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RESEARCH ARTICLE

Role of Cranial Neural Crest Cells in Visceral Arch Muscle Positioning and Morphogenesis in the Mexican Axolotl, *Ambystoma mexicanum*

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The role of cranial neural crest cells in the formation of visceral arch musculature was investigated in the Mexican axolotl, *Ambystoma mexicanum*. DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine, perchlorate) labeling and green fluorescent protein (GFP) mRNA injections combined with unilateral transplantations of neural folds showed that neural crest cells contribute to the connective tissues but not the myofibers of developing visceral arch muscles in the mandibular, hyoid, and branchial arches. Extirpations of individual cranial neural crest streams demonstrated that neural crest cells are necessary for correct morphogenesis of visceral arch muscles. These do, however, initially develop in their proper positions also in the absence of cranial neural crest. Visceral arch muscles forming in the absence of neural crest cells start to differentiate at their origins but fail to extend toward their insertions and may have a frayed appearance. Our data indicate that visceral arch muscle positioning is controlled by factors that do not have a neural crest origin. We suggest that the cranial neural crest-derived connective tissues provide directional guidance important for the proper extension of the cranial muscles and the subsequent attachment to the insertion on the correct cartilage. In a comparative context, our data from the Mexican axolotl support the view that the cranial neural crest plays a fundamental role in the development of not only the skeleton of the vertebrate head but also in the morphogenesis of the cranial muscles and that this might be a primitive feature of cranial development in vertebrates. *Developmental Dynamics 231:237–247, 2004.* © 2004 Wiley-Liss, Inc.

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INTRODUCTION

The neural crest is a transient embryonic structure unique to vertebrates. Cells migrate away from the neural crest during embryogenesis and give rise to many different cell types in a large variety of tissues and organs (for two recent book-length reviews see Hall, 1999 and LeDouarin and Kalcheim, 1999). In the head, unlike in the trunk, neural crest cells give rise to skeletal and dental tissues. In the chicken embryo, the migration and fate of cranial neural crest cells has been investigated very thoroughly, and it has been shown that most of the skull is neural crest derived. Data from other vertebrates indicate that this finding is a general vertebrate trait (Schilling, 1997; Osumi-Yamashita et al., 1997; Horigome et al., 1999; Manzanares et al., 2000; Kimmel et al., 2001). A direct contribution of neural crest cells to cranial musculature was first reported in 1975 by LeDouarin's group (LeLièvre and LeDouarin, 1975), and later confirmed by Noden (1983a,b) and Couly et al. (1992), who described the crest derivation of connective tissue components of several visceral arch muscles in quail–chick chimeras. More

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recent work has revealed that this additional role of the neural crest is only one component of a much more comprehensive mechanism of cranial development and patterning, in which positional relations among hindbrain segments (rhombomeres), the neural crest, and musculoskeletal derivatives are maintained throughout crest migration, pattern formation, and histogenesis (Graham et al., 1996; Köntges and Lumsden, 1996; Schilling, 1997; Schilling and Kimmel, 1997). The known complexity of cranial development increased even further with the discovery that the foregut endoderm is responsible for patterning the cartilages of the visceral arches (Couly et al., 2002; Ruhin et al., 2003, but first indications already in Hörstadius and Sellman, 1946). Several fate maps in chicken and mouse have shown that the myogenic cells originate from the paraxial mesoderm (Noden, 1983a, 1986; Couly et al., 1992; Trainor et al., 1994; Trainor and Tam, 1995; Hacker and Guthrie, 1998). Unlike in the trunk, the paraxial mesoderm in the head is not obviously segmented (Kuratani et al., 1999; Noden et al., 1999; Jouve et al., 2002, but see Jacobson, 1988). However, mesodermal and neural crest contributions to a visceral arch originate from approximately the same axial level (Noden, 1986; Couly et al., 1992; Schilling and Kimmel, 1994; Trainor et al., 1994; Trainor and Tam, 1995; Hacker and Guthrie, 1998). As most of the bones and cartilages in the head are neural crest-derived, the main contribution of the mesoderm is to muscles and blood vessels. Only a few studies have examined the relationship between cranial muscles and the cranial neural crest. Although several studies have included extirpations of neural crest cells, only a few have investigated the impact on cranial muscle development. Piatt (1938) included some comments on muscle development, and E.K. Hall showed (Hall, 1950) that the cranial muscles develop but are distorted when neural folds have been removed. The most recent study is that by Olsson et al. (2001) of the anuran *Bombina orientalis.* They performed extirpations when the neural crest cells had already started migrating, using the dark appearance of the neural crest cells. The results showed that cranial muscles were severely affected. The muscles seemed distorted and had often anastomosed with each other. Olsson and coworkers concluded that neural crest cells are crucial for correct morphogenesis of the visceral arch muscles. However, despite extirpations, muscles seem to appear more or less in their normal positions. The fate of the cranial neural crest cells is relatively unknown from a comparative perspective. With the exception of the chick–quail chimera technique, data are mostly derived from extirpations or vital dye experiments, including several classic studies on amphibians (Hall and Hörstadius, 1988). A direct contribution of the cranial neural crest to connective tissues in cranial muscles in amphibians, however, was not reported until 1987, when Sadaghiani and Thiébaud published results from chimeric *Xenopus* larvae. In analogy with the chick–quail studies, they had grafted neural fold material from *Xenopus borealis* into *Xenopus laevis* and used the fact that the nuclei could be distinguished between the species to undertake a fate mapping. They observed neural crest-derived cells in several cranial muscles (Sadaghiani and Thiébaud, 1987). Later, Olsson and coworkers, using extirpations and DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine, perchlorate) injections in the frog *Bombina orientalis,* concluded that there is a neural crest contribution to the connective-tissue component but not the myofibers of many larval muscles within the first two (mandibular and hyoid) branchial arches (Olsson et al., 2001). However, despite some preliminary data (Olsson et al., 2000), whether this finding is also true for salamanders has remained unclear. The aims of this study were to investigate the role of cranial neural crest cells in the formation of visceral arch muscles in the Mexican axolotl, and to see whether a role in muscle morphogenesis could be explained by the presence of neural crest cells in the

Fig. 1. A: Stage 25 axolotl embryo injected with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine, perchlorate). DiI at this stage is found only at the injection site (arrow). **B:** The same embryo as in A at stage 30. DiI-tagged cells are found in all the visceral arches (arrows). **C:** The same embryo as in A and B at stage 38. The DiI-tagged neural crest cells are still showing the pattern of the visceral arches (arrows). **D:** Horizontal section of DiI-injected stage 37 embryo. The section is through the visceral arches. DiI is found in the neural crest cells surrounding the mesodermal core of the visceral arches (arrowheads). **E:** Horizontal section of DiI-injected stage 39 embryo. The section is through the external gills of the branchial arches. At the base of the gill, DiI is found in the ceratobranchial cartilage (arrows). In the gill, DiI is found in connective tissue (arrowhead). Rostral is to the left. The green fluorescence is from a fibronectin staining. B1, first branchial arch; B2, second branchial arch; H, hyoid arch; M, mandibular arch. Scale bar = 500 μm in A–C, 200 μm in D,E.

Fig. 2. A: Lateral view of stage 28 axolotl embryo with migrating neural crest cells expressing green fluorescent protein (GFP) in the mandibular and hyoid arches. The part of the neural tube expressing GFP is the graft from the GFP-positive host. H, hyoid arch; M, mandibular arch. **B:** Horizontal section of stage 37 embryo with a GFP transplant. The section is through the mandibular and hyoid arches. GFP-expressing cells are found in the area surrounding the mesoderm core of the arches (arrowheads). **C:** Horizontal section of stage 38 embryo with GFP transplant. The section is through the first external gill. GFP-expressing cells are found in the connective tissue of the external gill and in the cells surrounding the mesoderm core (arrowheads). The orange color is from fibronectin staining. **D:** Lateral view of stage 36 embryo, stained for GFP (green fluorescence) and muscles (red fluorescence). Close-up of the branchial region. GFP-expressing cells are found in the dermis and surrounding the anlage of levator and depressor branchiarum ii (arrowheads). B1, B2, B3, branchial arches; ldba, anlagen of the levator and depressor branchiarum muscles. **E:** Ventral view of stage 40 embryo, stained for GFP and muscles. Close-up of the posterior head region. GFP-expressing neural crest cells are found in association with muscle fibers. The interhyoideus posterior and levator and depressor branchiarum are covered by a sheet of GFP-expressing cells (arrowheads). h, heart; ihp, interhyoideus posterior muscle; ldb, levator and depressor branchiarum muscles; rc, rectus cervicis muscle. Scale bar = 200 μm in A, 150 μm in B, C, 250 μm in D, E.

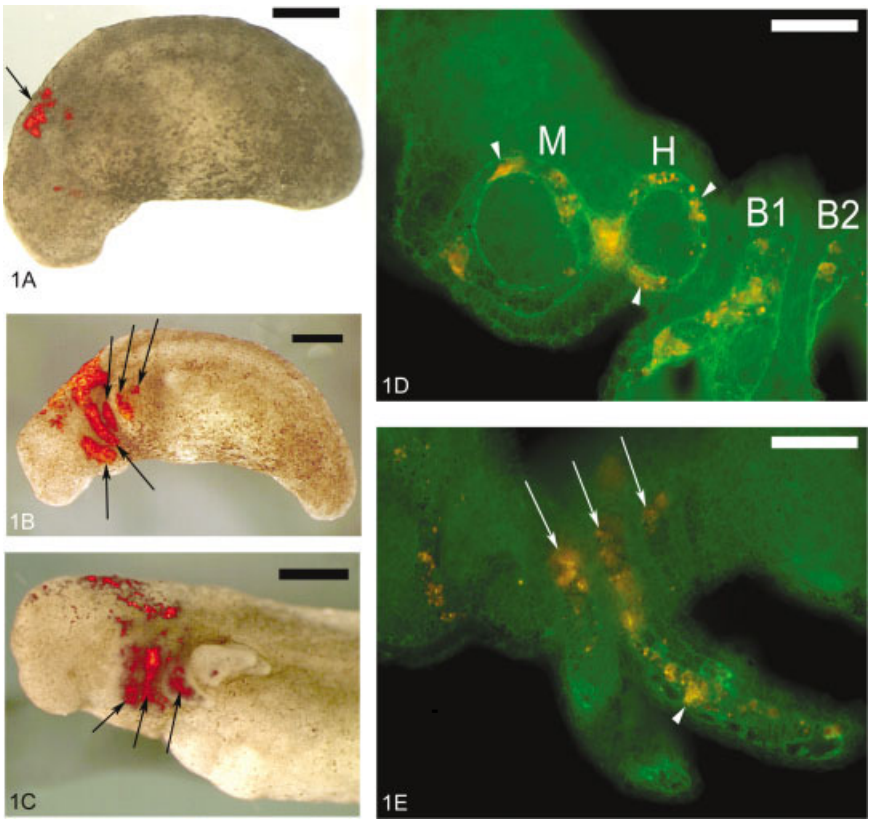


Fig. 1.

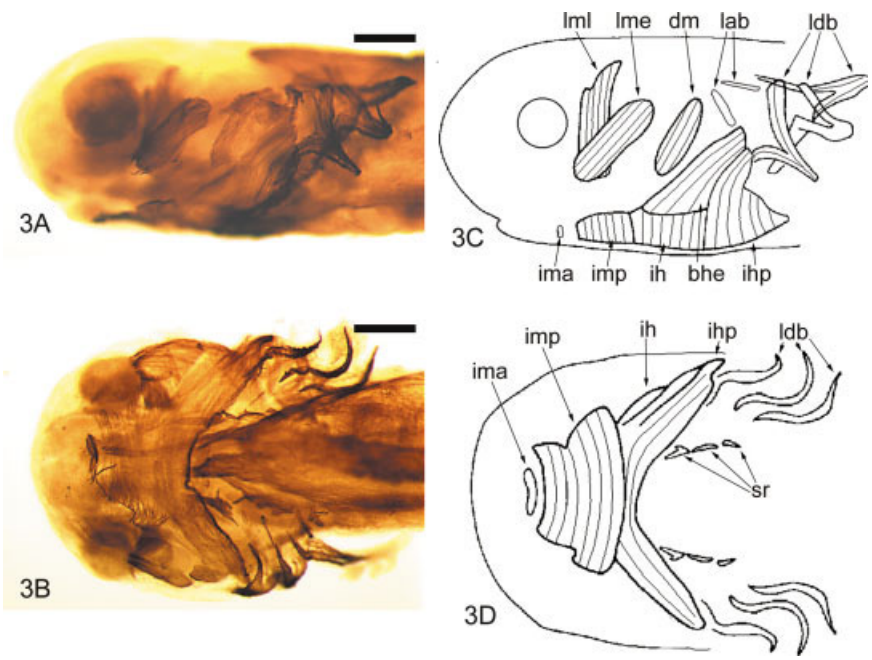


Fig. 3. **A:** Lateral view of stage 44 nonextirpated axolotl embryo. Muscles are stained with the 12/101 antibody and visualized with a horseradish peroxidase–conjugated secondary and diaminobenzidine. **B:** Ventral view of A. **C:** Schematic view of the visceral arch muscles of A. **D:** Schematic view of the visceral arch muscles of B. bhe, branchiohyoideus externus; dm, depressor mandibulae; ih, interhyoideus; ihp, interhyoideus posterior; ima, intermandibularis anterior; imp, intermandibularis posterior; lab, levator arcus branchiarum; ldb, levator and depressor branchiarum; lme, levator mandibulae externus; lml, levator mandibulae longus; sr, subarcualis rectus. Scale bars = 500 μm in A,B.

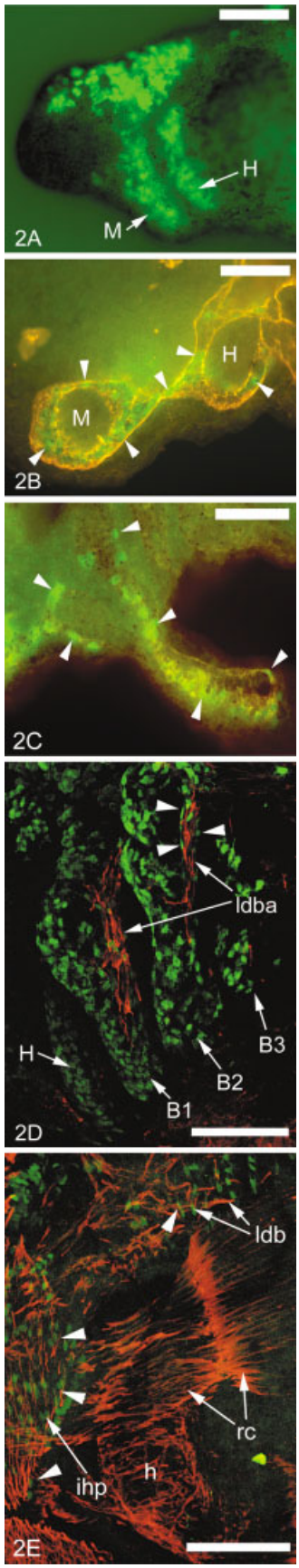


Fig. 2.

TABLE 1. Summary of the Results of the Neural Crest Extirpations

Extirpation	Cartilage staining	Muscle staining
Mandibular	Gaps in Meckel's cartilage Palatoquadrate distorted	Levator mandibulae longus and levator mandibulae externus are frayed and have extensions in the horizontal plane; intermandibularis posterior is frayed Intermandibularis anterior is shifted to the extirpated side
Hyoid	Missing hypohyal Ceratohyal appears as a small fragment attached to the caudalend of Meckel's cartilage	Depressor mandibulae is thin and fuses with interhyoideus posterior Branchiohyoideus externus is very faint and may fuse with interhyoideus posterior Interhyoideus binding to the ceratohyal fragment on Meckel's cartilage Interhyoideus posterior extends caudally and ends blindly
Branchial	Missing hypobranchial 1 and 2 All ceratobranchials missing	Levator and depressor branchiarum anastomose Interhyoideus posterior may shift its origin to the ceratohyal Branchiohyoideus externus may shift its origin to the ceratohyal Levator arcus branchiarum vague in appearance and ending blindly Subarcualis rectus muscles frayed, switched to a position dorsal to the pharynx or completely absent

connective tissues surrounding cranial muscles, as has been reported for other species (Noden, 1983b; Sadaghiani and Thiébaud, 1987; Köntges and Lumsden, 1996; Olsson et al., 2001). We found DiI-labeled cells in connective tissue surrounding muscle fibers and also between the muscle anlagen and the anlagen of the cartilages. The extirpation experiments showed that visceral arch muscles formed close to their origin in the absence of neural crest cells but failed to extend toward their normal insertions. We conclude that the cranial neural crest is not important for the correct early positioning of cranial muscles but is crucial for achieving the correct morphology. We interpret the complicated disturbances to muscle development induced by extirpations of each of the streams of cranial neural crest cells to be owing to the lack of directional guidance provided by neural crest derived connective tissues surrounding the myofibers.

RESULTS

Fate Mapping

The DiI-injected between the cranial neural crest and the overlying epidermis marked the migratory neural crest cells of the visceral arches, which migrated in the pattern documented in earlier studies (Hörstadius and Sellman, 1946; Epperlein et al., 2000). In many cases, only the two rostral-most of the neural crest cell streams were marked, but in some cases all the streams of cells were marked (Fig. 1A–C). DiI was visible in the neural crest cells until stage 40. In the sectioned material, DiI appeared in neural crest cells in the visceral arches, surrounding the mesodermal core (Fig. 1D). This position was stable throughout development, even as the mesoderm differentiated into myofibers. DiI was also present in the neural crest cells which form the visceral arch cartilages and in the connective tissues of the external gills (Fig. 1E). A more striking observation, as seen in the whole-mounts, was that neural crest cells were present in the area between the differentiating muscles and the anlagen of the visceral arch cartilages. The further development of visceral arch muscles is described in a separate study (Ericsson and Olsson, 2004). GFP-expressing cells from the transplanted neural folds were identified as neural crest cells by their position and migration pattern. The neural crest cells migrated into the visceral arches (Fig. 2A), showing the pattern seen in the DiI-injection experiments and reported by Hörstadius and Sellman (1946) and Epperlein et al. (2000). GFP was found in the neural crest cells that encircled the mesoderm in the visceral arches (Fig. 2B). In later stages of development, GFP-expressing cells were also found in the connective tissues of the external gills and in the visceral arch cartilages (Fig. 2C). In whole-mount immunostainings, GFP-positive cells were found in close proximity to visceral arch myofibers, confirming the data from the sectioned material (Fig. 2D,E).

Extirpations

The effect of neural fold extirpations on the cranial cartilages of *Ambystoma mexicanum* was described by Hörstadius and Sellman (1946). Our data are basically identical to those of Hörstadius and Sellman and were a control to test if the extirpations were successful (Table 1). The results of extirpations on muscle development varied between batches of embryos. The greatest variation in muscle defects was found among those embryos that took a longer time to heal the extirpation wound. In this group, the healed wounds also often contained clusters of undifferentiated cells, blocking normal morphogenesis. Embryos showing

this kind of malformation were omitted from the study (approximately 10% of the surviving embryos).

Mandibular stream.

Mandibular arch neural crest extirpations resulted in gaps in Meckel's cartilage and a distorted palatoquadrate. The mandibular arch muscles showed a wide range of malformations, from only slightly smaller levators to complete disarray, compared with the unextirpated embryos (Fig. 3). They did appear, however, generally in their normal positions. The levator mandibulae externus and longus, which normally develop in close proximity to each other, were now often further apart. In many cases, they extended not diagonally and rostroventrally but in the horizontal plane. They also showed a frayed appearance (Fig. 4). In some cases, one of the muscles extended all the way to the depressor mandibulae muscle. The levator mandibulae articularis muscle was not identified in any of the extirpated embryos, probably owing to its close proximity to the levator mandibulae externus. However, it is possible that this is one of the muscles described as ectopic in some cases. The intermandibularis posterior often had a frayed appearance and sometimes extended dorsally to the levator muscles (Fig. 4). In the most extreme cases, ectopic muscles appeared along the origin of intermandibularis posterior. This muscle was also affected on the nonextirpated side, especially along the insertion at the ventral midline. The intermandibularis anterior was intact but had shifted its position to be entirely on the extirpated side of the embryo (Fig. 4). Hyoid and branchial arch muscles were unaffected.

Hyoid stream.

The hyoid arch neural crest extirpations affected the formation of the ceratohyal, which was missing its proximal end. The distal end was often attached to the caudal end of Meckel's cartilage. The hypohyal was completely missing. The muscles of the hyoid arch had several deformities, but just like the mandibular and branchial arch muscles, they appeared in or close to their normal positions. Malformations were most common in the depressor mandibulae muscles (70 embryos), ranging from only shorter length to fusion with the interhyoideus (Fig. 5) or extension to levator mandibulae longus. The branchiohyoideus externus had similar malformations, ranging from shorter length to fusion with the interhyoideus posterior (Fig. 5). When fusing with interhyoideus posterior, the branchiohyoideus externus was very small and difficult to distinguish. Apart from common fusions with other hyoid arch muscles, the interhyoideus posterior was not much affected by the extirpations. However, in two cases, the origin of interhyoideus posterior had extended caudally, to a position lateral to the fourth ceratobranchial (Fig. 5D). The only malformation observed in the interhyoideus muscle was a shift in origin from the ceratohyal to, when present, the ceratohyal fragment on the caudal end of Meckel's cartilage.

Branchial stream.

In most cases (52 embryos), the branchial neural crest extirpations led to the absence of all ceratobranchials and hypobranchials. No external gills were formed on the extirpated side in the most severely affected embryos. Most muscles of the branchial arches formed in their normal positions, but the levator and depressor branchiarum had anastomosed with each other when no external gills were present (Fig. 6). The levator arcuum branchiarum were smaller than on the unextirpated side. Most of the subarcualis rectus muscles were frayed, had switched to a more dorsal position, or were missing altogether (Fig. 6). The branchial arch extirpations also affected some of the hyoid arch muscles. The branchiohyoideus externus was in many cases much smaller than normal and had shifted its origin to the ceratohyal. Interhyoideus posterior was shorter and ended more ventrally than in the unextirpated embryos. The other hyoid arch muscles as well as all mandibular arch muscles were unaffected.

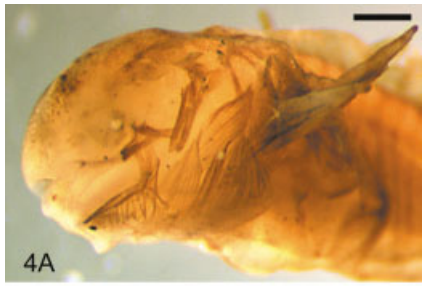
Controls.

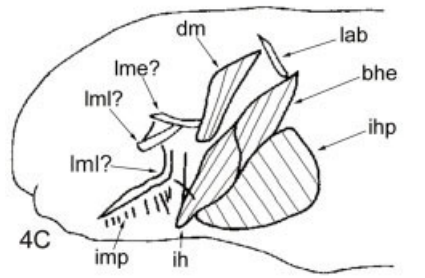
Several controls were made to test the accuracy of the extirpations. To find out if we managed to extirpate the majority of migrating neural crest, we extirpated the hyoid stream of DiI-injected embryos. No DiI-containing cells were observed migrating in the hyoid stream area upon extirpation (data not shown). Embryos in which only the ectoderm covering migrating neural crest cells was extirpated did not show any defects in either cartilage or muscles (data not shown). The internal control, the unextirpated right side of the embryo, was unaffected by the extirpations in all but a few cases. Cartilage on the unextirpated side was never affected, but in a few cases, the intermandibularis anterior muscle had shifted its position toward the extirpated side and the anterior part of intermandibularis posterior muscle did not reach the ventral midline.

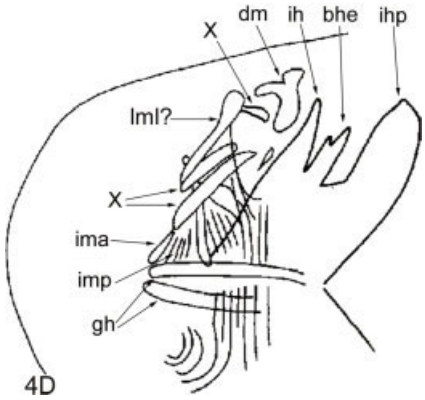
DISCUSSION

Fate of Cranial Neural Crest Cells

In addition to extirpations, we used two methods for tracing the fate of cranial neural crest cells directly: injection of the lipophilic dye DiI into premigratory cranial neural crest and transplantations of neural folds from a donor expressing green fluorescent protein (GFP) after injection of GFP mRNA. All three methods confirmed the neural crest derivation of most cranial cartilages, and both DiI- and GFP-labeled cells were found in connective tissues, between differentiating myofibers and visceral arch cartilages as well as in the external gills (Figs. 1, 2). As with earlier results in the domestic chicken (LeLièvre and LeDouarin, 1975; Noden, 1986; Couly et al., 1992; Köntges and Lumsden, 1996) and in the fire-bellied toad, *Bombina orientalis* (Olsson et al., 2001), the neural crest contribution was always localized to connective tissue components surrounding cranial muscle fibers and was not traced to contractile elements, viz., myofibers. The other study in an amphibian (Olsson et al., 2001), produced only indirect







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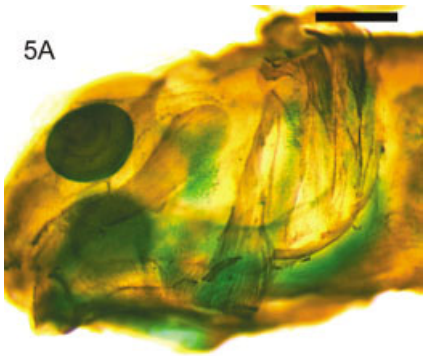

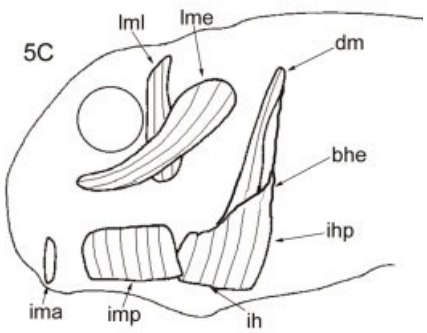

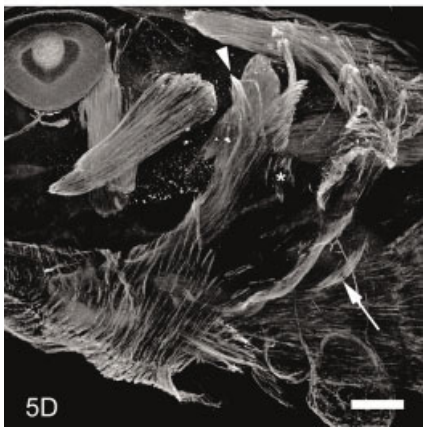

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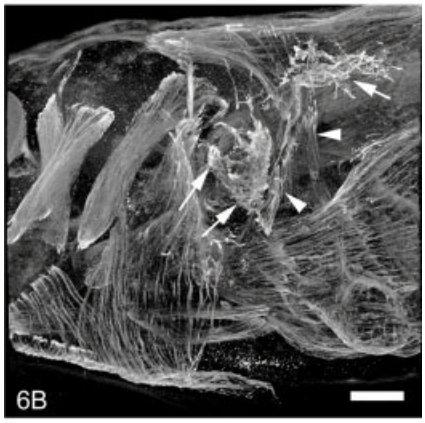

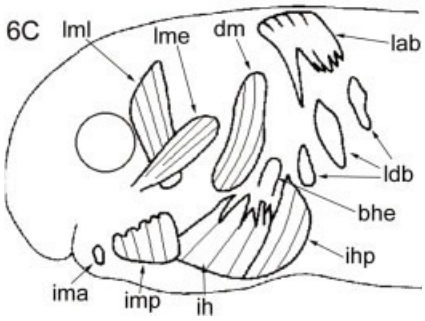

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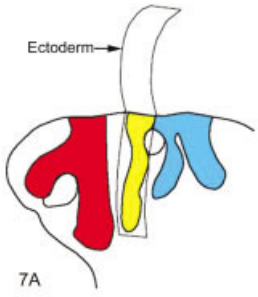

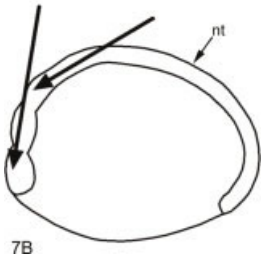

Fig. 7.

evidence for a neural crest contribution to gill skeletal and connective tissue elements, and recommended further studies on this topic in amphibians. Here, we present direct evidence for a neural crest contribution to these structures using both DiI and GFP labeling. Our results are very similar to those obtained by Olsson et al. (2001) in the fire-bellied toad. Extirpation of either the mandibular or the hyoid crest stream results in altered morphogenesis of muscles that vital labeling shows nor-

mally receive a contribution from that stream to their connective tissues. Muscles that do not normally receive a contribution from the extirpated stream are unaffected. The branchial stream extirpations also follow this pattern, with the added twist that the origin of the interhyoideus posterior and branchiohyoideus externus may be affected. Taken together, our data support a model in which the cells that form the connective tissue associated with cranial muscles are derived from the same visceral arch as the mesoderm cells that contribute the myofibers. Fidelity between individual branchial arch muscles and their connective tissue attachments is retained regardless of the segmental identity—or embryonic derivation—of associated skeletal components. This finding has been found also in anurans (Olsson et al., 2001), and a similar, more thoroughly investigated case has been made for chickens (Köntges and Lumsden, 1996). Studies in representatives of other vertebrate classes are needed to establish if this is a common theme in vertebrate development or a later innovation typical only for tetrapods.

Cranial Neural Crest and Positioning and Morphogenesis of Cranial Muscles

The mandibular arch extirpations resulted in a wide range of malformations. The most common are horizontal orientation of the levator muscles and their frayed appearance. In the hyoid arch, malformations range from only slight distortions of the depressor mandibulae to malformations in all hyoid muscles. The insertion of both depressor mandibulae and branchiohyoideus externus are more severely affected than their origins. The hyoid arch muscles are the only ones affected by extirpations of neural crest cells from another arch. Both interhyoideus posterior and branchiohyoideus externus normally have their origin on the first ceratobranchial. When it is missing, these muscles either attach to the ceratohyal or end blindly. This finding is also the only time when the origins of these muscles are affected. In the hyoid stream neural crest extirpations, the origins of both branchiohy-

Fig. 4. **A:** View of stage 44 embryo, subjected to mandibular arch neural crest extirpation. Muscles are stained with the 12/101 antibody and visualized with a horseradish peroxidase–conjugated secondary lateral antibody and diaminobenzidine, cartilage with Alcian blue. The muscles of the hyoid and branchial arches are normal. The levator mandibulae longus is almost horizontal, extending rostrally from its origin, with a branch extending ventrally. The levator mandibulae externus is extending caudally to the level of depressor mandibulae. Both muscles are smaller than normal. **B:** Ventral view of A. The intermandibularis anterior has shifted position to the extirpated side. The intermandibularis posterior has a frayed appearance on both sides of the ventral midline. A possible ventral extension of levator mandibulae externus has appeared. Several new ectopic muscles have appeared where the intermandibularis normally has its origin. **C:** Schematic view of the mandibular and hyoid muscles of A. **D:** Schematic view of the mandibular and hyoid muscles of B. bhe, branchiohyoideus externus; dm, depressor mandibulae; gh, geniohyoideus (not a visceral arch muscle); ih, interhyoideus; ihp, interhyoideus posterior; ima, intermandibularis anterior; imp, intermandibularis posterior; lab, levator arcus branchiarum; lme, levator mandibulae externus; lml, levator mandibulae longus; ?, indicates uncertainty of the identity; X, ectopic muscle. Scale bars = 500 μm in A,B.

Fig. 5. **A:** Lateral view of a stage 44 embryo, subjected to hyoid neural crest extirpation. Muscle are stained with the 12/101 antibody and visualized with a horseradish peroxidase–conjugated secondary antibody and diaminobenzidine. All muscles of the mandibular and branchial arches are normal in appearance. All of the hyoid muscles are affected. Depressor mandibulae has anastomosed with interhyoideus posterior, which has also anastomosed with the very small remnant of branchiohyoideus externus. The interhyoideus ends blindly. **B:** Lateral view of stage 43 embryo, stained for desmin and subjected to hyoid arch neural crest extirpation. Maximum projection of confocal image stack. The mandibular and branchial arch muscles are normal in appearance. Only the dorsal end of the depressor mandibulae is affected. The origin extends rostrally, medial to the levator mandibulae longus (arrow). A small extension anastomoses with branchiohyoideus externus (arrowhead). **C:** Schematic view of the mandibular and hyoid muscles of A. **D:** Lateral view of stage 43 embryo, stained for desmin, subjected to hyoid arch neural crest extirpation. Maximum projection of confocal image stack. The mandibular and branchial arch muscles are normal in appearance. The depressor mandibulae is short but in its normal position. The branchiohyoideus externus has acquired an extra origin, close to the origin of levator mandibulae externus (arrowhead). A small part of interhyoideus posterior is positioned in its normal origin (asterisk), whereas the major part extends caudally and ends blindly (arrow). bhe, branchiohyoideus externus; dm, depressor mandibulae; ih, interhyoideus; ihp, interhyoideus posterior; ima, intermandibularis anterior; imp, intermandibularis posterior; lme, levator mandibulae externus; lml, levator mandibulae longus. Scale bars = 500 μm in A, 250 μm in B, 250 μm in D.

Fig. 6. **A:** Lateral view of stage 44 embryo, subjected to branchial neural crest extirpation. Muscle are stained with the 12/101 antibody and visualized with a horseradish peroxidase–conjugated secondary antibody and diaminobenzidine. All muscles of the mandibular arch are normal in appearance. Of the hyoid arch muscles, the branchiohyoideus externus, is smaller than normal, and the interhyoideus posterior has split into an anterior and a posterior end with the branchiohyoideus externus in between. The branchial arch muscles show a wide range of malformations. However, they all appear in approximately the right position. The levator arcus branchiarum all attach to their origin at the otic capsule. They extend toward the nonexisting ceratobranchials, and either end blindly or anastomose with the levator branchiarum. The levator and depressor branchiarum are found in their normal positions but have anastomosed with each other and are very small. The ventral parts end blindly and the dorsal parts either end blindly or anastomose with levator arcus branchiarum. **B:** Lateral view of stage 44 embryo, stained for desmin, subjected to branchial arch neural crest extirpation. Maximum projection of confocal image stack. The mandibular and hyoid arch muscles are normal in appearance. The levator arcus branchiarum extend to new insertions medial to the levator and depressor branchiarum which are only tufts of fibers (arrows) anastomosed to each other. The subarcualis rectus muscles have shifted to a position dorsal to the pharynx and extend medially (arrowheads). **C:** Schematic view of A. bhe, branchiohyoideus externus; dm, depressor mandibulae; ih, interhyoideus; ihp, interhyoideus posterior; ima, intermandibularis anterior; imp, intermandibularis posterior; lab, levator arcus branchiarum; ldb, levator and depressor branchiarum; lme, levator mandibulae externus; lml, levator mandibulae longus. Scale bars = 500 μm in A, 250 μm in B.

Fig. 7. **A:** Schematic view of cranial neural crest streams in the Mexican axolotl. From left to right: red denotes the mandibular, yellow the hyoid, and blue the branchial streams. The extirpations were performed by removing the ectoderm covering the migrating neural crest cells and then the crest cells themselves. **B:** Schematic view showing how the DiI-injections were made in a stage 19 axolotl embryo. The entire cranial neural crest was tagged in two injections. nt, neural tube.

oideus externus and interhyoideus posterior are still close to the first ceratobranchial. Upon extirpation of branchial stream neural crest, the levator and depressor branchiarum form in their normal positions, even though the entire branchial basket is missing. The formation of the external gills seems to be dependent upon neural crest cells, because they do not form when the branchial stream neural crest is extirpated. The subarcualis rectus muscles are more severely affected by the lack of neural crest cells, as in many cases they do not form at all. As already shown in an earlier study by Ericsson and Olsson (2004), the anlagen of the visceral arch muscles appear close to their future origins. From the anlagen, the muscles slowly extend to the insertions. Our DiI and GFP data show that there is a sheet of neural crest cells covering the muscle anlagen and continuing to the anlagen of the visceral arch cartilages. We suggest that these neural crest cells act as a guide for the developing muscles to find their primary insertions. The result of extirpating neural crest cells is that the muscles appear close to their origins and then, when starting to extend and develop fibers, we suggest that, because there is nothing to guide them to their normal insertions, they may extend in other directions. The lack of connective tissue surrounding the myofibers will also cause them to split easily. Therefore, although the positioning of cranial muscles is not dependent on the cranial neural crest, the proper morphogenesis is. This finding is in line with the study by Noden (1993), wherein he notes that the position of some extraocular muscles can be specified before contact with neural crest cells. If the visceral arch muscles are going to attach to cartilages that are not derived from the same arch as the muscles, the muscles will have to form in a primary position and then gradually shift their attachment sites to the final origin and insertion. We interpret this finding as an effect of the absence of mixing between neural crest cells of different visceral arches leading to the pattern described by Köntges and Lumsden (1996), where the attachment points of visceral arch muscles originate from neural crest cells of the same arch. The results of this study are very similar to those of E. K. Hall (Hall, 1950). Hall performed bilateral extirpations of neural folds on neurula stage *Ambystoma maculatum* (then called *Ambystoma punctatum*) embryos and documented malformations similar to those seen in the present study. Hall observed that the levator and depressor muscles were normal at their origin and increasingly malformed toward the insertion. He also saw a connection between malformations in the palatoquadrate and the levator muscles. Lacking only Meckel's cartilage was not enough to produce malformed levator muscles. Malformed muscles were only produced when the palatoquadrate was affected by his extirpations. The palatoquadrate is positioned alongside the path on which the levator muscles are extending to reach their insertions on Meckel's cartilage. This finding indicates that the levator muscles are following a path, rather than responding to an attractant produced at the insertion. Several fate maps in amphibians by Hörstadius and Sellman (1946), Chibon (1967), Sadaghiani and Thiébaud (1987), and Olsson and Hanken (1996), in chicken by LeLièvre and LeDouarin (1975), Noden (1983a), Couly et al. (1992), and Hacker and Guthrie (1998), and in mouse by Trainor and Tam (1995) and by Trainor et al. (1994) have shown the regional specificity of the neural crest cells. Köntges and Lumsden (1996) showed that this regional specificity is shared by the cranial nerves and the mesoderm. In the chicken, muscles originating from the mesoderm of one visceral arch attach to cartilage derived from neural crest cells of the same arch and are innervated by cranial nerves from the corresponding rostrocaudal level (Köntges and Lumsden, 1996). The neural crest could then be considered to be the carrier of the pattern information from the hindbrain to the rest of the tissues in the head during development. Overexpression of *Hoxa2* in the head region leads to homeotic transformation of mandibular arch cartilages into hyoid arch cartilages in both chicken and *Xenopus* (Grammatopoulos et al., 2000; Pasqualetti et al., 2000). The mandibular arch muscles in these embryos were distorted and in some cases even completely lost. Malformations were also observed in the branchial muscles (Pasqualetti et al., 2000). Overexpression in the neural crest cells or mesoderm alone was not enough to cause homeotic transformations, indicating other sources of signaling. The expression of specific Hox genes does not seem to be fixed in migratory neural crest cells but is dependent on the environment. Trainor and Krumlauf (2000) showed in several experiments that transposing migrating neural crest cells from one visceral arch to another, in many cases caused a switch in Hox gene expression to that of their new neighboring cells. In recent studies (Couly et al., 2002; Ruhin et al., 2003), pieces of endoderm were rotated in chicken embryos, which caused the visceral arch cartilages to form with the same degree of rotation. Unfortunately, the state of the visceral arch muscles was not included in these studies, but it is likely that the mesoderm will respond in a similar way to endodermal modifications. Apart from possible signals from the neural crest and endoderm governing visceral arch muscle development and identity, the mesoderm itself may hold some clues. A study in mouse showed that the development of the masticatory muscles of the mandibular arch, specifically, is controlled by two genes: *MyoR* and *capsulin* (Lu et al., 2002). No other muscles were affected by knocking out these two genes, neither in the head nor in the trunk. The two genes encode transcription factors involved in muscle differentiation. The knock-out showed that the myocytes had a normal migration into the mandibular arch, but failed to differentiate and instead underwent apoptosis. Thus, specific developmental programs exist for at least some cranial muscles, and it is an important task for future studies to find the mechanisms responsible for the identity and correct morphogenesis of muscles in other visceral arches.

In conclusion, we show that, in the Mexican axolotl, as in other tetrapods, there is a contribution from the cranial neural crest not only to the cartilages of the larval skull but also to the connective tissues, which surround muscle fibers and form the attachment points to the skeleton. Our data indicate that the pattern observed also in anurans and in birds, where the neural crest cells associated with myofibers from a certain visceral arch originate from the same arch as the mesoderm cells in the muscles, is independent of the origin of the skeletal elements to which the muscles attach. The extirpation experiments show that visceral arch muscles form close to their origins in the absence of neural crest cells but fail to extend toward their normal insertions. Thus, the cranial neural crest has an important role, not so much for the early positioning of muscle anlagen, but for the proper morphogenesis of the muscles later in development. It is also clear that other tissues, for example the endoderm, must be important for laying down the correct architecture of the head in vertebrate embryos.

EXPERIMENTAL PROCEDURES

Wild-type embryos of the Mexican axolotl, *Ambystoma mexicanum* were obtained from the Axolotl colony, Indiana University (Bloomington, IL) or the colony at the Institut für Anatomie, Technische Universität (Dresden, Germany). The embryos were reared by using standard procedures and were dejellied manually with watchmaker's forceps. Stages were determined according to Bordzilovskaya et al. (1989).

Individual neural crest streams were extirpated when the cranial neural crest cells are migrating ventrally in streams (Fig. 7A). Embryos were placed in trenches cut in 2% agar-coated Petri dishes, which were filled with Steinberg's solution (Steinberg, 1957) plus antibiotic (50 mg of gentamicin sulfate per liter; Sigma G-1264). Surgery was performed using watchmaker's forceps and tungsten needles. The mandibular stream was extirpated at stages 19 to 22. However, this timing resulted in complete regeneration and, therefore, the neural crest of the mandibular area was removed at stages 19 to 20. The hyoid and branchial neural crest cell extirpations were performed at stages 23 to 24. The covering ectoderm was removed together with the neural crest cells. In some embryos, as a control, only the ectoderm covering the migrating neural crest cells was removed. The embryos were then reared in Steinberg's solution to stage 44–46, when they were killed by using 3-aminobenzoic acid ethyl ester (MS222, Sigma) and then fixed in Dent's fix (1 part dimethyl sulfoxide (DMSO) and 4 parts methanol; Dent et al., 1989). For bleaching and removal of endogenous peroxidase activity, the embryos were incubated in Dent's bleach (1 part 30% H_2O_2 and 2 parts Dent's fix; Dent et al., 1989). A total of 280 embryos were operated upon. Each type of extirpation was performed on 100 (hyoid and branchial) or 50 (mandibular) embryos, and 30 embryos served as controls. A total of 229 crest-extirpated embryos (45 mandibular, 96 hyoid, and 88 branchial), and 30 controls survived and were preserved as described above.

Immunohistochemistry

Whole-mount immunohistochemistry was performed according to Klymkowsky and Hanken (1991). Embryos were washed in "saline cocktail" (phosphate-buffered saline (PBS), 0.4% Triton X-100) and incubated with primary antibodies overnight in "BSA cocktail" (PBS, 0.4% Triton X-100, 1% bovine serum albumin (BSA), 5% DMSO). The embryos were then washed 5 × 30 min in BSA cocktail before overnight incubation with the secondary antibody. For visualization of muscles, an antibody against desmin (Monosan, PS031; Fuerst et al., 1989; Lin et al., 1994; Capetanaki et al., 1997; Capetanaki and Milner, 1998; Hacker and Guthrie, 1998) or myosin (12/101, Developmental Studies Hybridoma Bank; Schlosser and Roth, 1997a,b) was used together with either immunofluorescence (secondary antibodies with Alexa 488, Molecular Probes) or horseradish peroxidase–conjugated secondary antibodies and diaminobenzidine (DAB). To visualize cartilage, Alcian blue or type II collagen antibodies (II-II6B3 from the Developmental Studies Hybridoma Bank) and Alexa 568 secondaries (Molecular Probes) were used. The embryos stained with DAB and Alcian blue were cleared in glycerol and studied with a Leica stereomicroscope. Images were acquired by using a Leica DC100 digital camera. The embryos stained with Alexa antibodies were cleared in BABB (1 part benzyl alcohol, 2 parts benzyl benzoate; Dent et al., 1989) and studied with a Leica confocal scanning laser microscope (CLSM). Optical sections were taken at an interval of 3 to 7 microns, depending on the magnification and size of the embryo and combined into stacks of images. The image stacks were postprocessed with ImageJ and Photoshop. Maximum projections of the stacks were used to create two- and three-dimensional images of the embryos. In the maximum projection of the stacks, the brightest points of all images in the stack were collapsed into one image.

DiI Injections

Dejellied and decapsulated embryos were immobilized in shallow trenches cut into 2% agar gelled at the bottom of Petri dishes. A 0.5% stock solution of the lipophilic dye CM-DiI (Molecular Probes) was prepared in 100% ethanol and stored at 4°C. Immediately before injection, it was diluted in 0.3 M sucrose to a working concentration of 0.1%. An Inject+Matic microinjector was used to inject DiI in the entire neural crest of the mesencephalon and rhombencephalon. Instead of the conventional method of focal point injection, we penetrated the ectoderm and pushed the syringe underneath it, on top of the neural crest, from approximately the midbrain–hindbrain junction to the level of the optic placode (Fig. 7B). The DiI was injected when pulling out the syringe. The procedure was repeated further caudally to inject the hindbrain neural crest. A total of 60 embryos were injected at stages 19–20 on the left side. All injections were made by hand. After reaching the

proper stage, 37 embryos were killed with MS222 (Sigma), mounted in 2% agarose, and Vibratome-sectioned. The sections were counterstained with fibronectin antibodies and fluorescein isothiocyanate–coupled secondaries. To examine the accuracy of the extirpations, some of the DiI-injected embryos were extirpated in the same way as the uninjected embryos. The development to stage 40 was documented using a Leica MZ-FLIII stereomicroscope with epifluorescence and a Leica DC100 digital camera. Images were postprocessed by using Photoshop.

GFP mRNA Injections

Myc-tagged GFP mRNA was injected into dejellied one- or two-cell stage embryos with an Inject+Matic microinjector. Neural folds of 18 embryos expressing GFP at stage 16 were transplanted into noninjected hosts with tungsten needles. The embryos were then reared in Steinberg's solution (Steinberg, 1957) plus antibiotic until they had reached the required stage. Some embryos were Vibratome-sectioned at 100 μm and subsequently stained for fibronectin. Images were acquired with an Olympus BH-2 microscope and a SPOT RT (Diagnostic Instruments, Inc.) digital camera. The rest of the embryos were stained for c-myc by using the 9E10 antibody (Developmental Studies Hybridoma Bank) and for desmin (Monosan, PS031). After bleaching in 10% H_2O_2, secondary antibodies coupled to ALEXA 488 or 568 were added. The embryos were then dehydrated and transferred into BABB. A Leica confocal scanning microscope was used to acquire image stacks. The stacks were postprocessed with ImageJ and Photoshop.

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