Dorsal hindbrain ablation results in rerouting of neural crest migration and changes in gene expression, but normal hyoid development

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SUMMARY

Our previous studies have shown that hindbrain neural tube cells can regulate to form neural crest cells for a limited time after neural fold removal (Scherson, T., Serbedzija, G., Fraser, S. E. and Bronner-Fraser, M. (1993). Development 188, 1049-1061; Sechrist, J., Nieto, M. A., Zamanian, R. T. and Bronner-Fraser, M. (1995). Development 121, 4103-4115). In the present study, we ablated the dorsal hindbrain at later stages to examine possible alterations in migratory behavior and/or gene expression in neural crest populations rostral and caudal to the operated region. The results were compared with those obtained by misdirecting neural crest cells via rhombomere rotation. Following surgical ablation of dorsal r5 and r6 prior to the 10 somite stage, r4 neural crest cells migrate along normal pathways toward the second branchial arch. Similarly, r7 neural crest cells migrate primarily to the fourth branchial arch. When analogous ablations are performed at the 10-12 somite stage, however, a marked increase in the numbers of Dil/Hoxa-3-positive cells from r7 are observed within the third branchial arch. In addition, some Dil-labeled r4 cells migrate into the depleted hindbrain region and the third branchial arch. During their migration, a subset of these r4 cells up-regulate Hoxa-3, a transcript they do not normally express. Krox20 transcript levels were augmented after ablation in a population of neural crest cells migrating from r4, caudal r3 and rostral r3. Long-term survivors of bilateral ablations possess normal neural crest-derived cartilage of the hyoid complex, suggesting that misrouted r4 and r7 cells contribute to cranial derivatives appropriate for their new location. In contrast, misdirecting of the neural crest by rostrocaudal rotation of r4 through r6 results in a reduction of Hoxa-3 expression in the third branchial arch and corresponding deficits in third arch-derived structures of the hyoid apparatus. These results demonstrate that neural crest/tube progenitors in the hindbrain can compensate by altering migratory trajectories and patterns of gene expression when the adjacent neural crest is removed, but fail to compensate appropriately when the existing neural crest is misrouted by neural tube rotation.

Key words: neural crest, hindbrain, Hox genes, chicken, cell migration

INTRODUCTION

The developing hindbrain displays a segmental organization in the form of eight undulations called rhombomeres. A two-rhombomere periodicity of gene expression, neural crest migration and ganglion formation is exhibited in the hindbrain region. For example, cranial ganglia develop and are associated with even-numbered rhombomeres (Keynes and Lumsden, 1990). In addition, three distinct streams of neural crest cells arise from the hindbrain and exit lateral to rhombomeres 2, 4, and 6 (Lumsden et al., 1991; Sechrist et al. 1993). The first stream is composed of neural crest cells from r2 and 3 that emanate lateral to r2 to eventually populate the first branchial arch. A second stream of neural crest cells originating from r3, 4, and 5 migrate lateral to r4 and enter the second branchial arch. The third stream comprises neural crest cells primarily from r5 and 6, emanating lateral from r6 to the third branchial arch (Sechrist et al., 1993; Birgbauer et al., 1995).

Segmentation of the hindbrain coincides with the expression of many transcription factors, most notably the genes within the four vertebrate Hox gene clusters (Akam, 1989; Wilkinson et al., 1989a,b; Hunt et al., 1991a,b; McGinnis and Krumlauf, 1992). Within the neuroepithelium, the rostral borders of expression of many Hox genes coincide with rhombomere borders and are colinear with their 3′ to 5′ sequence along the chromosome (McGinnis and Krumlauf, 1992). Results of loss of function and ectopic expression experiments suggest that vertebrate Hox genes may confer segment identity similar to the HOM-C homeotic selector genes of Drosophila (Mark et al., 1993; Dolle et al., 1993; Carpenter et al., 1993; Zhang et al., 1994). Many Hox genes are expressed both in rhombomeres and neural crest cells populating the branchial arches. In addition, other transcription factors including Krox-20 are expressed in particular rhombomeres and neural crest cells populating the branchial arches. These results demonstrate that neural crest/tube progenitors in the hindbrain can compensate by altering migratory trajectories and patterns of gene expression when the adjacent neural crest is removed, but fail to compensate appropriately when the existing neural crest is misrouted by neural tube rotation.
However, the observations that neural crest cells from multiple rhombomeres contribute to a single branchial arch (Sechrist et al., 1993; Birgbauer et al., 1995; Kontges and Lumsden, 1996) and that some neural crest cells rapidly down-regulate Hox gene expression (Prince and Lumsden, 1994; Nieto et al., 1995; Saldivar et al., 1996) upon leaving the neural tube suggests that this scenario may be overly simplified. These results argue that environmental factors may play an important role in the regulation and maintenance of gene expression in neural crest populations derived from particular rhombomeres. Furthermore, the same gene can be differentially regulated within distinct rhombomeres (Nonchev et al., 1996).

Removal of the putative neural crest by the ablation of a small portion of the dorsal neural tube at mesencephalic, occipital or cervical levels results in an embryo with normal face and neck morphology (Yntema and Hammond, 1945; McKee and Ferguson, 1984). This has been taken as evidence that neighboring neural crest cells, both rostral and caudal to the ablated region, repopulate the extirpated segments. However, previous results have shown that ablation of the dorsal hindbrain prior to stage 9 results in the remaining hindbrain cells regulating to reform the neural crest (Scherson et al., 1993; Hunt et al., 1995; Sechrist et al., 1995; Buxton et al., 1997). Furthermore, normal Hoxa-3 and Hoxb-4 expression patterns are observed after regeneration of neural crest cells following hindbrain neural fold ablations (Hunt et al., 1995). Thus, for a limited time, neural tube cells possess the ability to regenerate the neural crest. Following a critical period, however, the neural tube loses this capacity, although it retains the ability to regenerate parts of the neural tube itself (Diaz and Glover, 1996). It is possible that, after the regenerative period has passed, neighboring neural crest cells repopulate the sites adjacent and ventral to the extirpated neural tube, raising the intriguing question of how cells adjust patterns of gene expression when exposed to different environments.

In this study, we use DiI labeling in combination with in situ hybridization to examine the normal and compensatory behavior of hindbrain neural crest/neural tube cells and their patterns of Hoxa-3 and Krox-20 gene expression in response to ablation of neighboring neural crest populations. Dorsal neural tube ablations from r5 and r6 were performed after the regenerative capacity of the neuroepithelium was diminished but prior to the onset of neural crest migration at that level (stage 10; Hamburger and Hamilton, 1951). Some Dil-labeled neural crest cells located caudal (r7) and sometimes rostral (r4) to the ablated region of the hindbrain diverted from their normal migratory pathways and invaded the depleted hindbrain region and associated branchial arches. A subset of the r4 cells expressed Hoxa-3, although these cells do not normally do so. Furthermore, a subpopulation of neural crest cells migrating caudal as well as rostral to r3 expressed prevalent Krox-20 transcripts after dorsal r5-6 ablation. Remarkably, the resultant craniofacial skeleton appeared normal after late neural crest ablation. In contrast, misrouting neural crest migration by means of rotating rhombomeres 4-6 resulted in deficits in Hoxa-3 expression in the third branchial arch and a corresponding absence of hyoid apparatus structures. These results suggest that, after the period of neural tube regenerative potential has passed, adjacent neural crest cells and their precursors can alter their migratory trajectories and patterns of gene expression to compensate for crest-depleted regions of the hindbrain.

**MATERIALS AND METHODS**

**Embryos**

White Leghorn chicken eggs were incubated in a rocking incubator at 37°C with constant humidity until stages 8 to 11 (4 to 12 somite stage) of development (Hamburger and Hamilton, 1951). Eggs were rinsed with 70% ethyl alcohol and approximately 3 ml of albumin was removed using a 5 ml syringe with an 18G needle. A small window was made in the shell and a solution of 10% India Ink (Pelikan Fount) in Howard Ringers solution was injected beneath the blastoderm to visualize the embryo. The vitelline membrane was cut and deflected away using a fine tungsten needle to expose the embryo.

**DiI labeling**

To label embryonic cells with a fluorescent dye that is preserved during in situ hybridization, we used a fixable form of DiI, Cell Tracker CM-DiI (Molecular Probes, Inc.). For other experiments, the original form of DiI was used (Serbedzija et al., 1989). Glass micropipettes connected to a pressure injection apparatus were back-filled with CM-DiI (diluted 1:10 in 100% ethanol) and a small amount of dye was injected into the dorsal neural tube of the desired rhombomere. In the case of r3, r4 and r7 injections, DiI labeling was most often performed prior to ablation of the neural tube, though similar results were observed when dye labeling was performed following the ablation. In other experiments, DiI was injected onto the cut edge of the neural tube at the level of r5 or r6 following ablation of the dorsal region.

**Dorsal neural tube ablations**

Bilateral ablations of the dorsal neural tube were performed using a fine glass needle as described in Sechrist et al. (1995). Using specific rhombomeres as landmarks, an incision was made between the rhombomere borders as landmarks, an incision was made between the rhombomere borders and adjacent mesoderm on both sides of the embryo. The neural tube plus notochord were teased away from the underlying endoderm. Then, perpendicular cuts were made in the neural tube at the r3/r4 border and the r6/7 border, freeing r4-6 from the rest of the embryo. r4 to r6 were rotated 180° rostrocaudally, while maintaining dorsoventral polarity, and reinserted. r4-to-r5 rotations were accomplished by a similar manipulation, except that the caudal cut was made at the r5/6 border. In some cases, a focal injection of DiI was made into r4 prior to rotation. The eggs were sealed with adhesive tape and incubated for 12 to 36 hours. For long-term survival experiments, embryos were allowed to develop for 10-12 days.

**Rhombomere rotation**

A fine glass needle was used to make a slit between the neural tube and adjacent mesoderm on both sides of the embryo. The neural tube plus notochord were teased away from the underlying endoderm. Then, perpendicular cuts were made in the neural tube at the r3/r4 border and the r6/7 border, freeing r4-6 from the rest of the embryo. r4 to r6 were rotated 180° rostrocaudally, while maintaining dorsoventral polarity, and reinserted. r4-to-r5 rotations were accomplished by a similar manipulation, except that the caudal cut was made at the r5/6 border. In some cases, a focal injection of DiI was made into r4 prior to rotation. The eggs were sealed with adhesive tape and incubated for 2-3 or 10-12 days.

**Fixation and in situ hybridization**

Embryos were allowed to develop to the stages indicated above, removed from the egg and rinsed in Howard Ringer’s solution prior to fixation in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 4°C overnight. A 900 bp Hoxa-3 cDNA fragment (kindly provided by Dr Robb Krumlauf) linearized with BamHI and T7 transcription was used to produce a digoxigenin-labeled antisense riboprobe (Saldivar et al., 1996). The Krox-20 probe (Nieto et al. 1995) was kindly provided by Dr David Wilkinson.
Whole mount in situ hybridization was performed essentially as described by Wilkinson and Nieto (1993). To visualize the expression patterns of Hoxa-3 and Krox-20 in whole embryos, the forebrain and trunk regions were removed in PBS using fine forceps and a tungsten needle. The remaining tissue, including the hindbrain, was placed in a welled slide with PBS. Bright-field photomicroscopy was accomplished using either a Zeiss SV6 or Olympus Vanox microscope. Dil-epifluorescence was visualized in the same embryos using an Olympus Vanox microscope with a rhodamine filter set. Images were processed using Adobe Photoshop.

**Cryostat sectioning**

Following in situ hybridization, embryos were stored in methanol, rehydrated through a series of PBS/methanol washes (75%, 50%, 25%) and then rinsed in PBS. They were placed in 5% sucrose/PBS, followed by 15% sucrose in PBS for 2-4 hours each at 4°C. The embryos were immersed in a 15% sucrose/PBS and 7.5% gelatin solution at 37°C for 4 hours prior to embedding in fresh gelatin and freezing in liquid nitrogen. Embryos were sectioned at -25°C at a thickness of 18 μm. Sections of the embryo were then coverslipped with Gel/Mount (Biomeda Corp.).

**Cartilage staining**

Embryos were allowed to develop to embryonic day 10 through 12, collected in Howard Ringer’s solution and fixed in 5% trichloroacetic acid. Subsequently, they were stained overnight in 75% ethanol/25% glacial acetic acid containing 0.15 mg/ml of Alcian blue and dehydrated with ethanol. Following rehydration through a series of washes, embryos were stained with 0.2 mg/ml Alizarin red in 0.5% potassium hydroxide, cleared with 0.5% KOH, and transferred to glycerin.

**RESULTS**

Previous studies have demonstrated a remarkable capacity of the hindbrain neural tube/neural crest to recover after ablation of the presumptive neural crest, generating normal craniofacial morphology (Scherson et al., 1993; Sechrist et al., 1995; Hunt et al., 1996; Buxton et al., 1997). To clarify the cellular and molecular mechanisms underlying this response, we examined the effects of removing the dorsal hindbrain at the levels of r5-6 on patterns of neural crest migration, Hoxa-3 and Krox-20 gene expression and craniofacial development. The majority of experiments were performed after the regenerative ability of the neural tube to form neural crest cells had declined (Sechrist et al., 1995).

**Bilateral ablation of dorsal r5-6 does not overtly alter Hoxa-3 expression in the branchial arches**

To examine whether obvious alterations in Hox gene expression occur in response to ablations of the dorsal neural tube at hindbrain levels, we examined the expression of Hoxa-3 in the neural tube and branchial arches in operated embryos. Ablation of the dorsal neural tube at r5-6 failed to alter the overall pattern of Hoxa-3 expression. Transcripts were expressed throughout the neuroepithelium of r5 and more caudal rhombomeres (Figs 1, 2), with the exception of the floor plate region. Immediately after ablation, Dil-labeled cells in r4 were Hoxa-3-negative (Fig. 1). When the dorsal neural tube was ablated at the levels of rhombomeres 5-6 at either ‘early’ stages (prior to the 10 ss; n = 10; Table 1) or at ‘later’ stages (10-12 ss; n = 78; Table 1), the pattern of Hoxa-3 expression in the branchial arches was indistinguishable from that observed in unoperated embryos. In embryos fixed at stages 10-19 after early or late ablation, Hoxa-3 expression was observed within neural crest cells in the third and fourth branchial arches (Fig. 2). These results are in agreement with those of Hunt and colleagues (1995), who ablated the dorsal hindbrain at early stages only.

Some differences in neural tube morphology were noted between early and late ablations. Far less compensation by remaining neural tube cells was evident after loss of the dorsal hindbrain at later times. The neural tube was constricted and sometimes crimped at the level of the otic vesicle, such that some Hoxa-3-expressing neural tube cells expanded dorsolaterally immediately rostral to the otic vesicle. This may be due to a small number of r5 cells crossing the rhombomere border after ablation (Birgbauer and Fraser, 1994) and/or enhanced migration of the Hoxa-3-positive cells normally destined to populate cranial ganglion VII. However, Hoxa-3 expression was never observed in the menenchyme of the second branchial arch, but remained restricted to the third and fourth branchial arches. Caudal to the otic vesicle, the neural tube often remained open.

**Table 1. r4 and r7 neural crest migration and Hoxa-3 expression after ablation of dorsal r5-6**

<table>
<thead>
<tr>
<th>n</th>
<th>Operation</th>
<th>St. of operation</th>
<th>St. of fixation</th>
<th>Location of Dil-cells (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>bilateral ablation</td>
<td>3 ss</td>
<td>15</td>
<td>midbrain - BA 1</td>
</tr>
<tr>
<td>2</td>
<td>r5-6 ablation</td>
<td>5-6 ss</td>
<td>16</td>
<td>r5 (0)</td>
</tr>
<tr>
<td></td>
<td>r5-6 ablation</td>
<td>8-9 ss</td>
<td>14-18</td>
<td>r5 (1)</td>
</tr>
<tr>
<td></td>
<td>r5-6 ablation</td>
<td>10-12 ss</td>
<td>18-19</td>
<td>r5 (0)</td>
</tr>
<tr>
<td></td>
<td>r5-6 ablation</td>
<td>10 ss</td>
<td>18-19</td>
<td>r5 (0)</td>
</tr>
<tr>
<td>10</td>
<td>r5-6 ablation</td>
<td>10-12 ss</td>
<td>18-19</td>
<td>r6 (10)</td>
</tr>
<tr>
<td>11</td>
<td>r5-6 ablation</td>
<td>13 ss</td>
<td>18-19</td>
<td>r6 (10)</td>
</tr>
<tr>
<td></td>
<td>r5-6 ablation</td>
<td>14-17</td>
<td>18-19</td>
<td>r6 (10)</td>
</tr>
<tr>
<td></td>
<td>r5-6 ablation</td>
<td>15 ss</td>
<td>18-19</td>
<td>r6 (10)</td>
</tr>
<tr>
<td>46</td>
<td>r5-6 ablation</td>
<td>10-12 ss</td>
<td>14-20</td>
<td>r5 (34)</td>
</tr>
<tr>
<td></td>
<td>r5-6 ablation</td>
<td>10-11 ss</td>
<td>17-19</td>
<td>r6 (6)</td>
</tr>
<tr>
<td>2</td>
<td>r3-6 ablation</td>
<td>10-12 ss</td>
<td>17-19</td>
<td>r6 (13)</td>
</tr>
<tr>
<td></td>
<td>r7* ablation</td>
<td>10-12 ss</td>
<td>17-19</td>
<td>r6 (13)</td>
</tr>
<tr>
<td>16</td>
<td>r5-6 ablation</td>
<td>10-12 ss</td>
<td>17-19</td>
<td>r7* ablation</td>
</tr>
</tbody>
</table>
We previously demonstrated that the regulative ability of the hindbrain to form neural crest cells after ablation declined with time (Scherson et al., 1993; Sechrist et al., 1995). In the present study, we repeated these observations at the level of r5 and r6 by placing a small spot of DiI on the cut edge of the neural tube after ablation. When ablations were performed at the 10–12 ss (n = 10 for r6; n = 6 for r5; Table 1), DiI-labeled cells made little or no contribution to the neural crest cells within the branchial arches. Only a few DiI-labeled r5 neural crest cells (mean ± s.d. = 2±3 cells per embryo; n = 6) were observed when ablations were performed at these late stages. In contrast, analogous ablations performed at the 2-7ss resulted in DiI-labeled cells derived from r4 migrated along or within the dorsal neural tube to the level of r5 (top arrow) and toward the second branchial arch as well as caudally to the r5/6 border (bottom arrow). Scale bar in A for all panels, 100 μm.

**Some r4- and r7-derived neural crest cells migrate to the third branchial arch after late bilateral ablation of dorsal r5 and r6**

The finding that the regulative time period is largely over by the 10-12 ss raises the intriguing possibility that neural crest cells from rostral and/or caudal to the ablated neural tube reroute their migratory patterns to compensate for missing neural crest cells following late ablations. To test this possibility, we labeled premigratory neural crest cells immediately rostral or caudal to the ablated region (the dorsal portion of r4 or r7) prior to ablation of dorsal r5-6.

Following early ablations, DiI-labeled r4 neural crest cells (n = 9; Table 1) migrated along their normal pathways to the second branchial arch and VIIth cranial ganglion (Fig. 2A). Some DiI-labeled cells were observed within the neural tube of both r4 and r5, suggesting that some r4-derived cells shifted caudally into the r5 level (Birgbauer and Fraser, 1994) after ablation.

After late ablations performed at the 10-12 ss, DiI-labeled r4 neural crest cells migrated to the second branchial arch in all embryos; however, DiI-labeled cells also were observed within the third branchial arch in approximately one third of the embryos (n = 15/46; Table 1; Fig. 2B). Labeled cells were observed within cranial ganglia VII (associated with the second branchial arch) and IX (associated with the third branchial arch). Some DiI-labeled cells also were observed deep within the distal portion of the branchial arches. This deviation of migrating neural crest cells after ablation is not unique to...
ablations in the caudal hindbrain; we observed a similar deviation in migration of r2-derived neural crest cells after ablation of r3-5 (data not shown).

The pathways followed by neural crest cells adjacent to r6 and r7 have been delineated in detail previously by HNK-1 immunocytochemistry and quail/chick chimerae (Kuratani and Kirby, 1991; Miyagawa-Tomita et al., 1991), and recently by DiI-labeling (Suzuki and Kirby, 1997). We also examined the normal patterns of migration from these two rhombomeres, 1-4 days following focal DiI injections (data not shown). At the level of r6, neural crest cells followed a dorsolateral pathway adjacent to the ectoderm, reaching the ventral aorta via the third branchial arch by the 35 ss. The relative concentration of DiI-labeled cells in the third, fourth or sixth branchial arches depended upon the rostrocaudal level of the injection. DiI applied at r7 labeled cells predominantly in the fourth arch, with a few cells in the third and sixth arches. DiI-labeling near the r6/7 junction (close to the first somite level) labeled cells in branchial arches 3, 4 and 6 with more labeling in the fourth branchial arch, whereas rostral r6 injections labeled primarily third arch neural crest.

After late ablation of the dorsal neural tube at the level of r5-6, there was a marked increase in the numbers of r7-derived neural crest cells after ablation of r3-5 (data not shown).

To analyze the distinct contributions of r4 and r7 cells to the Hoxa-3-expressing populations within the third branchial arch after late neural fold ablations, we combined labeling of either r4 or r7, by focal injections of fixable CM-DiI, together with in situ hybridization for Hoxa-3 transcripts. The CM-DiI remains visible following hybridization, permitting identification of Hoxa-3 transcripts and fluorescent signal in the same cell.

After early ablations, DiI-labeled r4 cells migrated only into the second branchial arch which was devoid of Hoxa-3 transcripts, as seen in whole mount (Fig. 2A). In contrast, after late ablations, DiI-labeled r4 cells were located adjacent to and intermingled with the stream of Hoxa-3-expressing cells between the dorsal neural tube and the ventral portions of the third branchial arch (Fig. 2B; Table 1; n = 15/46 embryos).

Transverse sections through these double-labeled embryos were made to evaluate the potential colocalization of DiI with Hoxa-3 transcripts at single-cell resolution. Within 4 hours after ablation, DiI-labeled r4 cells were observed in the roof of the dorsal neural tube over r5 and ventral to the otic vesicle, suggesting that they migrated caudally within and/or along the neural tube ventrolateral to the otic vesicle, and were beginning to migrate dorsoventrally. These cells failed to express Hoxa-3 in the third branchial arch after ablation

**Fig. 3.** Whole-mount views of embryos hybridized with Hoxa-3 probe after DiI-injection at r7 and ablation of dorsal r5-r6. Both panels contain bright-field images with superimposed fluorescence. (A) An embryo after ablation at the 10 ss, fixation at stage 19. Hoxa-3 expression is observed in the third (III) and fourth (IV) branchial arches, but not in the second arch (II). DiI-labeled cells (arrow) are entering the fourth and more caudal branchial arches. Other labeled cells were observed in cranial ganglion X (data not shown). (B) The embryo pictured in A shows r7-derived DiI-labeled cells (arrows) at the junction of r5/6 and migrating laterally. OV, otic vesicle. Scale bar in A for both panels, 70 μm.

**Table 2. Summary of Results: r7 neural crest migration after dorsal r5-6 ablation**

<table>
<thead>
<tr>
<th>Manipulation</th>
<th>at (ss)</th>
<th>Fixed (st)</th>
<th>No. of cells in BA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>r7* unablated</td>
<td>9</td>
<td>16</td>
<td>48</td>
</tr>
<tr>
<td>12</td>
<td>19</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>16</td>
<td></td>
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<tr>
<td>12</td>
<td>20</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>20</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>19</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>23±16 s.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r5-6 ablated</td>
<td>11</td>
<td>19</td>
<td>237</td>
</tr>
<tr>
<td>r7*</td>
<td>11</td>
<td>19</td>
<td>283</td>
</tr>
<tr>
<td>11</td>
<td>19</td>
<td>251</td>
<td></td>
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<td>10</td>
<td>19</td>
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</tr>
<tr>
<td>10</td>
<td>20</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>233±36 s.d.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

r, rhombomere; BA3, third branchial arch; ss, somite stage; st, stage; * indicates site of DiI-labeling.
3 transcripts, even 12 hours after ablation (Fig. 4C,D). However, some Dil-labeled cells that had migrated away from the neural tube were faintly Hoxa-3 positive 12-14 hours (18 ss) following ablation (Fig. 4A,B). At subsequent stages, a few Dil-labeled r4-derived cells were confirmed to express high levels of Hoxa-3 transcripts (Fig. 4E,F). In addition, most r7-derived cells were Dil/Hoxa-3-positive within branchial arches three and four (Fig. 5) of ablated embryos. Thus, r7 and, to a lesser extent, r4 contribute to the population of Hoxa-3-positive cells within the third branchial arch after ablation.

**Bilateral ablation of dorsal r5-6 alters Krox-20 expression in neural crest cells emerging from r3**

To assay whether other possible changes occur in gene expression in remaining neural crest/neural tube cells after late ablations, we examined the distributions of Krox-20 transcripts. The transcription factor Krox-20 is normally expressed in r3 and r5, as well as transiently in a subpopulation of neural crest cells that migrate caudal to the otic vesicle (Fig. 6A,B; Wilkinson et al., 1989b; Nieto et al., 1995). In addition, we noted a few Krox-20-expressing neural crest cells migrating rostral and caudal to r3 (Fig. 6A,C) in normal stage 12-13 embryos. These are likely to correspond to neural crest destined to form branchiomotor nerve ‘boundary caps’ (Wilkinson et al., 1989b; Niederlander and Lumsden, 1996). When the dorsal neural tube was ablated at the levels of r5-6 at ‘early’ stages (prior to the 10 ss; n = 9), an increased number of Krox-20-expressing neural crest cells was noted. Similarly, ablations of dorsal r5-6 performed at ‘later’ stages (10-12 ss; n = 9) resulted in a high level of Krox-20 transcripts in neural crest cells migrating caudally from r3 (Fig. 6B,D). Normally, Krox-20-positive neural crest cells are limited in number and transcripts are rapidly down-regulated as neural crest cells leave the neural tube (Fig. 6C). Thus, dorsal r5-6 ablation results in a pronounced increase in the numbers of Krox-20-expressing cells in the neighboring neural crest population. Dil-labeling of either r3 (Fig. 6E) or r4 (Fig. 6F) demonstrated that both rhombomeres contribute to the Krox-20-positive population of neural crest cells. Krox-20 expression in the neural tube was unchanged and the Krox-20-positive cells that normally migrate caudal to the otic vesicle at the level of the ablation were either missing or greatly reduced. At the time of surgery, those Krox-20-positive neural
Changes in neural crest after ablation

crest cells that have emigrated from the neural tube at the level of r5 and r6 remain closely associated with the dorsal neural tube and, therefore, are largely removed by ablation. In a few cases, we observed Krox-20-positive cells migrating adjacent to caudal r6 or r7. These are likely to originate from the unablated region of the caudal hindbrain. In several embryos, we noted a few Krox-20-positive cells migrating caudal to the otic vesicle after ablation.

Development of the facial skeleton after late ablation

The hyoid apparatus or ‘tongue skeleton’ of avian embryos consists of a body with an arrowhead-shaped entoglossus that extends into the tongue and a horn-shaped cornua that extends caudolaterally. The entoglossus and remaining body of the hyoid are derived predominantly from first and second branchial arch neural crest, while the cornua derives primarily from third branchial arch neural crest (Couly et al., 1996). Previous studies have shown that the skeleton forms normally after neural fold ablation prior to the 7 somite stage (Hunt et al., 1995). To examine the status of hyoid apparatus develop-

Fig. 6. Krox-20 expression is prevalent in neural crest cells migrating caudal and rostral to r3 after dorsal r5-6 ablation. (A) A whole-mount dorsal view of a normal embryo at the 16-18 ss (stage 12) showing Krox-20 expression in r3 and r5, as well as a small subpopulation of neural crest cells migrating caudal and rostral (arrows) to the otic vesicle; dashed line indicates the level of section shown in C. (B) A whole-mount view of an 18ss embryo after ablation of dorsal r5-6 at the 8ss. In addition to the normal pattern of Krox-20 expression in r3 and r5, more Krox-20-positive neural crest cells are present both rostral and caudal to r3 (arrows), but largely absent caudal to r5; dashed line indicates the level of section in D. (C) Transverse section close to the r3/r4 border of a normal embryo, as seen in A, shows that only a few Krox-20-positive neural crest cells (arrow) are adjacent to the neural tube. (D) A transverse section at the level of r4 through the embryo in B shows a prominent increase in cells with Krox-20 expression adjacent to r4. (E) Transverse section through an 18-20 ss embryo that received a focal injection of Dil into r3 followed by ablation of dorsal r5-6. Some Dil-labeled r3 neural crest cells (arrow) express Krox-20 transcripts. (F) Transverse section through another 18-20 ss embryo that received a focal injection of Dil into r4 followed by ablation of dorsal r5-6. Some Dil-labeled r4 neural crest cells (arrow) also express Krox-20 transcripts. Scale bar in A for all panels: A, 285 μm; B, 320 μm; C,D, 50 μm; E,F, 30 μm.
ment following ablations performed at later times (10-12 somite stages), we allowed embryos to survive until E10-12 after dorsal neural tube ablation at the level of r5-r6. We found that the hyoid apparatus appeared normal in size and shape (n = 12; Fig. 7B). Thus, despite the ingression of cells into the third branchial arch that were initially derived from inappropriate rhombomeres, the craniofacial skeleton developed normally after ablation.

**Hoxa-3 expression and development of the facial skeleton after rostrocaudal rotation of r4 through r6**

The results described above show that late ablation of the dorsal neural tube leads to misrouting and up-regulation of *Hoxa-3* expression in r4-derived neural crest cells that migrate to the third branchial arch. Does this change in gene expression occur as a result of the environmental influences within the third branchial arch or does neural fold ablation uniquely challenge the normal developmental pathway of r4-derived neural crest cells? To test between these possibilities, we intentionally misrouted r4 neural crest cells by a different manipulation: rhombomere rotation. We previously demonstrated that rotating r4 through r6 leads to r4 neural crest cells migrating to the third branchial arch (Saldivar et al., 1996). Here, we examined the *Hoxa-3*-expression of r4-derived neural crest cells after r4-to-r6 or r4-to-r5 rotation by labeling them with DII and subsequently processing them for in situ hybridization with a *Hoxa-3* probe. In whole mounts of r4-to-r6 rotated embryos (n = 12), *Hoxa-3* expressing cells were present in both the second and the third branchial arches. In transverse sections through these embryos (n = 12), we found that DII-labelled r4-derived neural crest cells entered the third branchial arch but failed to express *Hoxa-3* transcripts (Fig. 8). Thus, this surgical manipulation results in a substantial population of *Hoxa-3*-negative cells populating the third branchial arch, effectively decreasing the size of the *Hoxa-3*-expressing population in this territory. In contrast, r4-to-r5...
rotation did not change the pattern of \textit{Hoxa-3} expression in the branchial arches.

To examine the long-term effects of rhombomere rotation on craniofacial development, we allowed embryos to survive until E10-12 after either r4-to-r5 or r4-to-r6 rotation and then analyzed cartilage and bone formation. Marked abnormalities were noted in the hyoid apparatus after r4-to-r6 rotation (Fig. 7D; \( n = 12 \)). Interestingly, the defects were strictly associated with structures derived primarily from the third branchial arch. The entoglossus and body, which are both derived from the first and second branchial arches, appeared normal in all cases; however, the cornua, derived primarily from the third branchial arch, was either reduced to a single horn or was largely missing (Fig. 7D). In contrast, following r4-to-r5 rotation, the hyoid apparatus appeared normal (Fig. 7C; \( n = 14 \)). Thus, a decrease in \textit{Hoxa-3} expression in the third branchial arch correlates with hyoid apparatus abnormalities, whereas ectopic \textit{Hoxa-3} expression in the second branchial arch does not.

**DISCUSSION**

Cranial neural crest cells give rise to a wide range of craniofacial derivatives including much of the connective tissue of the face and skull, skeletal structures of the neck, cranial ganglia and the cardiac septum. Some evidence exists for early spatial specification of neural crest populations, since grafting the neural plate prior to neural crest emigration from one axial level to another results in duplication of skeletal structures (Noden, 1983). This has led to the idea that the neural tube and corresponding neural crest cells may possess inherent patterning information that is established early in neural development. Consistent with the idea of cell autonomy of gene expression, transplantation of r4 into ectopic sites in the rostral hindbrain resulted in maintenance of \textit{Hoxb-2} expression (Guthrie et al., 1992; Kuratani and Eichele, 1993).

\textit{Hox} gene expression in the hindbrain as well as the neural crest may be less cell autonomous than originally postulated. Recent evidence has demonstrated that clonally-related cells originating from one rhombomere can cross into an adjacent rhombomere (Birgbauer and Fraser, 1994) which expresses a different complement of transcription factors. A similar situation occurs in the neural crest, where neural crest cells arising from rhombomeres 3 and 5 migrate to multiple branchial arches (Sechrist et al., 1993; Birgbauer et al., 1995) which differ in their patterns of gene expression. Furthermore, when rostral hindbrain is grafted caudally to postotic positions, rostral gene expression is lost (Itasaki et al., 1996) and more caudal genes are expressed (Grain-Botton et al., 1995; Itasaki et al., 1996). Interestingly, this transformation appears to occur via an interaction with the neighboring paraxial mesoderm (Itasaki et al., 1996).

Late dorsal neural tube ablation leads to changes in migratory behavior in adjacent neural crest populations. By ablating the rhombencephalic neural folds after the period when the neural tube regulates (Scherson et al., 1993; Hunt et al., 1995; Sechrist et al., 1995), we created embryos with putative neural crest deletions in the hindbrain. In these embryos, neural crest cells derived from neighboring rhombomeres that were either rostral (r4) or caudal (r7) to the ablated region were able to reroute their migration to branchial arches that they would not normally enter. There appeared to be more compensatory migration from neural crest derived from caudal rhombomeres with the appropriate \textit{Hox} expression. Accordingly, the patterns of \textit{Hoxa-3} expression in these arches appeared relatively normal. The second branchial arch was devoid of \textit{Hoxa-3} expression whereas the third branchial arch appeared to contain a near normal complement of \textit{Hoxa-3}-positive cells.

Some alterations in gene expression were noted in adjacent neural crest populations after late dorsal hindbrain ablation. Using a technique that allows concurrent visualization of fluorescent Dil and in situ hybridization signals, we confirmed that cells derived from r7 that had migrated ectopically to the third branchial arch expressed \textit{Hoxa-3} transcripts. In addition, some r4 cells within the third branchial arch were \textit{Hoxa-3}-positive, suggesting that they up-regulated a transcript in their new environment that they would not normally express. Our data suggest that r4-derived neural crest do not express \textit{Hoxa-3} within the neuroepithelium of either r4 or r5, but only during their migration laterally and caudally. We also observed numerous \textit{Hoxa-3}-positive cells that were not Dil-labeled. It is likely that these derived from unlabeled portions of r7 or other rhombomeres. Analysis of \textit{Krox-20} expression after dorsal r5-6 ablation revealed a surprising increase in the number of \textit{Krox-20}-expressing neural crest cells emerging from r3 and r4. \textit{Krox-20} is normally down-regulated in the majority of neural crest cells that emerge from the neural tube with the exception of a small population of ‘boundary cap’ cells that remain tightly associated with nerve exit points (Wilkinson et al., 1989b; Niederlinder and Lumsden, 1996). Following ablation, however, neural crest cells derived from both r3 and r4 maintain high levels of \textit{Krox-20} expression during the early stages of their migration as they intermingle rostrally with the r2 stream and caudally with the r4 stream. Unlike r4- and r7-derived neural crest cells, which undergo a rerouting of migration after r5-6 ablation, the r3- and r4-derived \textit{Krox-20}-expressing cells continue to migrate along their normal pathways (Sechrist et al., 1993; Birgbauer et al., 1995). Thus, the differences in gene expression cannot be elicited by entry into a novel environment. Rather, ablation of the dorsal portion of r5-6 is likely to send a long-range signal resulting in a change in gene expression in these cells prior to their emigration from the neural tube. This signal may maintain \textit{Krox-20} expression in neural crest cells emerging from r3 and/or increase the number of neural crest cells emerging from this rhombomere.

Our results show a temporally limited ability of the neural tube to compensate for neural crest ablations in agreement with those of other investigators (Hammond and Yntema, 1958; Hunt et al., 1995), but differ somewhat from the recent results of Couly et al. (1996). Using quail/chick neural tube transplantations combined with dorsal hindbrain ablation, these investigators suggest that the neural tube is unable to regenerate neural crest cells. We previously demonstrated (Sechrist et al., 1995) that the regenerative ability of the neural tube to form neural crest decreases with time and depth of ablation and varies with rostrocaudal location. The timing, depth of ablation and differences in surgical techniques used by Couly and colleagues (1996) is likely to explain differences between their results and those of other investigators (present results; Sechrist et al., 1995; Hunt et al., 1995; Buxton et al., 1997). Our
bilateral ablations involved the dorsal third of the neural tube, with optimal regeneration occurring prior to the 4 somite stage (Sechrist et al., 1995); by contrast, Couly et al. (1996) ablated the dorsal half of the neural tube at the 5 somite stage, by which time the regenerative ability has begun to decline. Moreover, quail/chick chimeric embryos demonstrate some healing time after grafting (Miyagawa-Tomita et al., 1991), which has been shown to delay cardiac neural crest migration compared with unoperated embryos (Thompson and Fitzpatrick, 1979). Finally, quail cells have been documented to be more invasive than chick cells (Bellairs et al., 1981), perhaps leading to an apparently higher level of ‘filling in’ by adjacent populations when grafted from quail donors.

Curiously, after r4-to-r6 rotation, r4 cells failed to up-regulate Hoxa-3 expression, suggesting that there is a differential response of r4 neural crest to the third arch environment depending upon the type of manipulation performed. There are a number of possible explanations for the differences in these results. After ablation, a relatively small number of r4 cells enter the third branchial arch compared with the large number that enter after rhombomere rotation. Therefore, it is possible that there is a ‘community effect’ resulting from the presence of numerous r4 cells after rhombomere rotation which prevents adjustment of gene expression. Alternatively, the depletion of r6-derived neural crest cells from the third branchial arch after rhombomere rotation may preclude alterations in gene expression. Another possibility is that the time required for healing after rhombomere rotation and prior to neural crest emigration impedes the ability of r4-derived neural crest to up-regulate Hoxa-3 expression, whereas r4 neural crest emigrate on schedule following dorsal r5-6 ablation. Although it remains formally possible that there is some change in gene expression in r4 cells after ablation as they shift caudally within the dorsal neural tube and prior to emigration of neural crest cells, our analyses failed to detect Hoxa-3-positive r4 cells within the neural tube. Finally, it is intriguing to speculate that the main differences between dorsal rhombomere ablation and rhombomere rotation stems from the disparate nature of the experimental challenges. In the case of neural fold ablations, some existing neural crest cells reroute their migration into a region lacking the normal neural crest complement; in the case of rhombomere rotation, all putative neural crest cells are present but in an inappropriate location. Perhaps neural crest cells modulate gene expression in response to the absence of neighboring neural crest cells but fail to respond in the presence of neighboring neural crest cells, even if they possess an ‘inappropriate’ Hox code. A similar situation may have occurred in the neural plate transplantations of Noden (1983): when neural plate destined to form first branchial arch derivatives was transplanted to the level of the second arch, duplication of first arch derivatives was observed, suggesting that the neural crest cells maintained their original identity.

There are likely to be multiple mechanisms for regenerating the neural crest after ablation. Previous experiments showed that neural tube cells were able to reform the neural crest after surgical neural fold ablation in the midbrain or hindbrain (Scherson et al., 1993; Sechrist et al., 1995; Hunt et al., 1995; Buxton et al., 1997). Most neural crest derivatives were reformed after these surgeries (Sechrist et al., 1995), although significant size reductions in some structures were noted after extensive ablations (Hammond and Yntema, 1958; McKee and Ferguson, 1984). The regenerative ability of the neural tube declines with time as well as with the depth of ablation (Sechrist et al., 1995). In the present study, ablations were performed beyond the time of optimal regulation of the neural tube into neural crest. We found that regenerative growth occurred, although embryos having normal-sized branchial arches and hyoid apparatus, derived from the second and third branchial arches. Neural crest cells within the third branchial arch (adjacent to the ablated neural tube) were derived from regions caudal and rostral to the ablated neural tube, demonstrating that neural crest cells from these axial levels are able to migrate into and compensate for the missing neural crest cells (Couly et al., 1996). It is interesting to note that the neural tube often appears much more abnormal than the neural crest after such surgeries (Yntema and Hammond, 1945; McKee and Ferguson, 1984), suggesting that neural crest regeneration may occur at the expense of neural tube regeneration.

While dorsal neural tube ablation in the caudal hindbrain failed to affect craniofacial development, rhombomere rotation caused deficits in third arch-derived hyoid bones which are derived from r5 and r6 neural crest. After rotation of r4-to-r6, Hoxa-3 expressing cells from r6 populated the second branchial arch whereas r4 cells that failed to express Hoxa-3 populated a portion of the third branchial arch. Interestingly, no defects were noted in second arch derivatives after this operation despite the presence of ectopic Hoxa-3-expressing cells (Saldivar et al., 1996). Instead, defects were observed in the third arch which was missing its normal complement of Hoxa-3-expressing cells. Thus, the defects correlate with a deficit of Hoxa-3-expressing cells in the third branchial arch rather than an excess of Hoxa-3-expressing cells in the second branchial arch.

In summary, our experiments demonstrate a remarkable preservation of the neural crest and its derivatives during craniofacial development at the hindbrain level. There are at least two overlapping phases of neural crest regulation: (1) an early phase in which the neural tube regulates to reform the neural crest (Hammond and Yntema, 1958; Scherson et al., 1993; Sechrist et al., 1995; Hunt et al., 1995; Buxton et al., 1997) and (2) a later phase in which neural crest cells from rostral and caudal axial levels divert their migration and sometimes alter patterns of gene expression to repopulate the neural crest. We demonstrate that neural crest cells from neighboring levels of the neural tube adjust their migratory trajectories to compensate for missing neural crest populations and subsequently differentiate into craniofacial structures appropriate for their new locations. Such ‘filling in’ has previously been reported after surgical ablation of trunk (Yntema and Hammond, 1945) and cranial (McKee and Ferguson, 1984; Couly et al., 1996) neural crest and half or whole rhombomeres (Diaz and Glover, 1996). These results suggest that regional specification along the neural axis may not be absolute, but can be over-ridden by environmental influences at least over short distances. These influences may emanate from the adjacent mesoderm (Itasaki et al., 1996), neighboring neural tube cells and/or ectoderm.

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