, analysis of dye coupling with microelectrode impalements into presumptive neural crest cells in E8.5 embryos showed that gap junction communication was reduced greatly in the wnt1 knockout embryos to a level similar to that of the Cx43α1 or N-cadherin knockout mouse embryos (Table VII; Huang et al., 1998a). These results indicate that although wnt1 is not required for Cx43α1 protein expression nor Cx43α1 gap junction formation, it nevertheless may have a role in regulating gating of the Cx43α1 gap junction channel.

A possible role for p120 catenin in neural crest cell motility

To further investigate the mechanism by which N-cadherin and Cx43α1 gap junctions may modulate neural crest cell motility, we considered the potential role of p120ctn, an Armadillo protein which binds to the juxtamembrane region of cadherin and is known to modulate cell motility through the Rho GTPases (for review see Anastasiadis and Reynolds, 2000). Studies using various tissue culture cells have shown that p120ctn can be found distributed between the cell membrane, cell cytoplasm, and the nucleus (Anastasiadis and Reynolds, 2000). Analysis by immunohistochemistry showed that p120ctn is expressed abundantly in mouse cardiac neural crest cells. It is membrane localized at regions of cell–cell contact, and it is also present diffusely in the cell cytoplasm (Fig. 5 B). When the neural crest cells were fixed in the presence of 0.3% Triton X-100 to extract the cytoplasmic p120ctn, localization of p120ctn in the nucleus also can be discerned (Fig. 5 E and Fig. 6 A). The level of nuclear staining varied somewhat between explants with most being at very low levels (Fig. 5 E and Fig. 6 B). However, in 1 of 15 wild-type neural crest explants examined a more prominent level of nuclear staining was observed (Fig. 6 A). The inclusion of Triton in the fixative also made the membrane-bound p120ctn more sharply delineated, even when viewed by standard immunofluorescence microscopy (see Fig. 5 E, with Triton treatment; compare with B, without Triton treatment). This is presumably due to the removal of cytoplasmic p120ctn. It is important to note that the overall pattern of p120ctn distribution at the cell surface in Triton-treated cells was identical to that seen in cells fixed without Triton, being associated mostly with regions of cell–cell contact. Note that Fig. 5 B shows a relatively low level of cytoplasmic p120ctn immunostaining, since the image was obtained from optical slices of only 0.2 μm in thickness. In contrast, Fig. 5 E is a standard immunofluorescent image, but most of the cytoplasmic p120ctn has been extracted due to the inclusion of Triton during fixation.

### Table V. Analysis of Cx43α1-deficient neural crest locomotion by time-lapse videomicroscopy

| Cx43α1 genotype | Speed        | Directionality | Persistence |
|-----------------|--------------|----------------|-------------|
|                 | μm/min       | μm/min-deg     |             |
| +/- (16/3)      | 36.88 ± 2.10 | 0.472 ± 0.022  | 0.136 ± 0.020 |
| +/- (39/5)      | 38.53 ± 1.63 | 0.457 ± 0.020  | 0.142 ± 0.011 |
| +/- (15/2)      | 36.88 ± 2.10 | 0.369 ± 0.030b | 0.142 ± 0.009 |

*The number in parentheses is the number of cells and the number of separate embryos analyzed, respectively.

### Table VI. Analysis of wnt1-deficient neural cell locomotion by time-lapse videomicroscopy

| Wnt1 genotype | Speed        | Directionality | Persistence |
|---------------|--------------|----------------|-------------|
|               | μm/min       | μm/min-deg     |             |
| +/- (17/3)    | 31.49 ± 1.06 | 0.573 ± 0.032  | 0.137 ± 0.011 |
| +/- (29/5)    | 31.67 ± 1.07 | 0.605 ± 0.022  | 0.129 ± 0.006 |
| +/- (30/4)    | 32.73 ± 0.68 | 0.615 ± 0.010  | 0.131 ± 0.005 |

*No significant differences were found for any of the parameters.

*The number in parentheses is the total number of cells and the total number of embryos analyzed, respectively.
Table VII. Dye coupling in neural crest cells from the Wnt1 knockout mouse embryos

| Wnt1 genotype | Number of embryos | Number of impalements | Dye transfer |
|---------------|-------------------|-----------------------|--------------|
| +/-           | 5                 | 37                    | 3.97 ± 0.16  |
| +/-           | 5                 | 34                    | 4.15 ± 0.16  |
| --            | 6                 | 53                    | 1.58 ± 0.13  |

*Dye coupling was carried out with impalements into presumptive neural crest cells in the E8.5 embryo. No significant differences were found between Wnt1 +/- and +/-.

To determine whether N-cadherin and p120ctn are colocalized at the cell surface, immunostaining was carried out using an N-cadherin mouse monoclonal antibody (Fig. 5 D) followed by an FITC-conjugated p120ctn mouse antibody (Fig. 5 E). The N-cadherin antibody binding was detected using Cy3-conjugated secondary antibody. This analysis showed extensive colocalization of N-cadherin with p120ctn using Cy3-conjugated secondary antibody. This analysis followed by an FITC-conjugated p120ctn mouse antibody (Fig. 5 E). The N-cadherin antibody binding was detected using Cy3-conjugated secondary antibody. This analysis showed extensive colocalization of N-cadherin with p120ctn at the cell surface (Fig. 5 F). Interestingly, double immunostaining of neural crest cells with Cx43α1 and p120ctn antibodies showed that Cx43α1 is also extensively colocalized with p120ctn at the cell surface (Fig. 5 C, white arrows). This same pattern of p120ctn colocalization with N-cadherin and Cx43α1 was observed in cells fixed with or without Triton (Fig. 5; data not shown).

In the N-cadherin- and Cx43α1-deficient neural crest cells, we observed distinct changes in the pattern of p120ctn distribution. In the N-cadherin-deficient neural crest cells, most of the cell surface expression of p120ctn was lost; although infrequently, some p120ctn can still be found along cell processes (Fig. 6 D). Very striking was the strong localization of p120ctn in the nuclei of the N-cadherin-deficient crest cells (Fig. 6 D). The increase in p120ctn immunostaining in the nuclei cannot be fully appreciated by the image in Fig. 6 D, since the immunofluorescence intensity was beyond the saturation limit of the camera. A parallel analysis of the Cx43α1-deficient neural crest cells revealed an apparent increase in p120ctn localization in the nucleus but not as striking as that observed in the N-cadherin knockout cells (Fig. 6 C). In addition, there was a reduction in cell surface expression of p120ctn (Fig. 6 C compare with A and B). Quantitative analysis showed that this is due to a reduction in both the length and area of cell surface p120ctn (Table VIII). Overall, these observations suggest that the loss of N-cadherin and Cx43α1 could potentially perturb p120ctn-mediated signaling in neural crest cells.

Discussion

Our studies showed that N-cadherin is expressed in cell processes extended between migrating mouse cardiac neural crest cells. Often N-cadherin was colocalized or closely juxtaposed with Cx43α1 gap junctions. In the N-cadherin-deficient neural crest cells, Cx43α1 gap junction contacts remained abundant at the cell surface. This is unexpected, since previous studies in tissue culture cells indicated that cadherin-containing adherens junctions may play an important role in gap junction formation (Keane et al., 1988; Mege et al., 1988; Musil et al., 1990; Jongen et al., 1991; Meyer et al., 1992; Frenzel and Johnson, 1996; Fujimoto et al., 1997). However, it is possible that another cadherin expressed in neural crest cells may help facilitate the formation of Cx43α1 gap junctions in the absence of N-cadherin (Kimura et al., 1995; Nakagawa and Takeichi, 1995). Despite the prevalence of gap junctions in the N-cadherin-deficient neural crest cells, gap junction communication was nevertheless reduced markedly to levels like that of the Cx43α1-deficient crest cells. Thus N-cadherin, though not essential for Cx43α1 gap junction formation, is required for dye coupling via Cx43α1 gap junction channels.

Our studies also revealed an essential role for N-cadherin in mouse neural crest cell migration. Analysis of neural crest outgrowth area in neural tube explant cultures indicated a significant reduction in the apparent rate of neural crest cell migration in the absence of N-cadherin. These results are similar to that seen previously in Cx43α1-deficient neural crest cells (Huang et al., 1998a). However, time-lapse videomicroscopy and an analysis of the migratory movement of individual neural crest cells revealed distinct differences in the motility changes elicited by the loss of N-cadherin versus Cx43α1. N-cadherin-deficient neural crest cells exhibited an increase in the speed of cell locomotion but a significant reduction in the directionality of cell movement. In contrast, Cx43α1 deficiency elicited only a reduction in the directionality of cell movement but no change in the speed of cell locomotion. Whereas the directionality of cell movement was reduced only in the homozygous N-cadherin and Cx43α1 knockout neural crest cells, the speed of cell locomotion was elevated in both heterozygous and homozygous N-cadherin knockout neural crest cells, thus indicating the latter cell motility parameter is subject to N-cadherin haploinsufficiency.

Modulation of neural crest cell motility by N-cadherin

Overall, our studies indicated that N-cadherin has an essential role in neural crest cell motility and that this cannot simply involve facilitating Cx43α1-mediated gap junction communication. We note that although the formation of cadherin-based adhesion contacts are generally associated with the cessation of cell locomotion, N-cadherin can stimulate the motility of breast cancer cells and also has been shown to facilitate tumor cell metastasis (Nieman et al., 1999; Hazan et al., 2000). In addition, ectopic expression of N-cadherin can cause cells to lose their tight cell–cell contact and convert into a spindle or fibroblastic morphology (Kintner, 1992; Islam et al., 1996). Although the precise mechanism by which N-cadherin facilitates cell migration is not known, previous studies have indicated that cadherin’s role in cell motility is separable from its role in cell adhesion. Thus, the β-catenin–binding domain of cadherin required for cell adhesion is not essential for cell motility (Chen et al., 1997). Studies with retinal ganglion cells also indicated that the β-catenin–binding domain of N-cadherin is not required for cadherin’s role in axonal outgrowth (Riehl et al., 1996). Rather, recent studies suggest that cadherin may modulate cell motility by mediating signaling via the p120ctn family of Armadillo proteins. p120ctn is bound to...
Neural crest cells show colocalization of N-cadherin and Cx43α1 with p120ctn. Neural crest cells derived from wild-type mouse embryos were double immunostained using Cx43α1 and p120ctn antibodies (A–C) or N-cadherin and p120ctn (D–F) antibodies. (A–C) Images of Cx43α1/p120ctn double immunostained cells were obtained by iterative deconvolution analysis of image stacks comprised of 0.2-μm slices. Merging of the Cx43α1 (A) and p120ctn (B) immunofluorescence images in C showed that Cx43α1 and p120ctn are extensively colocalized along cell processes (C, arrows). (D–F) For the N-cadherin/p120ctn immunostaining, the cells were fixed in the presence of 0.3% Triton X-100, and the sections were viewed by standard immunofluorescence microscopy. The removal of much of the cytoplasmic p120ctn by Triton extraction allowed clear delineation of the cell surface localized p120ctn and N-cadherin without deconvolution analysis. Note the colocalization of p120ctn and N-cadherin shown in F by merging the p120ctn (D) and N-cadherin immunofluorescence images (E) with a phase-contrast image of the cells. Note that some p120ctn immunostaining can be observed in the nucleus in the Triton extracted cells (E). All images are at the same magnification. Bar, 25 μm.
cadherin’s juxtamembrane region, a domain distinct from that mediating β-catenin binding (Yap et al., 1998; Anastasiadis et al., 2000). Studies in various cultured cell lines show that p120ctn is also found in the cytoplasm, where it can modulate the activity of the RhoGTPases and thus affect cell motility by changing the organization of the actin cytoskeleton (Anastasiadis et al., 2000; Grosheva et al., 2000; Noren et al., 2000). In addition, p120ctn can signal to the nucleus, since it has been shown to interact with the transcription factor Kaiso and translocate into the nucleus (Daniel and Reynolds, 1999).

We observed that p120ctn is expressed abundantly in migrating mouse cardiac neural crest cells. Its distribution is similar to that described for other cell types: being localized at the membrane, diffusely in the cytoplasm, and at a low level in the nucleus. The latter was most evident when the heavy cytoplasmic p120ctn immunostaining was reduced by including Triton in the fixative. In the N-cadherin–deficient

![Image](image_url)

**Figure 6. Immunolocalization of p120ctn in N-cadherin– and Cx43a1-deficient neural crest cells.** Neural crest cells from wild-type, Cx43a1, and N-cadherin knockout mouse embryos were fixed in the presence of Triton X-100 and then immunostained with a p120ctn antibody. In wild-type neural crest cells, p120ctn localized predominantly at the cell periphery, outlining regions of cell–cell contact found in cell processes (A and B). In such Triton extracted cells, some nuclear localization of p120ctn can be observed (A). In neural crest cells from the Cx43a1-deficient embryos (C), p120ctn expression in the cell periphery is reduced, and there is increased localization of p120ctn in the nucleus. In the N-cadherin–deficient neural crest cells (D), p120ctn expression at the cell periphery is generally not observed except in some cell processes. However, there is very strong localization of p120ctn in the nucleus. These images were obtained by standard immunofluorescence microscopy. All images are at the same magnification. Bar, 25 μm.

| Cx43a1 genotype | Number of cells/field | Area of cell surface p120ctn per field μm² | Length of cell surface p120ctn per field μm |
|-----------------|-----------------------|------------------------------------------|------------------------------------------|
| +/+ (9)         | 13.89 ± 1.18          | 496.59 ± 42.14                           | 2.631 ± 0.263                            |
| −/− (9)         | 13.56 ± 1.22          | 199.19 ± 20.20                           | 1.654 ± 0.060                            |

*Numbers in parentheses represent the number of fields analyzed.

\[ P = 0.8467. \]

\[ P < 0.0001. \]

\[ P = 0.0023. \]
neural crest cells, membrane-bound p120ctn was decreased, whereas p120ctn localization in nuclei was markedly increased. These results are consistent with a previous study showing that the exogenous expression of E-cadherin can downregulate the nuclear localization of p120ctn (Van Hengel et al., 1999). Since the Rho GTPases also have been shown to have a role in cell cycle regulation (Welsh and Assoian, 2000; Assoian and Schwartz, 2001), alterations in p120ctn signaling could potentially affect cell proliferation in the N-cadherin–deficient neural crest cells. We note that ectopic N-cadherin expression in breast tumor cells was reported to slow the growth of individual tumors, a result that is consistent with our finding of an elevation of cell proliferation in the N-cadherin–deficient neural crest cells (Hazan et al., 2000).

The role of connexins in neural crest cell motility

The present study together with our earlier work showed that Cx43 gap junctions play an essential role in neural crest cell motility. We proposed previously that Cx43-mediated gap junction communication might regulate neural crest cell migration by facilitating the cell-to-cell movement of second messengers and other cell-signaling molecules involved in cell locomotion (Huang et al., 1998a; Lo and Wessels, 1998). This model was based on our finding of an apparent correlation between changes in the level of dye coupling and alterations in the apparent rate of neural crest cell migration elicited by the gain or loss of Cx43 function. However, in the present study we showed that neural crest cells from the wnt1 knockout mouse embryos have normal cell motility even though they exhibited only a low level of functional coupling via gap junction channels. In addition, our studies showed no consistent correlation between cell motility changes and the stepwise decrease in dye coupling in neural crest cells derived from the heterozygous and homozygous N-cadherin or Cx43 knockout mouse embryos. This divergence between changes in neural crest cell motility and alterations in the level of dye coupling would suggest that carboxyfluorescein injection is not an appropriate method for examining the role of Cx43 in cell motility. Thus, carboxyfluorescein may not be a suitable gauge of the movement of molecules that are important in cell motility. Since wnt1-deficient neural crest cells continued to exhibit a low level of residual dye coupling, in principle this may be sufficient to mediate the cell-to-cell movement of molecules involved in modulating cell motility. However, this explanation is somewhat problematic, given that Cx43- and N-cadherin–deficient neural crest cells have dye coupling levels comparable to that of the wnt1-deficient neural crest cells.

Another possibility to consider is that the role of Cx43 in motility may involve a novel function. Since Cx43 is extensively colocalized with p120ctn, perhaps connexins like cadherins may have a role in modulating p120ctn signaling. It is of significance to note that the distribution of p120ctn is altered in the Cx43-deficient neural crest cells. In the wnt1-deficient neural crest cells, Cx43 gap junctions continued to be expressed in abundance at the cell surface. Since Cx43 gap junctions are also maintained in N-cadherin–deficient neural crest cells, presumably it is not merely whether Cx43 gap junctions are present but perhaps the modulation of Cx43 interaction with other proteins that is of critical importance in cell motility.

The integration of Cx43 and N-cadherin in cell motility

In light of the binding of p120ctn by Cx43 and N-cadherin and the colocalization or juxtapositioning of Cx43 and N-cadherin at the cell surface, we hypothesize that Cx43 and N-cadherin may be integrated within a cell surface complex that can modulate cell motility by transducing signals to the cytoskeleton and the nucleus. This would allow cell adhesion contacts and gap junctions to be regulated dynamically in conjunction with cell locomotion. Since p120ctn family members are subject to differential splicing and are typically expressed in multiple isoforms (Reynolds, et al., 1994; Staddon et al., 1995; Mo and Reynolds, 1996; Keirsebilck et al., 1998), it is possible that different p120ctn isoforms may bind Cx43 and thus differentiate the roles of cadherin versus connexin in cell motility. In preliminary studies, we observed that Cx43 coimmunoprecipitated with only one of two p120ctn bands expressed in NIH3T3 cells (unpublished data). In future studies, it may be interesting to examine another p120ctn family member, Armadillo repeat protein deleted in velocardiofacial syndrome (ARVCF; Mariner et al., 2000), a gene situated in a chromosome region deleted frequently in patients with velocardiofacial syndrome, a congenital condition involving cranial and cardiac neural crest defects (Young et al., 1980; Shprintzen et al., 1981; Sirokin et al., 1997). Studies in polarized epithelial cells have indicated that cadherin proteins play an important role in integrating components of the apical junctional complexes with signal transduction pathways (Yeaman et al., 1999). In this regard, it is interesting to note that the cadherin protein ZO-1 has been shown to bind Cx43 (Toyofuki et al., 1998); ZO-1 also has been observed to have a role in cadherin-based cell adhesion (Itoh et al., 1997). In future studies, it will be important to examine, on a biochemical level, the interactions of Cx43 and N-cadherin and their interactions with ZO-1, p120ctn, and other cytoplasmic proteins. However, these studies will likely necessitate the use of model tissue culture systems, since at best only a few thousand neural crest cells can be obtained from each postotic neural tube explant culture.

Modulation of gap junction communication by N-cadherin and wnt

Our finding that Cx43 gap junctions persisted even as dye coupling was virtually eliminated in the N-cadherin– and wnt1-deficient neural crest cells would suggest that wnt1 signaling and N-cadherin can modulate gating of the Cx43 gap junction channel. These results contrast with a previous study of mammalian tissue culture cells, indicating that Cx43 is transcriptionally regulated by the wnt1/B-catenin–signaling pathway (van der Heyden et al., 1998). Studies in the Xenopus embryo have shown that wnt signaling can modulate gap junction communication (Olson et al., 1991; Olson and Moon, 1992; Heasman et al., 1994), but this likely is posttranscriptionally regulated, since the ef-
fects of wnt on coupling occurs before the activation of zygotic transcription. It is interesting to note that more recent work suggests that the wnt modulation of gap junction communication in *Xenopus* embryos involves β-catenin but is independent of cadherin’s role in cell adhesion (Krufta et al., 1998). As β-catenin is extensively colocalized with Cx43 in neural crest cells, an interesting possibility is that the inhibition of dye coupling in the *wnt*- and N-cadherin–deficient neural crest cells may arise from the perturbation of β-catenin and Cx43 interactions. For example, β-catenin interactions could modulate conformation of the cytoplasmic tail of Cx43 and thus affect gating of the gap junction channel as proposed in the particle–receptor model (Homma et al., 1998). We note that the cell surface localization of β-catenin is greatly reduced in both *wnt*- and N-cadherin–deficient neural crest cells. A possible role for p120ctn in gating the gap junction channel also may be worth considering. In addition, as the cytoplasmic tail of Cx43 contains a consensus phosphorylation site for GSK3, alterations in wnt signaling could alter phosphorylation of the cytoplasmic tail of Cx43 and thus bring about conformational changes that may perturb channel activity directly or indirectly. In future studies, it will be important to define the cytoplasmic domain of Cx43 to which β-catenin and p120ctn bind and determine whether mutations that disrupt such interactions may affect gating of the gap junction channel.

Materials and methods

Mouse breeding and genotyping

Heterozygous N-cadherin, Cx43Δ1, and *wnt*1 knockout mice were interbred to generate litters of embryos that were then genotyped by PCR analysis of yolk sac DNA (Hanley and Merlie, 1991). The oligonucleotide primers and thermal cycle conditions used for PCR genotyping the *wnt*1 knockout mice are as described previously (McMahon et al., 1992). For genotyping the Cx43Δ1 knockout mouse embryos, the primers and conditions used are modified from those previously described (Reaume et al., 1993). For detecting the wild-type allele, the primer pairs used consisted of IMR5'-ACTTTTGGCCTGACTTACC-3' and IMR5'-CCCCACCTCTCACAATGTCTCC-3', which gave a 500-bp product, whereas the knockout allele was detected using primers IMR5'-ACTTTTGGCCTGACTTACC-3' and IMR5'-5'GGGCAAAATGTCGGTGGGAA-3', which gave a 1,000-bp product. The cycling parameters were 94°C for 45 s, 61.5°C (wild-type allele) or 60°C (knockout allele) for 30 s, and 72°C for 1 min for 35 cycles. For the N-cadherin knockout mouse embryos, two primer pairs were used: one set detected the N-cadherin knockout allele associated to the neo gene, 5'-CGTGTCTCGGCTACGCAAGC-3' and 5'-CAAAGCTATGGTCTGATACGCGTC-3', and the other primer pairs detected the wild-type N-cadherin allele associated to exons 10 and 11 of the N-cadherin gene, 5'-CACCCAAATGTTCTGAGGCAGG-3' and 5'-GGCAAGATGATTAGATGTTGACG-3'. Cycling parameters were 94°C for 1 min, 65°C for 2 min, and 72°C for 3 min for 30 cycles. PCR products were resolved in a 1.0–1.6% agarose gel.

Neural tube explant cultures

To obtain embryos for neural crest outgrowth cultures, mice were mated, and the day the vaginal plug was found was considered E0.5. Embryos were collected on E8.5, and the yolk sac from each embryo was harvested for genotyping by PCR analysis. The method used to establish the neural crest outgrowth cultures was described previously (Huang et al., 1998a) and was adapted from those described by Murphy et al. (1991) and Moi- seiwitsch and Lauder (1994). In brief, the hindbrain neural folds of E8.5 embryos were collagenase/dispase treated, and the dorsal ridge of the neu- roepithelium spanning the postotic region of the hindbrain neural fold was surgically removed from the surrounding tissue and cultured on a fibronectin-coated Petri dish in DME with high glucose and 10% FBS. The cultures were maintained for 24–48 h at 37°C with 5% CO₂. Lineage studies in mouse embryos have confirmed that neural crest cells from the postotic hindbrain neural fold give rise to the cardiac crest cells, as has been found in chick embryos (Fukishii and Morrisey, 1992; for review see Kirby and Waldo, 1995).

Neural crest dye coupling analyses in vitro and in vivo

To quantitate gap junction communication, dye coupling experiments were carried out in vitro and in vivo as described previously. For the in vitro studies, microelectrode impalements were made into neural crest cells in 24-h explant cultures (Huang et al., 1998a). Microelectrodes were filled with 5% carboxyfluorescein and dye was iontophoretically injected for 2 min. The number of dye-filled cells found at the termination of dye injection was recorded. To examine dye coupling in vivo, impalements and dye injections were carried out into the dorsal ridge of the postotic hindbrain neural fold, the region where presumptive cardiac crest cells are found, and the number of dye-filled cells recorded after 2 min as described above.

Analyses of neural crest cell migration and proliferation

Images of the neural tube explant cultures were acquired by videomicroscopy at 24 and 48 h, and the outgrowth area was measured as described previously (Huang et al., 1998a). In brief, this entailed using NIH Image software to measure the area and perimeter of the outgrowth to obtain the migration index, which is the outgrowth area (mm²) divided by the perimeter (mm) of the explant. This normalized for variations in the outgrowth area arising from differences in the shape of the explant. For monitoring cell proliferation, 48-h explant cultures were incubated with BrdU (10 μM/ml) for 2 h, then fixed, and processed for immunodetection using an anti-BrdU antibody as described previously (Huang et al., 1998a). To determine the total cell number in the outgrowth after BrdU immunostaining, the cultures were further stained with hematoxylin. Cell counting was carried out using NIH Image. The migration and proliferation obtained were analyzed statistically using Statview (SAS Institute, Inc.).

Time-lapse videomicroscopy and motion analysis

For time-lapse videomicroscopy, images of the explants were captured every 10 min over a 20-h interval using IPLab (Scanalytics) or Openlab (Im- provision, Inc.) imaging software. For these studies, the explant cultures were maintained at 37°C on a heated microscope stage in phosphate-buffered L-15 medium containing 10% FBS. The images were then converted to a Quicktime movie for viewing and motion analysis. The motion analysis was carried out using the Dynamic Image Analysis Software (Solltech, Inc.). Using this software, we determined the speed of cell locomotion, the directionality of cell movement (the net distance achieved divided by the total distance traveled), the direction change (the change in direction of cell movement in degrees), and the persistence of cell movement (the speed of cell locomotion divided by direction change in gradients). The data obtained was statistically evaluated by ANOVA using Statview (SAS Institute, Inc.).

Immunohistochemistry

For immunohistochemical staining of neural crest cells in explant cultures, neural tube explants were plated on glass coverslips coated with 15 μg/ml of fibronectin. After 24–48 h of culture, the cells were fixed in 4% parafomaldehyde in PBS for 20 min at room temperature. In some cases, cells were first incubated with 3.7% formaldehyde containing 0.3% Triton X-100 for 3 min followed by postfixation in 4% formaldehyde for an additional 15 min. For immunostaining, the coverslips were incubated for 2 h at room temperature with the primary antibody diluted in blocking buffer (1% BSA and 2% FBS with 0.3% Triton X-100 in PBS), then washed with PBS three times for 1 h or longer, and incubated with the secondary antibody for 45 min at room temperature. After washing, the coverslips were mounted with SlowFade antifade reagent (Molecular Probes) on glass slides and ex- amined by epifluorescence illumination using a 40 or 63× oil objective on a Leica DMIRE microscope. All of the images were captured using a 5 Mhz Micromax cooled CCD camera. For some samples, images were obtained as Z stacks comprised of 0.2-μm optical slices and then subjected to decon- volution analysis by volume neighbor or iterative deconvolution algorithms using the Openlab deconvolution software (Improvision, Inc.).

The antibodies used included mouse monoclonal anti-N-cadherin antibody from Zymed Laboratories (33-3900; used at 1:100 dilution) and mouse monoclonal anti–β-catenin IgG (C19220; used at 1:100 dilution), mouse mon- oclonal anti-p120ctn antibody (P17920; 1:500 dilution), and mouse monoclonal anti-p120- FITC–conjugated antibody from BD Transduction Laboratory (P17924; 1:50 dilution), and rabbit polyclonal Cx43 in anti- body 18-A8 made against the cytoplasmic tail of Cx43 in (1:150 dilution);
supplied by Dr. Elliot Hertzberg, Albert Einstein College of Medicine, Bronx, NY). The secondary antibodies used were Cy3- or FITC-conjugated goat anti-mouse or anti-rabbit IgG obtained from Jackson Immunoresearch Laboratories. For double immunostaining with the N-cadherin and p120ctn mouse antibodies, cells were first incubated with the N-cadherin antibody followed by Cy3-conjugated anti-mouse antibody. Then they were further incubated with the FITC-conjugated p120ctn antibody.

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