Graded potential of neural crest to form cornea, sensory neurons and cartilage along the rostrocaudal axis

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Summary

Neural crest cells arising from different rostrocaudal axial levels form different sets of derivatives as diverse as ganglia, cartilage and cornea. These variations may be due to intrinsic properties of the cell populations, different environmental factors encountered during migration or some combination thereof. We test the relative roles of intrinsic versus extrinsic factors by challenging the developmental potential of cardiac and trunk neural crest cells via transplantation into an ectopic midbrain environment. We then assess long-term survival and differentiation into diverse derivatives, including cornea, trigeminal ganglion and branchial arch cartilage. Despite their ability to migrate to the periocular region, neither cardiac nor trunk neural crest contribute appropriately to the cornea, with cardiac crest cells often forming ectopic masses on the corneal surface. Similarly, the potential of trunk and cardiac neural crest to form somatosensory neurons in the trigeminal ganglion was significantly reduced compared with control midbrain grafts. Cardiac neural crest exhibited a reduced capacity to form cartilage, contributing only nominally to Meckle’s cartilage, whereas trunk neural crest formed no cartilage after transplantation, even when grafted directly into the first branchial arch. These results suggest that neural crest cells along the rostrocaudal axis display a graded loss in developmental potential to form somatosensory neurons and cartilage even after transplantation to a permissive environment. Hox gene expression was transiently maintained in the cardiac neural tube and neural crest at 12 hours post-transplantation to the midbrain, but was subsequently downregulated. This suggests that long-term differences in Hox gene expression cannot account for rostrocaudal differences in developmental potential of neural crest populations in this case.

Key words: Neural crest, Cornea, Trigeminal ganglion, Branchial arch, Quail-chick chimera

Introduction

The neural crest is a uniquely vertebrate cell population that arises from the neuroectoderm. After formation of the neural tube, these cells migrate from their site of origin in the central nervous system to diverse regions in the developing embryo. They localize in numerous sites where they differentiate into diverse derivatives, including melanocytes, peripheral neurons and glia, as well as the cartilage, bone and connective tissue of the face.

Much of what we know about neural crest derivatives comes from elegant and now classic transplantation experiments performed in birds. Most notably, chimeric grafts in which the neural tube of a quail embryo was transplanted in place of a chick host neural tube were used to define the derivatives that arose from the neural crest at various axial levels (Weston, 1963; Le Douarin and Teillet, 1974) (reviewed by Le Douarin, 1982). By contrast, trunk neural crest cells contributed to some combinations thereof. We test the relative roles of intrinsic versus extrinsic factors by challenging the developmental potential of cardiac and trunk neural crest cells via transplantation into an ectopic midbrain environment. We then assess long-term survival and differentiation into diverse derivatives, including cornea, trigeminal ganglion and branchial arch cartilage. Despite their ability to migrate to the periocular region, neither cardiac nor trunk neural crest contribute appropriately to the cornea, with cardiac crest cells often forming ectopic masses on the corneal surface. Similarly, the potential of trunk and cardiac neural crest to form somatosensory neurons in the trigeminal ganglion was significantly reduced compared with control midbrain grafts. Cardiac neural crest exhibited a reduced capacity to form cartilage, contributing only nominally to Meckle’s cartilage, whereas trunk neural crest formed no cartilage after transplantation, even when grafted directly into the first branchial arch. These results suggest that neural crest cells along the rostrocaudal axis display a graded loss in developmental potential to form somatosensory neurons and cartilage even after transplantation to a permissive environment. Hox gene expression was transiently maintained in the cardiac neural tube and neural crest at 12 hours post-transplantation to the midbrain, but was subsequently downregulated. This suggests that long-term differences in Hox gene expression cannot account for rostrocaudal differences in developmental potential of neural crest populations in this case.

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Introduction

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Clear differences in the potential of neural crest cells to form these different derivatives exist along the rostrocaudal axis. This was first demonstrated by heterotopic grafting experiments in which cranial neural crest was grafted to trunk levels and vice versa. The results revealed that cranial neural crest cells could contribute to all truncal derivatives but also formed ectopic cartilage nodules after transplantation into the trunk levels (Le Douarin and Teillet, 1974; Le Lièvre et al., 1980). By contrast, trunk neural crest cells contributed to some
normal cranial derivatives such as the cranial ganglia, but were unable to form cartilage of the head (Noden, 1978a; Nakamura and Ayer-Le Lievre, 1982). This suggested that the cranial neural crest has a broader (or at least different) developmental potential than that of trunk neural crest. However, under appropriate culture conditions, trunk neural crest cells have been shown to acquire some properties of chondrocytes (McGonnell and Graham, 2002).

The fact that trunk neural crest cells can form cartilage under appropriate conditions raises the possibility that previous techniques may not have been sufficiently sensitive to detect all neural crest phenotypes. In the original quail/chick transplantation experiments performed over 25 years ago, quail cells were recognized by histological staining for heterochromatin (Le Douarin, 1973; Le Douarin, 1974). This was effective for groups of cells but not sufficiently sensitive to achieve single cell resolution. Furthermore, differentiated cell types were assigned by location rather than by means of cell type-specific markers that are available today. Thus, it is possible that differentiation of some trunk neural crest cells into appropriate cranial derivatives may have been missed. In addition, previous experiments concentrated on skeletal derivatives; thus, differentiation and survival of quail cells into the cornea and trigeminal neurons after heterotopic transplantation were not examined. Given the significance of these classic experiments to our understanding of neural crest developmental potential along the neural axis, it seems important to revisit the elegant grafting experiments of Le Douarin and colleagues using modern approaches.

In order to test the relative roles of intrinsic information versus environmental influences on neural crest cell fate, we have challenged the developmental potential of cardiac and trunk neural crest cells by transplanting them into an ectopic midbrain environment. We examined their long-term ability to contribute to the cornea, trigeminal ganglia and first branchial arch cartilage and bone using a combination of cell labeling techniques and molecular markers of differentiation and positional identity. The results show that all populations contribute equally well to non-neuronal cells of the cranial ganglion and to melanocytes. Despite their ability to properly migrate along appropriate midbrain neural crest pathways, there appears to be a progressive loss of ability to contribute to the endothelium and stroma of the cornea, somatosensory neurons of the trigeminal ganglion and branchial arch cartilage with distance along the rostrocaudal axis. Expression of Hoxa2 and Hoxa3 was transiently maintained in cardiac neural crest after transplantation to the midbrain but was subsequently downregulated. This suggests that long-term maintenance of Hox gene expression cannot account for rostrocaudal differences in developmental potential of neural crest populations in this case.

Materials and methods

Embryos

Fertilized White Leghorn chick (Gallus gallus domesticus) and Japanese quail (Coturnix coturnix japonica) eggs were obtained from commercial sources. Chick eggs were incubated at 38°C for 30 hours to obtain seven-somite or stage 9 (Hamburger and Hamilton, 1951) embryos. Quail eggs were incubated at 38°C for 27-40 hours to obtain 7- to 10-somite (stage 9-10) embryos. Embryos were prepared as previously described (Lwigale, 2001). Briefly, chick eggs were windowed and the vitelline membrane was removed from the region of surgery using pulled glass needles. Only stage 9 chick embryos (not showing neural crest migration in the midbrain region) were used as hosts. Quail-donor embryos between stages 9 and 10 were lifted from the eggs using filter paper rings, rinsed and kept at room temperature in sterile phosphate buffered solution (PBS) until needed.

Quail-chick grafts

Dorsal neural tube explants were ablated from the midbrain region (between posterior mesencephalon and anterior metencephalon) of stage 9 chick hosts and replaced with quail dorsal neural tubes of similar size from midbrain, cardiac or trunk axial levels removed from stage 9-10 donor embryos. Eggs were sealed with Scotch tape and chimeric embryos were reincubated for an additional 5 to 15 days. Corneas and trigeminal ganglia were analyzed from embryonic day 15 (E15) chimeras. Because bones had ossified by this stage, we chose E5-7 chimeras to look at contribution to cartilage.

Antibodies and immunostaining

E15 chimeric embryos were sacrificed and their heads collected in cold 4% paraformaldehyde. Corneas and trigeminal ganglia were dissected out and fixed further at room temperature for 2 hours with mild agitation then rinsed twice in 0.1 M PBS. Trigeminal ganglia and corneas were embedded in gelatin and cryosectioned at 10 or 12 μm. PBS containing 0.2% (w/v) bovine serum albumin, 0.2% (v/v) Triton X-100 and 5% (w/v) heat-inactivated goat serum (PBT) was used as an antibody buffer. Quail-specific neural antibody-QN (mouse IgG1) (Tanaka et al., 1990) and quail-specific nuclear marker-QCPN (mouse IgG1, Developmental Studies Hybridoma Bank [DHSB], University of Iowa) were used diluted 1:1 in PBT to label the gifted quail cells. Rabbit anti-neuron-specific class III β-tubulin antibody TuJ1 (IgG2a, BABCO) was used diluted 1:500 to label all nerves (quail and chick). TrkA rabbit IgG antibody was used diluted 1:1000 to detect neural crest-derived sensory neurons in the trigeminal ganglion. Rabbit anti-collagen II (IgG, BABCO) was used diluted at 1:200 to detect chondrocytes. Mouse anti-MF20 (IgG2b, DSHB) against heavy chain myosin was used diluted 1:3 to label the ciliary muscles of the eye. HNK-1 (IgM) was used diluted at 1:50 to label migrating neural crest cells. Fluorochrome-conjugated goat secondary antibodies were purchased from Molecular Probes (Alexa Fluor 488 anti-mouse IgG2b, Alexa Fluor 594 anti-mouse IgG1, Alexa Fluor 594 anti-mouse IgM, and Alexa-Fluor 488 anti-rabbit IgG1 and 2a) and used diluted 1:200. Immunostained sections were counterstained with DAPI, rinsed in PBS and mounted on slides using Perma Fluor (Immunon, Pittsburgh, PA). Fluorescent images were captured using a Zeiss Axioskop 2 microscope. Images were processed using Adobe PhotoShop (Adobe Systems).

In situ hybridization

Stage 12-18 embryos were harvested, trimmed, and fixed in 4% paraformaldehyde. In situ hybridization was carried out as previously described (Henrique et al., 1995).

Microscopy and imaging

Fixed trigeminal ganglia and corneas were photographed prior to sectioning and immunostaining using an Olympus DP10 digital camera mounted on a Zeiss Stemi SV11 microscope. Images of all trigeminal ganglia were taken at the same magnification. To determine the relative areas of trigeminal ganglia, images were imported into Adobe Photoshop, digitally trimmed by erasing the sensory root and maxillo-mandibular nerves at the point of bifurcation. The ophthalmic branch was always cut adjacent to the spinal cord during dissection. Trimmed images were imported into the NIH-Image program. All ganglia were traced using the threshold option, and their trimmed areas were measured. All area measurements were compared statistically using a Student’s t-test.
To determine the number of neurons, serial sections representing each entire ganglion were mounted on one slide and immunostained. Three representative sections with the highest numbers of neurons were chosen from each slide, and the region with the highest number of neurons was selected for imaging. The numbers of neurons from each selected region were averaged and used for statistical analysis using a Student’s t-test.

Results

In order to challenge the developmental potential of neural crest populations, dorsal neural tubes were ablated from the midbrain region and replaced with quail neural crest from either cardiac, trunk or midbrain axial levels. Three grafting procedures were done (Fig. 1A,C,E) using the following extirpated tissues: (1) midbrain neural crest \( n = 37 \), from a similar region of a quail donor (experimental control); (2) cardiac neural crest \( n = 51 \), between the region of the otic placode and somite 1, Kirby et al., 1985) taken from stage 9-10 quail donors; and (3) trunk neural crest \( n = 12 \), between somite 7-10) of stage 9-10 quail donors.

We examined quail-chick chimeras at E15 for incorporation of transplanted quail dorsal neural tube tissues from the midbrain, cardiac and trunk regions. Because neural crest cells from the entire body axis of avian embryos give rise to melanocytes (Le Douarin, 1982), we used the presence of quail (brown/black) melanocytes in White Leghorn chicks as an indication of incorporation of the grafted quail tissue into the chick host. Only the chimeras that showed incorporation of the grafted neural crest cells based on the pigmentation criteria were used for further analysis. For incorporation of grafted tissue into cartilage, embryos were examined at E6.

Transplantation of dorsal neural tubes from the cardiac and trunk regions resulted in embryos with visually similar quantities of brown/black quail melanocytes in the head region to those seen in the control chimeras that had received a midbrain graft (Table 1; Fig. 1B,D,F). In some cases, part of the beak was pigmented, as expected (Noden, 1983). Visual assessment of the morphology of the lower beaks, where most of the transplanted neural crest would normally migrate, did not show any size differences in the E15 chimeras.

Cardiac neural crest cells form ectopic pigmented masses on the cornea and make minimal contributions to keratocytes, corneal endothelial cells or ciliary muscle

We first examined the ability of cardiac neural crest cells to contribute to the cornea after heterotopic transplantation.

Table 1. Summary of graft experiments and relative abundance of neural crest derivatives in E15 chimeras

<table>
<thead>
<tr>
<th>Level on AP axis</th>
<th>Stage</th>
<th>( n )</th>
<th>Melanocytes</th>
<th>% forming ectopic mass</th>
<th>Trigeminal ganglion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
<td>9-10 to 9</td>
<td>37</td>
<td>++++</td>
<td>0</td>
<td>+++++</td>
</tr>
<tr>
<td>Cardiac</td>
<td>9 to 9</td>
<td>24</td>
<td>++++</td>
<td>0</td>
<td>++++</td>
</tr>
<tr>
<td>Trunk</td>
<td>10 to 10</td>
<td>27</td>
<td>++++</td>
<td>48</td>
<td>++++</td>
</tr>
</tbody>
</table>

Neural crest cells from the posterior mesencephalon normally migrate into the perioocular region (Couly et al., 1996), where they combine with prosencephalic neural crest (Johnston et al., 1979) to contribute to the stromal keratocytes and endothelial cell layer of the cornea, as well as to the skeletal and connective tissues of the eye. It was immediately obvious that cardiac neural crest chimeras were not normal, as they developed darkly pigmented masses of cells on the dorsal region of the corneas (Fig. 1D, arrow). To determine the contribution of transplanted neural crest cells to the stroma and endothelial cell layers of the cornea, chimeric E15 corneas were sectioned and immunostained with QCPN and MF20 antibodies, to recognize
All chimeric corneas were surrounded by a ring of melanocytes (Fig. 2A,E,I,M) in the region of the limbus. Control midbrain chimeric corneas showed numerous QCPN-positive cells in both the stroma and endothelial cell layers (Fig. 2B,C). Some of the QCPN-positive cells observed in the limbus and in the epithelial cell layer were darkly pigmented (Fig. 2D). Some QCPN-positive cells were located in the ciliary muscle and they were also MF20 positive (Fig. 2B,C). Corneas from cardiac neural crest chimeras were similar to midbrain controls, but had comparatively fewer QCPN-positive cells in the stroma and endothelial cell layer (Fig. 2J). Numerous QCPN-positive cells were also MF20 positive (Fig. 2J,K). Some of the QCPN-positive cells surrounding the cornea and in the epithelial cell layer were darkly pigmented (Fig. 2H,L). In addition, ectopic masses of cells formed on the dorsal part of corneas in cardiac neural crest chimeras (Table 1; Fig. 2E,I). These masses were observed when cardiac neural crest was transplanted from stage 10, 48% ($n=13$), but were not seen in stage 9 grafts into the midbrain region. They were darkly pigmented and of variable size (Fig. 2E,I). In some cases, feather buds developed on their surfaces (Fig. 2I, arrow). Sections through the darkly pigmented mass showed numerous pigmented and non-pigmented QCPN-positive cells covered by an epithelial layer of host origin (Fig. 2G,H).

The masses formed only on the dorsal side of the cornea. To determine whether this was due to spatial or temporal effects, we transplanted stage 9-10 cardiac neural crest into the forebrain region from which most of the periocular neural crest cells originate (Johnston et al., 1979). Dark masses similar to the ones observed after grafting into the midbrain formed in all cases $n=4$ (data not shown). Unlike grafts into the midbrain,
however, these masses were more extensively localized, not only on the dorsal cornea, but also around the entire posterior half of the corneal margin. These results suggest that some of the neural crest grafted into the midbrain region contribute to the dorsal region of the cornea. However, in this case, the cardiac neural crest cells fail to contribute to the developing cornea and instead, form an ectopic mass that is mostly comprised of melanocytes. These results show that cardiac neural crest has limited capacity to form appropriate corneal derivatives, even though they migrate appropriately to the periocular region.

**Trunk neural crest cells grafted to the midbrain only contribute melanocytes to the cornea**

In contrast to cardiac neural crest cells, which contributed to some corneal derivatives, trunk crest showed little or no ability to properly incorporate into the cornea after heterotopic transplantation. Sections through E15 trunk neural crest chimera corneas revealed no QCPN-positive cells in the stroma or endothelial cell layers (Fig. 2N,O). However, a few QCPN-positive cells were present in the limbus area, the epithelial cell layer and ciliary muscle (Fig. 2O,P). All the QCPN-positive cells were darkly pigmented (Fig. 2P), indicating they had differentiated into melanocytes, and none of the cells observed in the ciliary muscle were MF20 positive (Fig. 2O,P, arrow). These results indicate that trunk neural crest can migrate to the periocular region but fail to form normal corneal derivatives.

**Cardiac and trunk neural crest contribute fewer neurons to the trigeminal ganglion**

Grafting of cardiac and trunk neural crest to the midbrain resulted in the formation of trigeminal ganglia that were reduced in size and had fewer somatosensory neurons. The normal neural crest contribution to the trigeminal ganglion comes from the midbrain region (D’Amico-Martel and Noden, 1983) and rostral hindbrain (Lee et al., 2003). These cells migrate ventrolaterally before condensing in the dorsal region of the forming ganglion, where they continue to undergo cell division and give rise to neurons and supporting cells (D’Amico-Martel and Noden, 1980). Analysis of E15 midbrain chimera trigeminal ganglia sections showed numerous large and small QCPN-positive nuclei (Fig. 3A,B). All the large cells were TUJ-1 positive (data not shown) and had the morphological appearance of neurons. The cells with large QCPN-positive nuclei were restricted to the proximodorsal side of the ganglion whereas the small non-neuronal cells (which probably support cells and glia) were widely spread throughout the ganglion (Fig. 3A). In control chimera, many of the large cells were also trkA positive (Fig. 3C), suggesting that they were somatosensory neurons. In contrast to control transplants, trigeminal ganglia formed in embryos that received cardiac neural crest grafts showed a significant reduction in the number of large QCPN-positive cells, but the number of smaller QCPN-positive cells appeared unchanged (Fig. 3D,E). Few of the large cells were trkA positive (Fig. 3F). The number of large QCPN-positive neurons was even more reduced in grafts of trunk neural crest, although the numbers of small cells remained high (Fig. 3G,H). In most cases, the large cells in these ganglia were trkA negative (Fig. 3I). In some trunk neural crest chimeras, no QCPN-positive neurons were observed in the ganglia (data not shown), although numerous small QCPN-positive cells were present (Table 1). These results indicate that the ability to form trigeminal neurons decreases with the axial level of origin,
but that the ability to form support cells and/or glia is not affected.

To quantitate the reduction in neuronal number with transplants from progressively more caudal axial levels, we counted the number of QCPN-positive neurons in selected fields of representative sections through midbrain, cardiac, and trunk chimeric trigeminal ganglia. The number of large QCPN-positive cells was determined as a percentage of midbrain controls. We observed a significant decrease ($P<0.0001$) in the number of large cells in cardiac ($n=10$) and trunk ($n=6$) neural crest ganglia compared to the midbrain controls ($n=12$) (Fig. 3J). Similarly, there was a significant reduction ($P=0.0038$) between the numbers of large nuclei in cardiac versus trunk ganglia.

The trigeminal ganglion receives a dual, and approximately equal, contribution from the neural crest and ectodermal placode cells (Yntema, 1944; Hamburger, 1961; Lwigale, 2001). To determine whether the reduced number of chimeric neurons affected total ganglion size, the relative areas of trigeminal ganglia was compared between the different types of transplants (Fig. 4A for midbrain controls; $n=10$; Fig. 4B for cardiac, $n=13$; Fig. 4C for trunk, $n=10$). For all chimeras, the trigeminal ganglia had the appropriate shape, forming the ophthalmic and maxillomandibular branches. Relative areas of cardiac chimera ganglia were not significantly different ($P=0.0798$) from midbrain controls, but those of trunk chimera ganglia were significantly smaller ($P=0.0005$) than the midbrain controls (Fig. 4D).

The sizes of the ganglia in each experimental group correlated with the reduced number of neurons (Table 1). Nonetheless, normally shaped ganglia formed. This is probably due to compensation for the lost neurons by host neural crest cells from adjacent axial levels and/or from increased placodal contribution. Because the trigeminal ganglion derives from a large segment of the neuraxis ranging from the rostral midbrain to the hindbrain at the level of r4 (Lee et al., 2003), a sizeable proportion of the host cells in the ganglion are derived from adjacent neural crest populations; thus, our grafts represent only a fraction of the neural tube region that contributes to the trigeminal ganglion. However, it remains possible that the placodal contribution may increase after transplantation as placodes also contribute somatosensory neurons to the trigeminal ganglion. Accordingly, trunk neural folds were transplanted rostrally to the midbrain and the embryos were allowed to develop until the 12-somite stage (after neural tube closure but prior to ingress of placode cells). The surface ectoderm was then labeled with Dil. In embryos examined 5 days later, the trigeminal ganglion had formed and placodal neurons had become post-mitotic. We found no apparent alteration in the numbers of placode-derived neurons within the ganglion after this manipulation compared with midbrain controls (data not shown). This suggests that the placodes did not significantly alter their contribution to the trigeminal ganglion.

Fewer cardiac and trunk neural crest-derived nerves innervate the cornea

Sensory innervation of the cornea is derived from the trigeminal neural crest (Lwigale, 2001). To determine whether the reduced population of neurons derived from cardiac or trunk grafts form proper functional connections and innervate their normal peripheral targets, we immunostained chimeric corneas with a quail nerve-specific antibody (QN) and then counterstained with TuJ-1. After midbrain neural crest transplants, numerous QN-positive neurons were present in the corneas (Fig. 5A,B). The pattern was similar in cardiac neural crest chimera, although fewer QN-positive neurons were observed (Fig. 5C,D). Reflecting the large reduction in quail neurons, very few QN-positive axons were seen in the cornea after trunk transplants (Fig. 5E,F). In some cases no QN-positive axons were seen in trunk chimeric corneas (data not shown). The results show that cardiac and trunk neural crest-derived axons can make functional connections in the cornea but their numbers decrease progressively.

Cardiac but not trunk neural crest cells contribute to some first branchial arch derivatives after transplantation to the midbrain

Grafts of cardiac neural crest cells to the midbrain revealed a reduced potential to form cartilage but proper migration to the first branchial arch environment. Neural crest cells from the midbrain region normally migrate to the first arch, where they give rise to the skeleton and connective tissues of the lower jaw (Couly et al., 1993; Couly et al., 1996; Kontges and Lumsden, 2003; Schneider and Helms, 2003; Trainor, 2003). To determine the ability of the cardiac neural crest to contribute to the lower jaw tissues, E6 chimeras were sectioned and immunostained with QCPN and collagen type II antibodies to recognize quail cells and cartilage, respectively. As expected, midbrain controls showed numerous QCPN-positive cells dispersed throughout the mandibular process, with few cells migrating into the maxillary process ($n=3$, Fig. 6A). Numerous cells contributed to Meckel’s cartilage (Fig. 6B,C). Some
QCPN-positive cells were observed in the ciliary and trigeminal ganglia. After cardiac neural crest transplants, numerous QCPN-positive cells were observed in the mandibular processes, but they did not mix with the host cells (n=4, Fig. 6D,E). Meckel’s cartilage was mostly comprised of host cells, except for a few clusters of QCPN-positive cells on the ventral side (Fig. 6E,F,F’), some of which had formed cartilage, as assayed by collagen II expression. In contrast to Meckel’s cartilage, which had few cardiac crest cells, numerous QCPN-positive cells contributed to the quadrate bone (Fig. 6E,F). These results demonstrate that cardiac neural crest can form cartilage in some parts of the mandibular process, but appear to have reduced capacity compared to midbrain controls.

Grafts of trunk neural crest cells to the midbrain demonstrated little ability to populate the branchial arches and no ability to form cartilage. Transverse sections through trunk neural crest chimeras (n=3) revealed many QCPN-positive support cells in the ophthalmic nerve but few cells in the periocular region (Fig. 6G, arrowhead), mandibular process (Fig. 6G,H, arrow), and very few in Meckel’s cartilage (Fig. 6H,I). This result suggests that trunk neural crest cells either fail to form cartilage or cannot migrate properly into the first branchial arch environment.

**Trunk neural crest cells do not form cartilage when grafted directly into the first branchial arch**

Trunk neural crest cells have been shown to form bones and cartilage when cultured in media that promotes bone differentiation (McGonnell and Graham, 2002). To determine whether trunk neural crest cells can form cartilage when placed directly into the appropriate environment in vivo, we transplanted trunk dorsal neural tubes directly into the region of first branchial arch of stage 12-13 host embryos (n=5). As a control, similar grafts were carried out using midbrain dorsal
neural tubes ($n=2$). Sections through E6-7 midbrain control chimeras showed numerous QCPN-positive cells in the tongue, mandibular process and, in particular, in Meckel’s cartilage (Fig. 7A,B). Grafting trunk dorsal neural tubes directly into the first branchial arch increased the number of QCPN-positive cells in the mandibular process (Fig. 7C) compared with the previous experiment when they were transplanted into the midbrain dorsal neural tube (Fig. 6H). Some of the QCPN-positive cells formed aggregates directly adjacent to the cartilage-forming region indicated by the collagen II antibody. However, they never became incorporated into the cartilage-forming region or expressed collagen II. This result suggests that trunk neural crest cells do not form cartilage even when grafted directly in a conducive environment in vivo.

**Analysis of Hox gene expression after grafting cardiac neural folds to the midbrain**

A possible explanation for the reduced ability of cardiac neural crest to form somatosensory neurons and cartilage may be that, unlike midbrain neural crest, cardiac neural crest express Hox genes that may be important for positional identity (Prince and Lumsden, 1994; Saldivar et al., 1996). If their rostrocaudal Hox identity is maintained, the cardiac neural crest may lack the proper program to respond to signals in the new environment. To examine this possibility, we monitored the expression of Hox genes in the cardiac neural tube and neural crest after transposition.

At the level from which cardiac neural crest emigrates, the hindbrain neural tube expresses a number of Hox genes, including Hoxa2, Hoxa3, Hoxb3 and Hox4. Of these, whole-mount in situ hybridization revealed that Hoxa2 and a3 are also expressed in migrating cardiac neural crest cells (data not shown) (Prince and Lumsden, 1994; Saldivar et al., 1996). To look at the effects of transplantation of cardiac neural folds to the midbrain on expression of these markers of rostrocaudal identity, we analyzed embryos between 12 to 72 hours after transplantation; previous ablation experiments had observed that the Hox code in the branchial arches was restored by 48 to 72 post-surgery (Hunt et al., 1995).

At 12 hours post-transplantation, both Hoxa2 and Hoxa3 were maintained in the caudal hindbrain neural tube after transplantation to the midbrain (Fig. 8A,E). Migrating neural crest cells emerging from the graft also expressed Hoxa2 (Fig. 8B,C) and a3 (Fig. 8F,G), with the latter being more robustly expressed than the former. However, maintenance of Hox gene expression in the neural tube and neural crest appeared to occur only transiently after transplantation to the midbrain. After 24-72 hours post-grafting, we observed a complete absence of expression of either Hox gene in cardiac neural crest cells transplanted rostrally to the midbrain (Fig. 8D,H). Furthermore, staining was observed in the donor neural tube that became integrated into the midbrain only at 12 hours following grafting and became down-regulated thereafter. This contrasts with the robust expression of Hoxa2 and a3 in migrating cardiac neural crest and branchial arches in the normal environment. The apparent downregulation of Hoxa2 and a3...
...was surprising given that previous rostral transposition of rhombomere 4 to the rostral hindbrain resulted in maintenance of its Hox expression (Prince and Lumsden, 1994). These results suggest that Hoxa2 and a3 transcripts are maintained from 12 hours after transplantation but downregulated thereafter in both the cardiac neural tube and neural crest. Thus, aspects of rostrocaudal identity are only transiently maintained after transplantation.

**Discussion**

Our results show that there is a graded decrease along the rostrocaudal axis in the ability of neural crest cells to form corneal cells, trigeminal neurons and mandibular cartilage. Challenging cardiac and trunk neural crest cells via transplantation into an ectopic midbrain environment allowed us to distinguish the relative roles of intrinsic and extrinsic influences on neural crest differentiation. For example, our results reveal that cardiac neural crest have some potential to form cartilage albeit less than midbrain neural crest. By contrast, trunk neural crest failed to form cartilage in vivo even when directed transplanted into the branchial arches. Thus, the arch environment positively influences cardiac neural crest but had no detectable effect on trunk neural crest. Although previous studies have tested the ability of neural crest cells from hindbrain, vagal and trunk levels to contribute to the skeleton (Noden, 1978a; Nakamura and Ayer-Le Lievre, 1982) (reviewed by Chambers and McGonnell, 2002), little attention has been given to non-skeletal derivatives, nor has the long term survival of the transplanted cells been examined. By combining modern cell marking techniques with molecular markers of cell differentiation, our results have revealed previously unknown abilities of neural crest cells to contribute to distinct types of neurons and cartilaginous elements.

Hox genes are expressed in a segmented pattern in the hindbrain, that is colinear with their sequence along the chromosome, and thought to impart rostrocaudal positional information to the neural tube. Because migrating cardiac neural crest cells express a similar Hox code to their neural tube of origin, the difference in Hox gene expression between cardiac and midbrain crest offers a possible explanation for their reduced potential to contribute to normal midbrain crest derivatives. Surprisingly, our results show that Hox genes are maintained only transiently in the caudal hindbrain neural tube and neural crest after transplantation rostrally into the midbrain region. This suggests that long-term maintenance of their Hox identity cannot explain differences in developmental potential. It remains possible, however, that the early expression of Hoxa2 and Hoxa3 in the migrating cardiac neural crest is sufficient to account for developmental differences in their ability to populate midbrain crest derivatives.

**Potential to form corneal cells**

Cardiac neural crest contributes minimally to the cornea and forms ectopic cell masses

Neural crest cells from the midbrain, together with those from the posterior diencephalon region, normally migrate to the region of the eye, where they contribute to the pericocular mesenchyme (Kontges and Lumsden, 1996; Couly et al., 1996). During early development of the chick eye, ectoderm overlaying the newly formed lens synthesizes and assembles the primary stroma (Hays, 1980) that serves as a substratum for migration of neural crest cells from the diencephalon and posterior midbrain region (Johnston et al., 1979; Couly et al., 1996). These give rise to the innermost layer of the cornea, the endothelium (Johnston et al., 1979). Shortly thereafter, the presumptive cornea is invaded by a second group of neural crest cells that migrate directly into the extracellular matrix of the primary stroma between the epithelium and endothelium, differentiating into corneal stromal fibroblasts, called keratocytes (Johnston et al., 1979). Thus, two major cell populations of the cornea are of neural crest origin. Finally, the growth cones of sensory nerves whose cell bodies are located mainly in the trigeminal ganglion provide sensory innervation to the cornea (Arvidson, 1977; Morgan et al., 1978; Marfurt et al., 1989; Lwigale, 2001).

Grafting of quail midbrain neural crest isotopically into chick resulted in numerous pigmented feathers and dark rings around the corneas indicating that some of the neural crest cells differentiated into melanocytes by E15. Sections through the corneas revealed numerous quail cells, identified by the QCPN antibody, that had formed stromal keratocytes and endothelial cells, as expected (Johnston et al., 1979). In addition, a few darkly pigmented QCPN-positive cells were observed in the epithelium.

Both similarities and differences to these control grafts were noted after transplantation of cardiac neural crest to the midbrain. Similar to midbrain grafts, cardiac neural crest formed a dark ring around chimeric corneas and contributed to the ciliary muscle. However, ectopic and darkly pigmented masses were noted on the dorsal sides of the corneas (48%) when stage 10 cardiac neural crest was transplanted into stage 9 hosts. This indicates that grafted cardiac neural crest cells fail to mix with the host cells, and thus result in large ectopic aggregates on the dorsal side of the cornea that protrude externally. Sections through the ectopic masses revealed that they comprised pigmented and non-pigmented QCPN-positive cells. By contrast, very few QCPN-positive cells contributed to the normal portion of the corneal stroma or endothelial cell layer. These results suggest that cardiac neural crest can follow the normal migratory pathway from the midbrain region into the pericocular region, but only a few of those cells can respond to the cues in this environment and contribute to the corneal cell layers or ciliary muscle.

The observed location of the large masses of transplanted cardiac neural crest cells on the dorsal side of the cornea probably reflects the fact that neural crest from the caudal midbrain region migrate to the dorsal side before entering and populating the developing eye. When cardiac neural crest cells were transplanted into more anterior regions (data not shown), similar ectopic masses were observed protruding from the dorsal and entire posterior half of the cornea. Except for their pigmentation and location at the dorsal side of the cornea, these ectopic masses resemble the non-pigmented mass of tissue overgrowing the region of the cornea in the clinical condition known as pterygia (Coroneo et al., 1999). Others have observed aggregates of cells in the nasal septum when r4-r6 neural crest was transplanted into the diencephalic level where they still maintained their Hoxa2 expression (Couly et al., 2002). Overexpression of Hoxa2, or Hoxa3 and Hoxb4 in the diencephalic region has been shown to be associated with reduced cranial cartilage, but cornea formation was apparently
normal at E7, right after its formation (Creuzet et al., 2002). However, later problems in the ability to contribute to the cornea were not examined. Our results indicate that cardiac neural crest cells do not form normal cornea when grafted into the midbrain region, suggesting that there is an axial difference in potential to form corneal cells between midbrain and cardiac neural crest cells. However, our results suggest that they only transiently maintain Hox expression in this ectopic location.

Trunk neural crest cells grafted to the midbrain only form melanocytes in the cornea

Whereas the majority of midbrain and cardiac neural crest cells migrate into the pericocular region following grafting, only a few trunk neural crest cells were observed within the cornea. Chimeric corneas derived from trunk grafts formed a dark ring of cells surrounding the cornea resembling those of midbrain and cardiac chimeras. However, only a few QCPN-positive cells were observed within the periphery of the cornea (in the limbus region adjacent to the cornea), with little or no contribution to the stroma, endothelium, epithelium or ciliary muscle. Furthermore, all of the QCPN-positive cells in trunk chimeras differentiated into melanocytes, suggesting that they may lack the ability to respond to the corneal and ciliary environments, but retain the capacity to form melanocytes, even in an ectopic environment. Our results suggest that trunk neural crest cells can follow the correct migration pathway to region of the cornea and eye but fail to invade after this experimental manipulation. Preliminary results suggest that some trunk neural crest can populate the cornea if directly transplanted into the periorcular region (P.Y.L. and M.B.-F., unpublished). Without definitive cell markers, however, it is unclear whether or not they differentiate properly.

Cardiac and trunk neural crest cells have limited neurogenic ability within the trigeminal ganglion

Sensory innervation of the eye, face and mouth is derived from the trigeminal ganglion, which comprises both neural crest and ectodermal placode cells (for a review, see Noden, 1993). Unlike placode cells, which are specified to form neuroblasts shortly after undergoing epithelial-mesenchymal transition, neural crest cells continue to divide after aggregating in the trigeminal ganglion (D'Amico-Martel and Noden, 1980) giving rise to neuroblasts, glia and other support cells.

Consistent with previous studies, our control midbrain grafts gave rise to numerous QCPN-positive cells in the dorsoproximal region of the trigeminal ganglion (Lwigale, 2001), comprising both large (neurons) and small (support) cells. The majority of the large cells were trkA positive, suggesting that they are somatosensory neurons. When cardiac neural crest were transplanted into the midbrain region, significantly fewer QCPN-positive neurons were observed in the trigeminal ganglion, but the number of QCPN-positive support cells seemed unaffected. With trunk neural crest grafts, there was an even more significant decrease in the percentage of trkA-positive neurons in the ganglion. For cardiac and trunk grafts, almost the entire neural crest contribution to the ganglion was host derived. These results suggest that there is an axial difference in potential to generate trigeminal neurons, decreasing in a rostral to caudal order. Previously, it was reported that the neural crest cells from caudal rhombencephalic levels (similar to the cardiac neural crest reported in this study) can contribute to the neuronal derivatives of the first branchial arch (Coulb et al., 1998). However, these authors only examined the glial cells present in the trigeminal mandibular nerve. In agreement with their findings, our data show that the number of non-neuronal cells (probably glia) was similar to control grafts. However, when sections of trigeminal ganglia were carefully analyzed, reduced numbers of neurons were observed.

Not only the percentage of neurons, but also the size of the trigeminal ganglion at E15 were altered after heterotopic grafting. All chimeric ganglia had a normal shape, with both major branches (ophthalmic and maxillomandibular) morphologically similar to those formed in midbrain control chimeric embryos. Cardiac chimera ganglia were slightly smaller but not significantly different from controls. By contrast, the size of trigeminal ganglia derived from trunk chimeras was significantly smaller than midbrain controls, consistent with the result that very few trunk neural crest cells form neurons. Noden (Noden, 1975) showed that [3H]thymidine-labeled chick trunk neural crest cells transplanted into the midbrain region formed morphologically normal trigeminal ganglia. Later experiments using quail-chick chimeras (Noden, 1978b) revealed that although trunk neural crest cells can form neurons when transplanted in the midbrain region, they fail to aggregate with the ectodermal placode cells, instead forming separate ganglia. The present results using the quail-chick technique are in agreement with Noden's initial observation, as the ganglia in all of our grafts properly formed the major branches. The differences between our results and Noden's later results are likely to reside in the size of tissue grafted. In the present experiments, chick-host neural crest cells rostral and caudal to the grafted tissue appeared to contribute the bulk of neurons to the trigeminal ganglion (D’Amico-Martel and Noden, 1983; Couly and Le Douarin, 1985; Couly and Le Douarin, 1987) and appeared to compensate for normal ganglionic morphology even when few or no chimeric neurons formed.

Cardiac and trunk neural crest contribute little sensory innervation to the cornea

The cornea is highly innervated by neural crest-derived neurons (Lwigale, 2001) originating from the ophthalmic branch of the trigeminal ganglion (Arvidson, 1977; Morgan et al., 1978; Marfurt et al., 1989). Because cardiac and trunk neural crest formed some trigeminal ganglion neurons, we analyzed chimeric corneas for the presence of quail neural crest-derived sensory axons, using a quail nerve specific antibody-QN. Midbrain control corneas showed numerous QN-positive nerves in the cornea. By contrast, cardiac and trunk neural crest chimera corneas contained fewer QN-positive nerves, decreasing as the axial level of donor tissue became more caudal. These results indicate that the few neurons that differentiate in the trigeminal ganglion in cardiac and trunk chimeras are viable and form normal afferent projections to the cornea. These results contrast with those of Noden (Noden, 1978b), who showed that trunk neural crest cells form ectopic ganglia that fail to make normal afferent ophthalmic and maxillomandibular projections. As a consequence, Noden concluded that they did not innervate their designated targets. In the present study, our grafts resulted in normal-appearing ganglia, which may have facilitated the
proper formation of neuronal connections. In addition, the availability of a species-specific antibody for tracking projections made it possible to identify processes that previously may have been missed.

**Cardiac but not trunk neural crest contribute to a few first branchial arch derivatives**

Midbrain neural crest cells normally migrate into the branchial arch regions, where they form specific cartilages and bones of the jaw (Noden, 1978a; Lumsden et al., 1991; Konteges and Lumsden, 1996; Couly et al., 1996). In the first branchial arch region, neural crest cells give rise to cartilages and bones of the lower jaw, such as Meckel’s cartilage, quadrate, and squamosal bones (Couly et al., 1993; Couly et al., 1996; Konteges and Lumsden, 1996). Consistent with previous grafting experiments, our isotopic grafts into the midbrain region showed numerous QCPN-positive cells in the mandibular process. In these chimeras, Meckel’s cartilage was almost entirely derived from quail cells.

Cardiac neural crest cells (between r6 and somite 3) normally migrate into branchial arches 3, 4 and 6, where they contribute to blood vessels, heart and cardiac ganglia (Bockman et al., 1987; Kirby et al., 1983). When cardiac neural crest were heterotopically grafted into the midbrain region, the lower jaws of E15 chimeras appeared normal. Sections through E6 chimeras showed numerous cardiac neural crest cells in the mandibular process. However, unlike midbrain controls, few such heterotopic crest cells contributed to Meckel’s cartilage, which was mostly comprised of host cells. The few individual cardiac neural crest cells detected were scattered throughout the cartilage. In some cases, cardiac neural crest cells did not mix with the host cells and instead formed nodules on the ventral side of Meckel’s cartilage, similar to those observed in r1-7 rotation experiments (Hunt et al., 1998). Cardiac neural crest, however, contributed significantly to the quadrate bone. By contrast, transplantation of r4/r6 neural folds to the r1/r2 level (Couly et al., 1998) showed migration into the first branchial arch, but a failure to contribute to the cartilage and bones at that axial level. This suggests that r4/r6 neural crest cells were unable to differentiate into derivatives of the first branchial arch. In the present study, we grafted cardiac neural crest from a region that normally expresses Hoxa2 and Hoxa3. Our results reveal some degree of plasticity in this population of neural crest to form lower jaw bones. However, the cardiac neural crest cells did not maintain Hox gene expression in their new environment. It is possible that transient expression of Hoxa2 and Hoxa3 is sufficient to account for their reduced potential of the cardiac neural crest to form cartilage and trigeminal neurons. However, it is clear that the expression of Hox genes is subsequently downregulated, suggesting that the cells are also subject to environmental regulation. This may explain the mixed results obtained with cardiac populations such that some take on normal midbrain fates whereas others fail to respond appropriately to their new environment.

In contrast to cardiac crest, no differentiation of trunk neural crest cells into cartilage was observed after heterotopic grafting, consistent with results of the original transplantation experiments of Noden (Noden, 1978a) and Nakamura and Le Lievre (Nakamura and Le Lievre, 1982). A few trunk cells migrated into the arches, and those that did were sparsely spread throughout the entire mandibular process, which was mostly of host origin. Some of these trunk crest cells were detected in the region where Meckel’s cartilage formed. Although the host neural folds were extirpated prior to grafting the trunk neural folds, many host neural crest cells, probably derived from along the adjacent neural axes, were found within the first arch. These compensated for the loss of normal midbrain neural crest cells, contributing to the normal lower jaws seen at E15.

Owing to the very small number of trunk neural crest cells in the mandibular process, it is possible that they may have the ability to form cartilage or bone if a larger population had migrated into this region. In fact, similar grafts in axolotl revealed some capacity of trunk neural crest to form cartilage in those cases where the cells made their way to the proper destination even when by means of a circuitous route (Epperlein et al., 2000). To investigate this possibility, we grafted midbrain (control) and trunk dorsal neural tubes directly into the first branchial arch. The midbrain grafts produced QCPN-positive neural crest cells that migrated from the graft and populated most of the mandibular process including Meckel’s cartilage. Trunk grafts produced more neural crest cells when transplanted directly into the first branchial arch, but unlike the midbrain control grafts, trunk neural crest cells formed aggregates adjacent to Meckel’s cartilage but not directly contributing to it. Some of the differences between cranial and trunk neural crest have been attributed to their migration behaviors (Lallier et al., 1992). Therefore, by grafting dorsal neural tubes directly into the first branchial arch we were able to increase the number of neural crest in the vicinity of the developing cartilage, but they failed to differentiate appropriately. Altogether, these results suggest that it is not due to population effect that trunk neural crest cells fail to form cartilage, but other intrinsic reasons. Recently, it was shown that trunk neural crest can form both bone and cartilage when cultured in appropriate media (McGonnell and Graham, 2002), suggesting that trunk neural crest has skeletogenic potential in vitro. By grafting dorsal neural tubes directly into the first branchial arch, we challenged the trunk neural crest cells to respond to the bone-forming cues in that environment. As they failed to form cartilage even in a conducive environment, this suggests that, unlike the in vitro induction, the cues that are present in vivo are not sufficient to induce trunk neural crest to form cartilage.

**Conclusion**

In summary, our results show that there are axial differences between midbrain, cardiac and trunk neural crest in potential to generate corneal keratocytes and endothelial cells, ciliary muscle, trigeminal neurons and first branchial arch derivatives. Although both cardiac and trunk neural crest migrate appropriately to the periorcular region, they fail to make appropriate contributions to the cornea. Cardiac crest forms a large percentage of ectopic masses, but only a small number localize in the cornea. Trunk neural crest only forms melanocytes in this location. The trigeminal ganglia of chimeric embryos are morphologically normal, but reduced in size, and contain a significantly reduced number of somatosensory neurons, particularly for truncal grafts. Similarly, cartilage-forming ability is reduced for cardiac neural crest and absent for trunk neural crest after transplantation. These results suggest a loss in neural crest
capacity along the rostrocaudal axis to form somatosensory neurons and cartilage, even after transplantation to a permissive environment. Our results using more sensitive detection techniques confirm the inability of trunk neural crest to form cartilaginous derivatives in vivo, as originally described by Le Douarin and colleagues using quail-chick chimeric transplants. It was important to readdress this issue given the recent demonstration that trunk neural crest can form cartilage in vitro after long-term culture in rich medium (McGonnell and Graham, 2002). In addition to the previously reported effect on skeletogenesis, the present results show that transplanting neural crest from cardiac and trunk regions into midbrain levels also affects the formation of non-skeletal crest derivatives, such as the cornea and trigeminal neurons. Surprisingly, we show that transplanted cardiac neural folds only transiently maintain Hox gene expression, which is subsequently downregulated in the midbrain environment. This contrasts with previous findings where Hox genes are maintained for long time periods after transpositions of r4 to r2 level. Thus, long-term maintenance of Hox gene expression is not sufficient to account for differences in developmental potential between cardiac and midbrain neural crest. However, initial maintenance of Hoxa2 and a3 in the population transplanted to midbrain may be sufficient to bias the population to exhibit reduced ability to respond to the midbrain environment.

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References


