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Cranial Neural Crest Cells Contribute to Connective Tissue in Cranial Muscles in the Anuran Amphibian, *Bombina orientalis*

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The contribution of cranial neural crest cells to the development and patterning of cranial muscles in amphibians was investigated in the phylogenetically basal and morphologically generalized frog, *Bombina orientalis*. Experimental methods included fluorescent marking of premigratory cranial neural crest and extirpation of individual migratory streams. Neural crest cells contributed to the connective tissue component, but not the myofibers, of many larval muscles within the first two branchial arches (mandibular and hyoid), and complex changes in muscle patterning followed neural crest extirpation. Connective tissue components of individual muscles of either arch originate from the particular crest migratory stream that is associated with that arch, and this relationship is maintained regardless of the segmental identity—or embryonic derivation—of associated skeletal components. These developmental relations define a pattern of segmentation in the head of larval anurans that is similar to that previously described in the domestic chicken, the only vertebrate that has been thoroughly investigated in this respect. The fundamental role of the neural crest in patterning skeleton *and musculature* may represent a primitive feature of cranial development in vertebrates. Moreover, the corresponding developmental processes and cell fates appear to be conserved even when major evolutionary innovations—such as the novel cartilages and muscles of anuran larvae—result in major differences in cranial form.

Key Words: **neural crest; cell migration; cell fate; *Bombina*; extirpation; vital dye labeling; cranial muscles.**

INTRODUCTION

The neural crest is a distinct population of embryonic cells that is unique to vertebrates. It gives rise to a large and diverse array of adult tissues, including nerves, pigment cells, and much of the cranial skeleton (Hall, 1999). Many neural crest derivatives were first reported as the results of embryological studies conducted during the late 19th and early 20th centuries, yet several important features have been described only recently. The full extent of neural crest contribution to the vertebrate body is still to be defined.

A direct role of neural crest in cranial muscle development was first reported by Le Lièvre and Le Douarin (1975), and later by Noden (1983a,b) and Couly *et al.* (1992), who described the neural crest derivation of connective tissue components of several branchial arch muscles in quail–chick chimeras. This role is now known to be only one component of a 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 (Köntges and Lumsden, 1996; Graham *et al.*, 1996; Schilling, 1997; Schilling and Kimmel, 1997). One consequence of these relations is that the connective tissue components of a given muscle and its corresponding connective tissue (skeletal) attachment site(s) typically are

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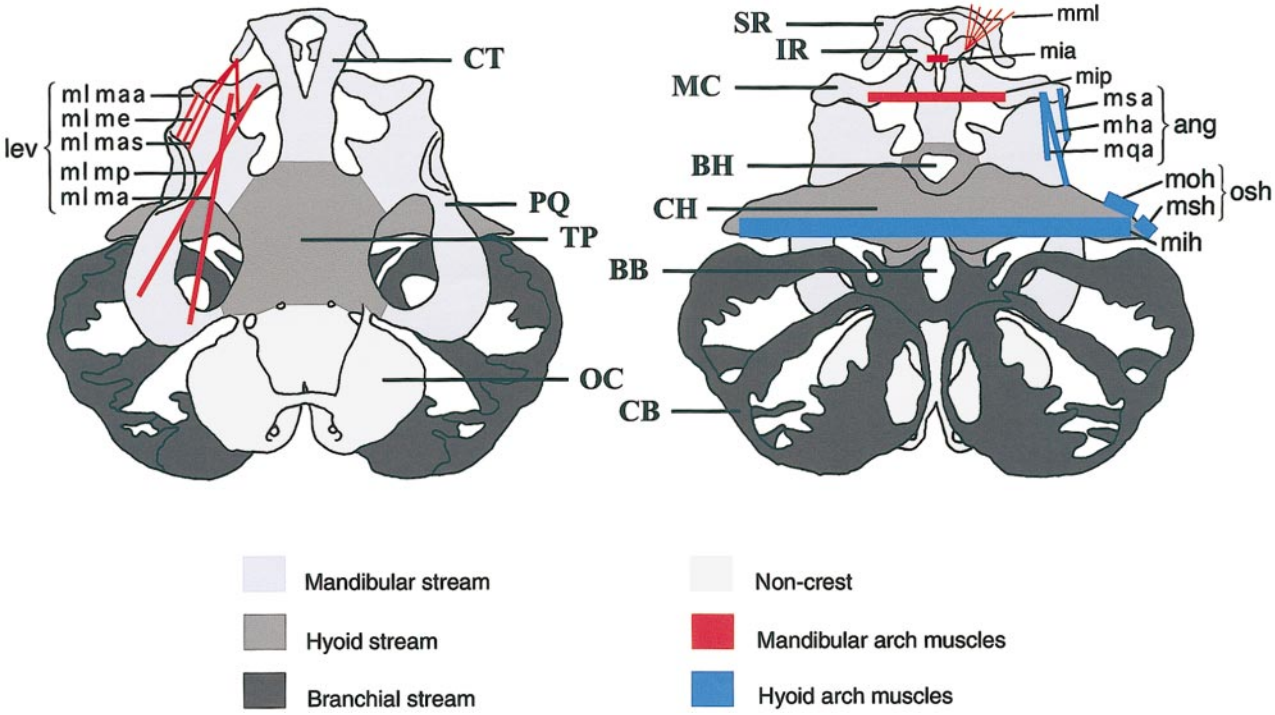

FIG. 1. Larval skull and cranial musculature of *Bombina orientalis*, depicted in dorsal (left) and ventral views. Neural crest-derived cartilages are shaded according to the migratory stream from which they originate (redrawn from Olsson and Hanken, 1996): light gray, mandibular stream; medium gray, hyoid stream; dark gray, branchial stream. The few noncrest-derived cartilages are lightly shaded. Cranial muscles are depicted schematically; only muscles of interest for the present study are shown. Mandibular (first) arch muscles are red, hyoid (second) arch muscles are blue. Paired muscles are depicted on one side only. Cartilages: BB, basibranchial; BH, basihyal; CB, ceratobranchials I–IV; CH, ceratohyal; CT, cornua trabecula (trabecular horn); IR, infrarostral; MC, Meckel's; OC, otic capsule; PQ, palatoquadrate; SR, suprarostral; TP, trabecular plate. Muscles: lev, levator mandibulae group—mlma, levator mandibulae anterior; mlmaa, levator mandibulae anterior articularis; mlmas, levator mandibulae anterior subexternus; mlmp, levator mandibulae posterior (comprising two parts; superficialis and profundus); ang, angularis group—mha, hyoangularis; mqa, quadratoangularis; msa, suspensorioangularis; hyoideus group—mih, interhyoideus; moh, orbitohyoideus; msh, suspensoriohyoideus; osh, orbito- and suspensoriohyoideus; others—mia, intermandibularis anterior; mip, intermandibularis posterior; mml, mandibulolabialis. Anatomical nomenclature follows Cannatella (1999).

derived from the same migratory crest stream. It also leads to segmental boundaries that may lie within individual skeletal or connective tissue elements, instead of coinciding with discrete anatomical boundaries. In chickens, for example, distal portions of the lower jaw are derived from the mandibular (first arch) neural crest stream, whereas the most proximal portion of the jaw is derived from the hyoid (second arch) crest stream (Köntges and Lumsden, 1996).

Much of the classical research assessing the embryonic origins of cranial tissues in vertebrates, including derivatives of the neural crest, is based on amphibians (reviewed in De Beer, 1937; Edgeworth, 1935; Goodrich, 1930; Hall and Hörstadius, 1988; Holtfreter, 1968). These early studies precisely and accurately define the extensive contribution of neural crest to many cranial tissues in both frogs and salamanders (e.g., cranial cartilages; Stone, 1926, 1929), yet none report a direct contribution of neural crest to cranial muscle in any species. This fact, in light of the recent studies in birds summarized above, immediately raises the possibility of variation among the major living groups of vertebrates with respect to the role of neural crest in cranial derivation and patterning.

At the same time, results of two separate studies provide evidence that neural crest might contribute to cranial muscle development in amphibians in a manner similar to that seen in amniotes, but that this important feature went undetected by the classical embryologists and anatomists. These results are rarely, if ever, cited in contemporary studies. In the axolotl, cranial muscle patterning is severely affected by neural crest extirpation and transplantation, and these effects cannot be explained simply as indirect consequences of altered skeletal morphology (Hall, 1950). Ablation of the hyoid crest stream, for example, severely disrupts patterning of the depressor mandibulae muscle, even though the (first arch) cartilages to which this muscle would normally attach are intact. These effects are exactly

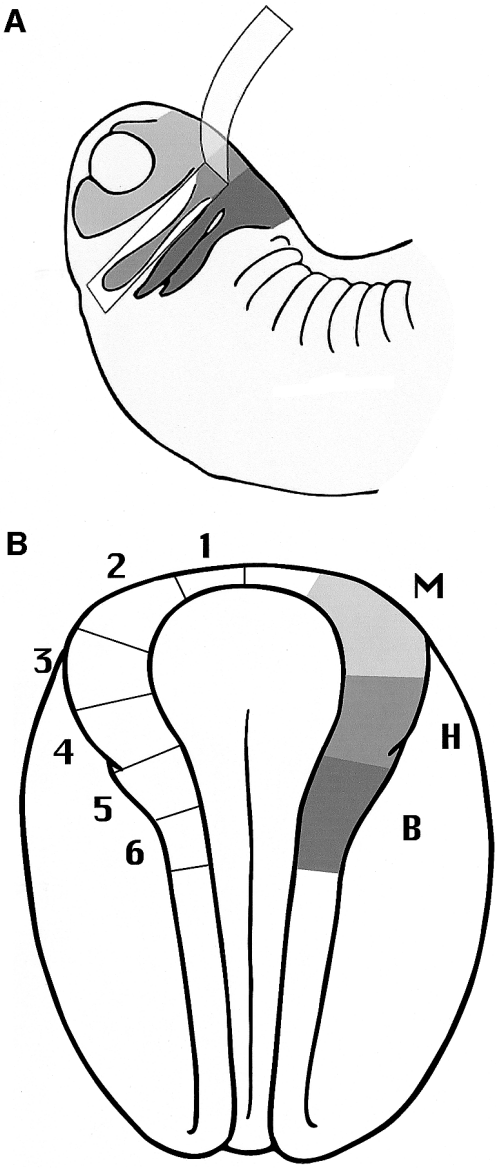

FIG. 2. (A) A late stage-16 *B. orientalis* embryo showing the extirpation technique. A flap of epidermis overlying the stream to be extirpated (here the hyoid stream) is folded up and the underlying neural crest stream removed using tungsten needles. Lateral view; anterior to the left. (B) Stage-14 (neurula) embryo of *B. orientalis*. The left side depicts six regions of the cranial neural fold; regions 2–6 were injected with DiI. The right side depicts the approximate origins of neural crest cells that contribute to the three cranial migratory streams—mandibular (M), hyoid (H), and branchial (B). Dorsal view; anterior at top.

what one would expect if neural crest is mediating cranial muscle patterning directly. In chimaeric *Xenopus* larvae that developed from embryos that received heterospecific grafts of cranial neural crest, labeled (crest-derived) cells were observed within several cranial muscles (Sadaghiani and Thiébaud, 1987). Thus, the possibility of a neural crest contribution to connective tissue components of cranial musculature in living amphibians remains unresolved and worthy of investigation. Anuran amphibians are especially interesting in this regard because of the extremely specialized larval head, which includes a unique, complex feeding apparatus comprised of novel upper and lower jaw cartilages and muscle configurations found in no other vertebrates (Cannatella, 1999; Hanken, 1999; Haas, 2001).

The primary goals of the present study are to determine: (1) whether neural crest contributes to the development of connective tissue components of larval cranial musculature in amphibians, and (2) if it does, whether the pattern of crest contribution to branchial arch musculoskeletal and connective tissues reveals the same segmental relations described in amniotes (chicken). We focus on mandibular and hyoid arch components in larval anurans because of their importance to cranial function and evolution (Cannatella, 1999; De Jongh, 1968; Hanken *et al.*, 1997), because their post-hatching (metamorphic) development is relatively well known (Alley, 1989; Alley and Omerza, 1999), and because of novel musculoskeletal configurations that offer convenient means of assessing underlying developmental relations. We employ two different experimental methods, neural crest ablation and fate mapping with a vital dye. These experiments provide direct evidence of neural crest contribution to connective tissue components of branchial arch muscles in amphibians, including the connective tissue attachments to cranial cartilages. They also implicate a prominent role for neural crest in cranial muscle patterning. Furthermore, as in amniotes, the connective tissue components of individual muscles and their associated connective tissue (skeletal) attachments are derived from the same neural crest migratory stream, which need not be the same crest stream that gives rise to the corresponding skeletal component (cartilage). This suggests that these relations are an evolutionarily primitive and conserved feature of cranial development in vertebrates.

MATERIALS AND METHODS

Choice of Species

We assessed neural crest contribution to cranial musculature in the fire-bellied toad, *Bombina orientalis*. This is a phylogenetically basal taxon among living frogs, and both adults and larvae have a relatively generalized morphology (Cannatella, 1999; Cannatella and de Sá, 1993; Fig. 1). Both features make it an especially attractive subject for investigation of presumed ancestral, or primitive, features of anurans. The neural crest contribution to the cartilaginous larval skull was evaluated in an earlier study (Olsson and Hanken, 1996).

Embryos

Eggs were obtained from laboratory matings among wild-caught adults (Charles D. Sullivan Co., Inc.), which were maintained as a

breeding colony at the University of Colorado. Breeding and husbandry followed established procedures (Carlson and Ellinger, 1980; Frost, 1982). Adults received dorsal subcutaneous injections of chorionic gonadotropin (Sigma, cat. no. CG-5) and were allowed to spawn overnight in the dark at room temperature. Fertilized eggs were reared in 10% Holtfreter solution (Hamburger, 1960) at 10–25°C. Embryos were dejellied either chemically (0.63 g cysteine HCl, 0.12 g NaCl, 24 ml H_2O, buffered to pH 8.0 with 5 N NaOH) or manually with watchmaker's forceps. Embryos and tadpoles were staged from external morphology according to the scheme of Gosner (1960), which defines a total of 46 stages from fertilization to metamorphosed froglet.

Surgical Procedures

Neural crest extirpations were performed at late stage 16 (neural tube, gill plates), when the cranial neural crest cells are migrating ventrally in four main streams (mandibular, hyoid, and two branchial, Fig. 2A). Embryos were placed in trenches cut in 2% agar-coated Petri dishes, which were filled with 10% Holtfreter solution plus antibiotic (50 mg gentamicin sulfate per liter; Sigma, cat. no. G-1264). Surgery was performed by using watchmaker's forceps and tungsten needles. A flap of ectoderm was prepared by cutting through three sides of a rectangular portion overlying the targeted neural crest stream and leaving the dorsal side intact. The flap was folded up dorsally, and the mandibular, hyoid, or branchial stream removed (Fig. 2A). In many anuran species, and especially *Bombina orientalis*, neural crest cells at this stage are more darkly pigmented than neighboring cells and are readily seen against the lighter, underlying tissues (Stone 1932; Olsson and Hanken, 1996). This makes possible complete removal of all crest cells, and only crest cells. Finally, the flap of ectoderm was pressed back into place and the extirpated embryos allowed to heal for 1–2 h before being transferred to agar-coated dishes filled with 10% Holtfreter solution plus antibiotic. Additional sham-operated embryos received the same surgery, except for removal of the neural crest, and served as a control for crest extirpation. All operations were unilateral, usually on the right side of the embryo; the opposite side was left intact and served as an additional control.

Embryos were reared until larval stage 26 or 27, killed in 30% aqueous chloretone (1,1,1-trichloro-2-methyl-2-propanol; Sigma, cat. no. T-5138), fixed for 2 h in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS), run through a graded methanol series (25, 50, and 75%; 5 min each), and fixed overnight or longer in Dent fixative [1 part dimethyl sulfoxide (DMSO): 4 parts methanol; Dent *et al.*, 1989]. After immersion in Dent bleach (1 part 30% hydrogen peroxide: 2 parts Dent fixative; Dent *et al.*, 1989) for 4 days to lighten skin pigmentation, specimens were stored in 100% methanol at −20°C.

Each type of extirpation (mandibular, hyoid, or branchial stream) was performed on 20 embryos, and 26 additional embryos served as controls. Thirty-four of 60 crest-extirpated embryos (9 mandibular, 11 hyoid, and 14 branchial) and 25 of 26 controls survived and were preserved as described above.

Cartilage and Muscle Double Staining

Preserved larvae were first stained for cartilage as whole mounts (Klymkowsky and Hanken, 1991). Specimens were immersed in Alcian blue solution [20 mg Alcian blue 8GX (C.I. 74240), 30 ml absolute ethanol, 70 ml glacial acetic acid] for about 3 h and returned to Dent fixative. The same specimens were then immunostained according to the technique of Klymkowsky and Hanken (1991), which was slightly modified to minimize nonspecific background staining, as follows. After rehydration in a graded methanol series, specimens were washed three times for 5 min in "saline cocktail" [0.1 M Niu-Twitty saline, 0.1 M phosphate buffer (K/Na; pH 7.4), 0.4% Triton X-100], preincubated for 1–2 h in "serum cocktail" (saline cocktail containing 5% newborn-calf serum, 5% DMSO, and 0.1% thimerosal), and incubated for 20–24 h with a monoclonal antibody for newt skeletal muscle (12/101; Kintner and Brockes, 1984) that was diluted 1:500 with serum cocktail. After six washes in serum cocktail (5 h total), specimens were incubated for 20–24 h in secondary antibody [horseradish peroxidase (HRP)-conjugated, goat anti-mouse IgG, diluted with serum cocktail at 1:1000]. Specimens were washed two times for 30 min and then overnight in serum cocktail, further washed three times for 1 h in saline cocktail, and then reacted for 1–2 h with 0.5 mg/ml diaminobenzidine (DAB) in saline cocktail containing 0.02% hydrogen peroxide. The reaction was stopped by dehydration with methanol two times for 5 min. Finally, larvae were cleared by stepwise transfer from 100% methanol to BABB (1 part benzyl alcohol: 2 parts benzyl benzoate) and examined with a Wild M5 dissecting microscope. The 12/101 monoclonal antibody cross-reacts widely with striated muscle from a wide variety of vertebrates (e.g., other amphibians, rodents and chicken; Klymkowsky and Hanken, 1991). It does not bind to smooth muscle, and binds only weakly to cardiac muscle. Monoclonal antibody 12/101, developed by Dr. J. P. Brockes, was obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the NICHD and is maintained by The University of Iowa, Department of Biological Sciences (Iowa City, IA).

Vital Labeling

Embryos were labeled with vital dye at stage 14 (neurula; Fig. 2B), immediately before the onset of cranial neural crest cell migration. 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 DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine, perchlorate; Molecular Probes, Inc., cat. no. D-282) was prepared in 100% ethanol and stored at 4°C. Immediately before injection, it was diluted in 0.3 M sucrose to working concentrations of 0.1 and 0.05%. Micropipets pulled from thin-walled, 1.2-mm diameter glass microfilaments were filled with dye and attached to a Picospritzer II (General Valve). Micropipet tips were broken to a diameter of ca. 20 μm. DiI was injected into any of five different sites in the left neural fold (Fig. 2B, sites 2–6) by inserting a micropipet and expelling a small amount of dye solution. Injections were made by hand or with a micromanipulator. The right side was not injected and served as a control. Following injection, embryos were reared individually in 24-well tissue culture plates in 10% Holtfreter solution plus antibiotic at 25°C.

All neural crest and presumptive neural tube cells within a given site appeared to be labeled by the injection procedure. A total of six sites were injected in an earlier study that assessed the chondrogenic fate of cranial neural crest cells in this species (Olsson and Hanken, 1996; Fig. 2B). Because the first site (transverse neural fold) did not produce neural crest, we injected only sites 2–6 in the present study. These five sites span all three cranial neural crest streams. Great care was taken to avoid labeling any mesoderm cells. Fortunately, in *Bombina orientalis* the unpigmented (white) mesoderm is readily distinguished from the pigmented (gray) cranial neural crest, and eggs and embryos are large (relative to

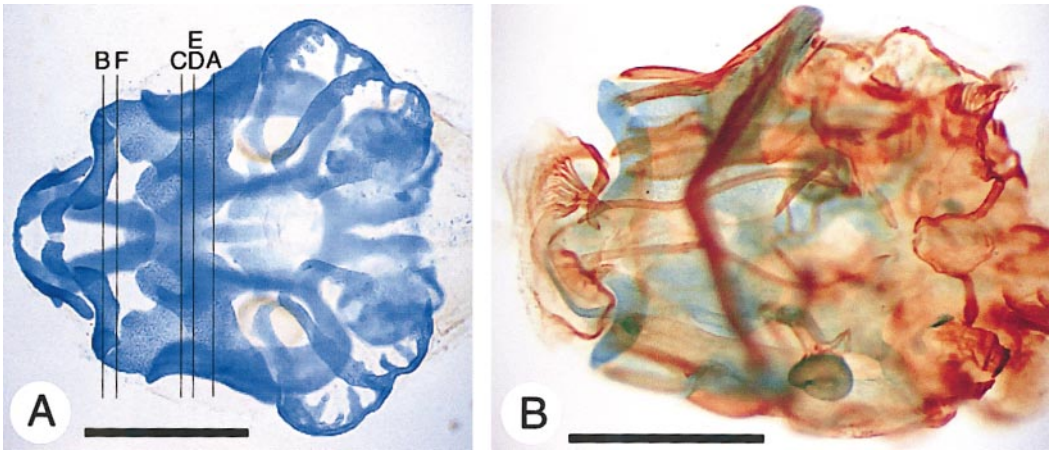

FIG. 3. Larval *Bombina orientalis* (stage 26). Control larvae showing (A) the normal configuration of cartilages, or (B) cartilages and muscles. The thin vertical lines in (A) depict the planes of section in Fig. 7. The scale bars equal 1.0 mm.

those of other anurans, e.g., *Xenopus laevis*). Also, cranial neural crest migration begins relatively early in *Bombina*, when neural folds and neural crest lie dorsomedial to, and distinct from, paraxial mesoderm (Olsson and Hanken, 1996). Thus, it is relatively easy to inject DiI into neural crest at this stage without accidentally labeling paraxial mesoderm.

Fluorescence Microscopy of Living Embryos and Cryostat Sections

For fluorescence imaging, living embryos were mounted temporarily in rectangles cut into gelled agar at the bottom of custom-made brass slides with coverslip floors (M. Klymkowsky, University of Colorado). Frozen sections of older specimens (stage 26) were prepared by immersing tadpoles in 30% aqueous chloretone and fixing them overnight or longer in 4% paraformaldehyde/0.25% glutaraldehyde in PBS at 4°C. Specimens then were washed thoroughly in PBS, soaked overnight in 15% aqueous sucrose, transferred through two changes of a solution of 15% sucrose/7.5% gelatin at 37°C for a total of at least 5 h, and embedded in fresh sucrose/gelatin solution at 4°C. Once set, the specimens were frozen and stored at −20°C. Sections (20 μm) were cut with a Leica CM 1800 cryostat. Live whole mounts and sections were viewed with a Leitz Dialux 20 or Leica DMRXE epifluorescence microscope equipped with a rhodamine (N2) filter block. Sections also were viewed with Nomarsky differential interference contrast (DIC) microscopy.

Documentation

Photographs and digital images were obtained with either a Wild MPS55 Photoautomat and Kodak T-MAX P3200 or Ektachrome P1600 film, or a Photonic Science Colour Coolview digital video camera. Images were analyzed and enhanced with Adobe Photoshop on Macintosh computers. Corresponding images obtained from fluorescence and DIC microscopy were combined to document the exact histological location of the fluorescent signal in a given section.

RESULTS

Musculoskeletal Patterning Following Neural Crest Ablation

The following sections report the results of embryonic neural crest ablation on larval musculoskeletal patterning in *Bombina orientalis*. Each set of experiments involved unilateral ablation of a single cranial migratory stream—mandibular, hyoid, or branchial (Fig. 2A). Ablated and control larvae were double-stained for cartilage and muscle with Alcian blue and a muscle-specific antibody, respectively. The cranial morphology of control specimens was normal and did not differ from that in intact, unoperated larvae (Figs. 1, 3A, and 3B).

Mandibular stream. Unilateral extirpation of the mandibular neural crest stream severely affected the morphology of first (mandibular) arch cartilages and muscles. Paired suprarostral, infrarostral, Meckel's, and palatoquadrate cartilages, as well as the trabecular horns, were all severely malformed or missing on the operated side in all specimens (Fig. 4A). There was no significant variation among cases. All muscles of the levator mandibulae group were similarly affected. In the absence of Meckel's cartilage, to which they normally insert, these muscles instead fused either with the second (hyoid) arch muscles of the angularis group (Fig. 4B, arrow) or with the m. mandibulolabialis (Fig. 4D, arrow). Ventrally, both the anterior and posterior intermandibularis muscles were severely malformed and lacked their normal attachments to the lower jaw (infrarostral and Meckel's cartilages, respectively). Instead, the muscles appeared to "float" freely or to possibly attach to the larval integument. Their configuration was highly variable among specimens.

In contrast, cartilages and muscles of the hyoid and more posterior branchial arches were largely unaffected by mandibular crest extirpation. The m. interhyoideus, which

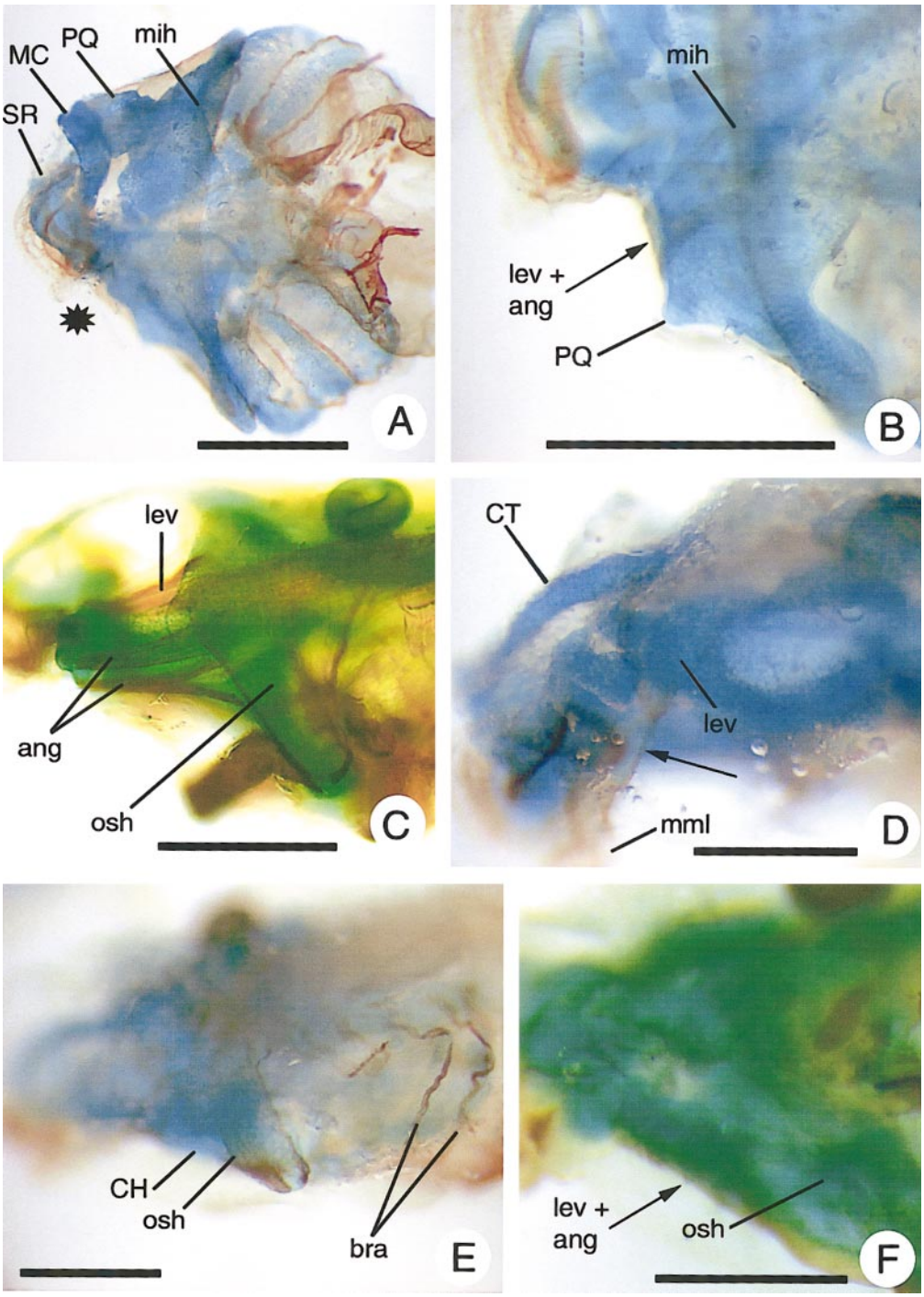

FIG. 4. Larval *Bombina orientalis* (stages 26 and 27) following ablation of the mandibular stream of cranial neural crest at embryonic stage 16. Specimens are prepared as stained whole mounts; cartilages are blue, muscles are dark brown. Anterior is always to the left. (A, B) Ventral views; (C–F) Lateral views. (A) Unilateral ablation results in loss or severe malformation of the palatoquadrate (PQ), Meckel's (MC), and suprarostral (SR) cartilages on the operated side (asterisk). All three cartilages are components of the first (mandibular) arch. The m. interhyoideus (mih), a second (hyoid) arch muscle, is unaffected. (B) In another specimen, seen at higher magnification, the levator mandibulae group (lev) of cranial muscles have fused with the angularis (ang) muscles (arrow) in the absence of Meckel's cartilage, to which they would normally attach. (C) A control larva depicting the normal configuration of levator mandibulae muscles (lev), which are components of the first (mandibular) arch. The angularis group muscles (ang) belong to the second (hyoid) arch, as do the orbitohyoideus and suspensoriohyoideus muscles (osh). (D) A novel muscle slip derived from the levator (lev) musculature (arrow) extends ventrally

connects paired ceratohyal cartilages, was normal (Figs. 4A and 4B). The m. orbitohyoideus and m. suspensoriohyoideus appeared largely intact, even though the palatoquadrate cartilage from which they both originate was severely malformed (cf. Figs. 4C and 4E). Muscles of the angularis group also were surprisingly normal in appearance, despite their frequent fusion with the levator mandibulae group (Fig. 4F, arrow; for control configuration, see Fig. 4C).

Hyoid stream. In all 11 cases, extirpation of the hyoid neural crest stream caused severe malformation of the ceratohyal cartilage and the cartilaginous trabecular plate, and of several of their associated muscles. Both cartilages were reduced on the operated side; only a small fragment remained of the ceratohyal, leaving a big gap in the ventral skeleton in this area (Fig. 5A, arrow). All three paired muscles of the angularis group retained their normal insertion on Meckel's cartilage, but their respective origins were abnormal: instead of originating from the ceratohyal (m. hyoangularis) or palatoquadrate (m. quadratoangularis and m. suspensorioangularis) cartilages, each muscle fused to the m. interhyoideus (Fig. 5D) and, occasionally, to the m. orbitohyoideus and m. suspensoriohyoideus. The m. interhyoideus normally attaches to the lateral end of each ceratohyal cartilage on either side of the head. In the absence of the ceratohyal on the operated side of treated embryos, the m. interhyoideus instead projected anteriorly to attach to Meckel's cartilage (Fig. 5C). The m. interhyoideus also frequently fused to both the m. orbitohyoideus and m. suspensoriohyoideus (Fig. 5B, arrow). Finally, the m. orbitohyoideus and m. suspensoriohyoideus retained their normal origin from the palatoquadrate cartilage. However, instead of inserting on the (now absent) ceratohyal cartilage, each muscle fused to the m. interhyoideus. Cartilages and muscles of the mandibular and posterior branchial arches were normal.

Branchial stream. Unilateral extirpation of the branchial neural crest stream led to absence of the entire cartilaginous branchial basket (arches 3–6) on the operated side (Fig. 6A, asterisk). Yet, despite the loss of their skeletal attachments, the corresponding branchial arch muscles were present and, although shrunken (Fig. 6B, asterisk), retained their basic segmental configuration (cf. Figs. 6C and 6D). The muscles appeared to originate from dorsal fascia, but there were no obvious insertions at the opposite ends. Mandibular and hyoid arch cartilages and muscles were intact (Figs. 6A and 6B).

DiI Fate Mapping

DiI injected into the left cranial neural fold of stage-14 (neurula) embryos bound to the cell membranes of neural crest cells, which emerged to form three migratory streams (Fig. 2B). Later, DiI was readily identified in cryostat sections of early larvae (stage 26), where it labeled several structures within the head. The pattern of neural crest contribution to cranial cartilages is identical to what we reported earlier (Olsson and Hanken, 1996) and is identical to that inferred from the results of ablation experiments described above. The following account, therefore, emphasizes results that pertain to the contribution of neural crest to muscle connective tissue. In Fig. 7, results are presented as DIC micrographs overlain by the DiI stain taken from fluorescence images of the same section. Whereas this imaging technique obviates the need to show both images side by side, the connective tissue morphology (which is clearly visible with DIC microscopy alone) is somewhat obscured by the DiI staining.

The mandibular neural crest stream originates from region 2 and the anterior portion of region 3 (Fig. 2B). In larvae, DiI-labeled cells from this stream were seen in the palatoquadrate and Meckel's cartilages (Figs. 7A–7D), two prominent skeletal components of the first (mandibular) arch. Moreover, neural-crest contributions to the attachment points of the levator mandibulae muscles were seen at both their origins from the palatoquadrate (Fig. 7A, arrow) and their insertions on Meckel's cartilage (Fig. 7B, arrows). Consistently, only cartilages derived from the mandibular neural crest stream and first (mandibular) arch muscles, such as the m. levatores mandibulae (Fig. 7D), ever contained DiI. Second (hyoid) arch muscles, e.g., m. orbitohyoideus (Figs. 7C and 7D), never displayed the marker. Not all mandibular muscles, however, were labeled. No neural crest contribution was detected in any attachment point of the m. intermandibularis anterior or posterior, two transverse, ventral muscles that attach exclusively to the mandibular skeleton (infrarostral and Meckel's cartilages, respectively). Also, DiI was never visible within the tiny attachment points of anterior slips of levator mandibulae muscles that insert on the suprarostral cartilage, or within connective tissue at the origin of the m. mandibulolabialis.

The hyoid neural crest stream originates from the posterior portion of region 3 and all of region 4 (Fig. 2B). DiI injected into these cells in embryos labeled connective

to fuse with the m. mandibulolabialis (mml). (This embryo was operated on the right side and the image has been reversed so that anterior is to the left.) (E) The operated (left) side of a larva, following unilateral ablation of the mandibular stream. Second (hyoid) arch muscles are unaffected by the treatment. Orbitohyoideus and suspensoriohyoideus muscles (osh), for example, retain their normal insertions on the ceratohyal cartilage (CH). Branchial muscles (bra) also are normally developed. (F) Operated (left) side of a larva following unilateral ablation of the mandibular stream. The angularis (ang) group muscles fuse with those of the levator mandibulae (lev) group (arrow) in the absence of Meckel's cartilage, to which they would normally attach. The scale bars in (A), (B), and (D) equal 1.0 mm, those in (C) and (E) are 0.5 mm, and the scale bar in (F) is 0.3 mm.

tissue in several cranial muscles in larvae. Connective tissue at both the origin (palatoquadrate) and insertion (ceratohyal) of the m. orbitohyoideus contained the marker, as did connective tissue surrounding the corresponding myofibers (Fig. 7E). Similar results were seen in the m. suspensoriohyoideus and m. interhyoideus (not illustrated). DiI was also detected within connective tissues at the origin and insertion of two of the three muscles of the angularis group, the m. hyoangularis and m. quadratoangularis (Fig. 7F). No label was detected within the m. suspensorioangularis, the third member of the angularis group, or in any first (mandibular) arch muscle.

DiI injected into neural fold regions 5 and 6 was carried within the branchial crest stream or in trunk neural crest cells. No contribution to any muscle attachment points or associated connective tissue components was seen following these injections.

DISCUSSION

Neural Crest Contribution to the Connective Tissue Component of Cranial Musculature

Our results provide both direct and indirect evidence that embryonic neural crest is important for the development of connective tissue components of cranial musculature in at least one species of amphibian, the fire-bellied toad, *Bombina orientalis*. Following injection of the lipophilic dye DiI into premigratory cranial neural crest, fluorescent label was readily localized in the connective tissue component of several cranial muscles and associated skeletal attachments of larvae prepared as frozen sections. These contributions were derived from two of the three principal migratory streams of cranial neural crest, and they included musculoskeletal components of both the first and second (mandibular and hyoid) branchial arches (Table 1). As with earlier results in the domestic chicken (Le Lièvre and Le Douarin, 1975; Noden, 1986; Couly *et al.*, 1992; Köntges and Lumsden, 1996), the neural crest contribution was always localized to connective tissue components within and adjacent to cranial muscles and was not traced to contractile elements, viz., myofibers. The corresponding pattern of neural crest contribution to cranial cartilages is concordant with earlier accounts for several anuran species, including *B. orientalis* (Olsson and Hanken 1996; Hanken, 1999; Fig. 1), as well as other vertebrates (reviewed in Hall, 1999 and LeDouarin and Kalcheim, 1999). This further validates the precision of our labeling procedure.

Extirpation of individual streams of migrating cranial neural crest cells resulted in predictable and complex changes in cranial muscle patterning. Such results must be interpreted cautiously because of the experimental artifacts that may follow neural crest ablation and because of the possibility of crest regeneration (Scherson *et al.*, 1993; Vaglia and Hall, 1999). Nevertheless, when combined with analyses that are less sensitive to such experimental artifacts, such as fate mapping using vital dyes, ablation may be valuable and informative. In this study, results following neural crest ablation are entirely concordant with those from vital labeling in terms of the evidence they provide of a neural crest contribution to connective tissue components of cranial musculature. Ablation of either the mandibular or hyoid crest stream results in altered patterning of muscles that vital labeling shows normally receive a contribution from that stream. Muscles that do not normally receive a contribution from the ablated stream are unaffected. Moreover, these effects on muscle patterning cannot be explained simply as artifacts of altered skeletal morphology following neural crest ablation, since in many instances of altered muscle patterning the associated skeleton is largely, if not completely, intact. Finally, both ablation and labeling data in *Bombina* are consistent with the hypothesis that neural crest is helping to mediate cranial muscle patterning (Noden, 1983a, 1986; Schilling and Kimmel, 1997), although such a patterning role in this or other species of amphibians awaits more explicit proof.

Evidence for neural crest contribution to muscle connective tissue is most compelling for the first two cranial migratory streams, but weaker for the third, or branchial stream. Unilateral extirpation of the branchial stream led to disrupted development of the gill musculature on the operated side, such that the muscles were small and lacked proper attachments. Yet, no fluorescent label was observed within any posterior branchial arch muscle following vital labeling of the branchial stream, which is the principal source of the associated skeleton (ceratobranchial cartilages; Olsson and Hanken, 1996). Because our analysis of frozen sections focused on musculoskeletal components of the first two branchial arches, components of more posterior arches were not assessed comprehensively; thus, fluorescent label in the connective tissue components of these muscles may have gone undetected. The full extent of neural crest contribution to gill musculature in amphibians remains to be adequately documented.

Developmental Relations between Musculature and Skeleton

Combined data from extirpation and fate-mapping experiments in *Bombina* reveal that the neural crest-derived connective tissue components of individual larval muscles of the first two branchial arches originate from the particular crest migratory stream that is associated with each arch. In other words, connective tissues of first (mandibular) arch muscles are derived from mandibular stream neural crest, whereas connective tissues of second (hyoid) arch muscles are derived from the hyoid crest stream. These developmental relations, which are established during embryonic development, define a pattern of segmentation in the head of larval anurans that is similar to that previously described in the domestic chicken, the only vertebrate that has been thoroughly investigated in this respect (Köntges and Lumsden, 1996; Graham *et al.*, 1996).

Fidelity between individual branchial arch muscles and

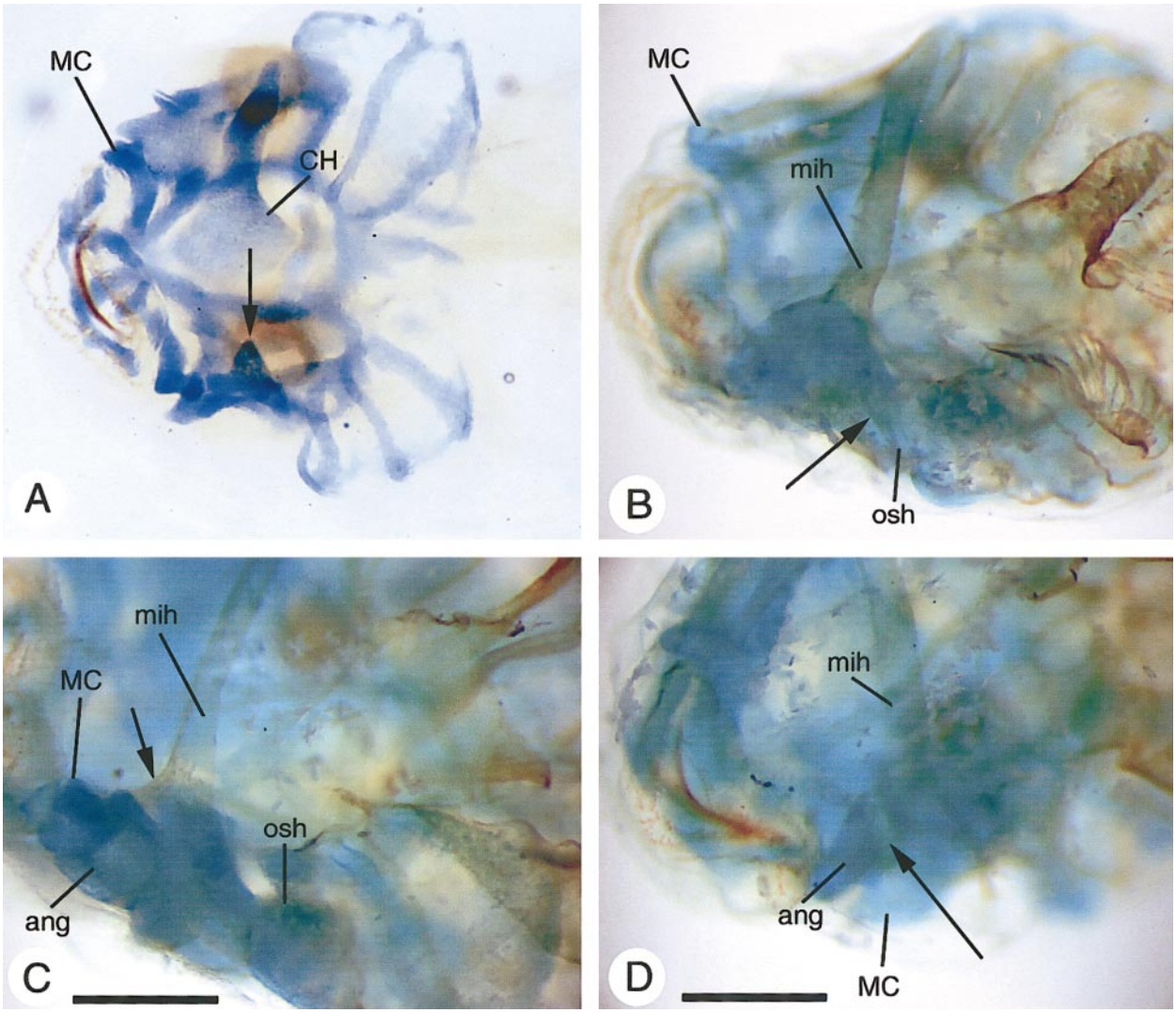

FIG. 5. Stained, larval whole mounts following unilateral ablation of the hyoid stream of cranial neural crest at embryonic stage 16. Specimens are seen in ventral view. Anterior is always to the left. (A) Severe malformation of the ceratohyal (CH) cartilage and trabecular plate follows unilateral ablation of the hyoid stream of cranial neural crest. Arrow points to the remnant of the ceratohyal on the operated (right) side. Mandibular and branchial stream-derived cartilages develop normally. (B) In the absence of the ceratohyal cartilage, its normal attachment site, the m. interhyoideus (mih) on the operated (right) side extends anteriorly to form several novel attachments. The novel, complex insertion of the m. interhyoideus includes a posterior slip, which fuses with the orbitohyoideus and suspensoriohyoideus muscles (arrow). (C) Operated (right) side of a double-stained larva, following unilateral ablation of the hyoid stream. Novel attachments of the m. interhyoideus (mih) include an anterior slip (arrow), which inserts on Meckel's cartilage (MC). (D) Operated (right) side of a double-stained larva, following unilateral ablation of the hyoid stream. The m. interhyoideus (mih) extends anteriorly, instead of transversely, and its rostral part has fused to mandibular muscles of the angularis (ang) group (arrow). Scale bars equal 1.0 mm in (A) and (B), and 0.5 mm in (C) and (D).

their connective tissue attachments is retained regardless of the segmental identity—or embryonic derivation—of associated skeletal components. The m. quadratoangularis, for example, is a larval-specific muscle unique to frogs. While it is appropriately considered a second (hyoid) arch muscle based on its pattern of innervation (facial nerve, N. VII) and its anatomical derivatives in the adult frog, the m. quadratoangularis in larvae is associated exclusively with first (mandibular) arch skeletal elements (De Jongh, 1968; Cannatella, 1999). These skeletal elements—palatoquadrate and Meckel's cartilages—are both derived from first arch (mandibular stream) neural crest, yet the connective tissue attachments for the m. quadratoangularis on the same cartilages are derived from second arch (hyoid stream)

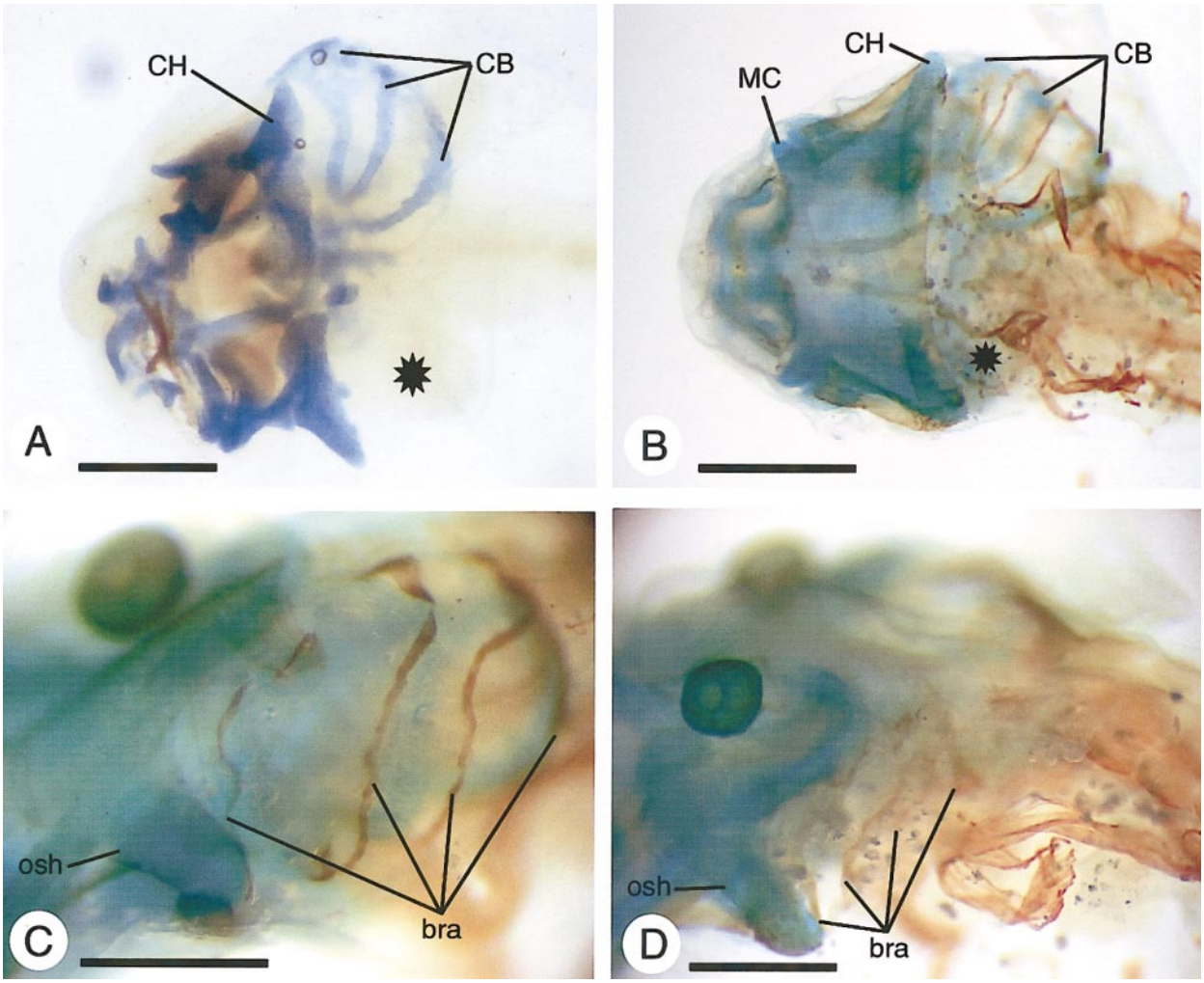

FIG. 6. Stained, larval whole mounts following branchial cranial neural crest ablation at embryonic stage 16. (A, B) Ventral views. (C, D) Lateral views; anterior is to the left. (A) Cartilage-stained larva, following unilateral ablation of the branchial neural crest stream. Ceratobranchial (CB) cartilages I–IV, which correspond to branchial arches 3–6, are missing on the operated (right) side (asterisk). First (mandibular) and second (hyoid) arch cartilages are unaffected. (B) Double-stained larva, following unilateral ablation of the branchial neural crest stream. The collapsed appearance of the operated (right) side causes the head to tilt in the direction of the missing ceratobranchial cartilages (asterisk). (C) Double-stained control larva, illustrating the normal, segmented morphology of posterior branchial arch muscles (bra), which are associated with successive ceratobranchial cartilage arches. (D) Double-stained larva, following unilateral ablation of the branchial neural crest streams. The posterior branchial arch muscles (bra) retain their basic segmental configuration but lack normal skeletal attachments. Scale bars equal 1.0 mm in (A) and (B), and 0.5 mm in (C) and (D).

neural crest (Table 1). The same relation exists for the m. orbitohyoideus at its origin from the palatoquadrate cartilage (cf. Figs. 7D and 7E). Thus, cranial neural crest from a given migratory stream is not the sole source of skeletal and other connective tissues that come to be associated anatomically with the corresponding branchial arch, as has been traditionally assumed. Rather, the pattern of neural crest derivation of individual muscles and cartilages corresponds to their respective sites of embryonic origin, regardless of the extent to which these muscles and cartilages are anatomically and functionally linked at later stages. Finally, these developmental relations underscore the need for a detailed mapping of the embryonic origin of the myogenic component of cranial muscles in amphibians, to complement data now available for the connective tissue component and for associated cartilages.

Comparison with Earlier Studies of Amphibians

Embryologists and anatomists have been mapping neural crest contributions to cranial tissues in caudate and anuran amphibians for more than a century (reviewed in Hall, 1999).

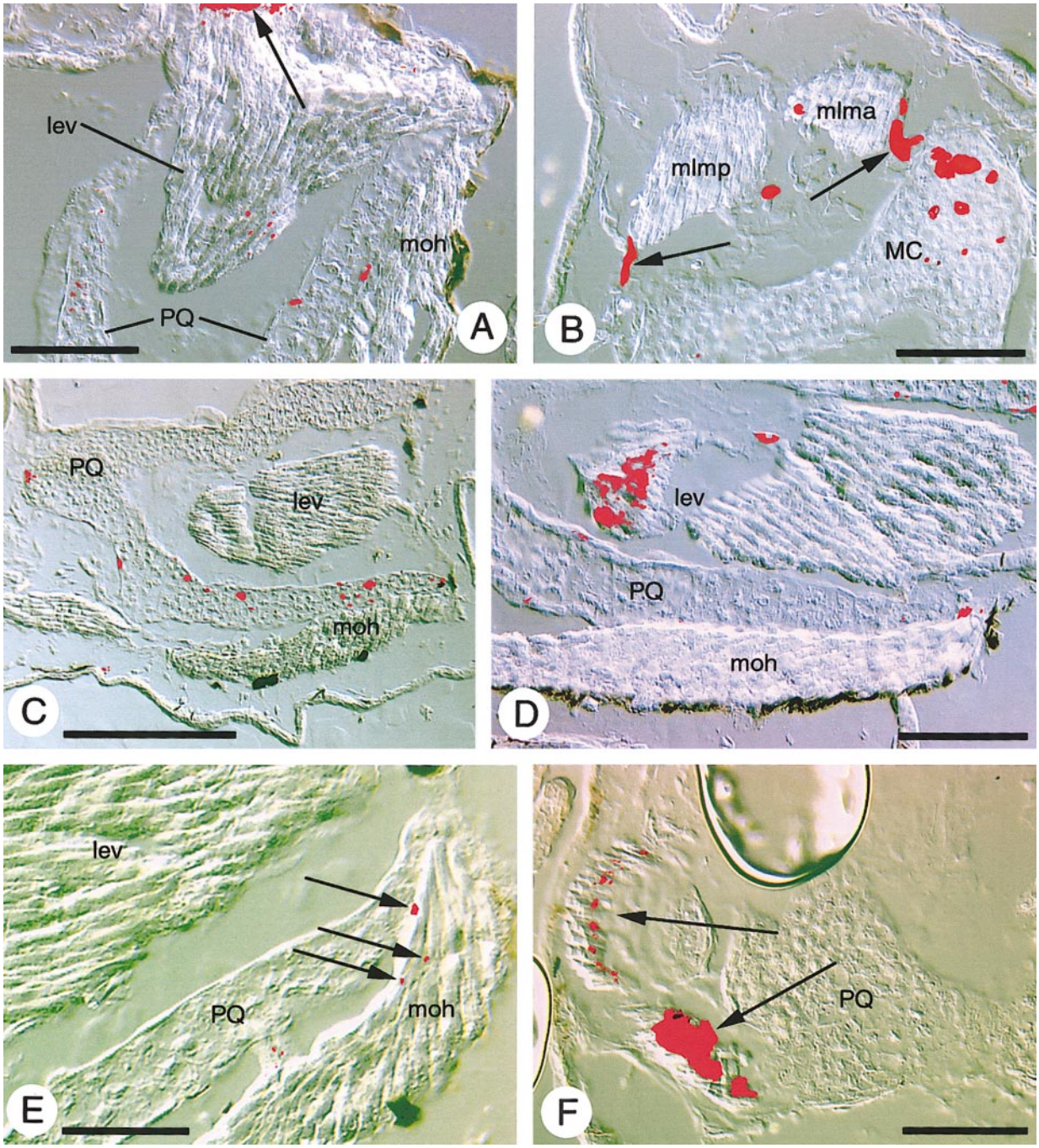

FIG. 7. DiI labeling of larval cranial cartilages and musculature, following unilateral injections into either the mandibular (A–D) or the hyoid (E, F) neural crest stream at embryonic stage 14. A given histological section was viewed sequentially with DIC and fluorescence microscopy, and the corresponding pair of images superimposed to yield each individual image seen here. All are transverse views; corresponding planes of section are depicted in Fig. 3A. Dorsal is either at the top (A, B) or to the right (C–F). Each image depicts the left (injected) side. No labeled cells were seen on the uninjected side. (A) DiI is present in the U-shaped palatoquadrate cartilage (PQ) and in the m. levatores mandibulae (lev). The arrow points to the DiI-labeled insertion of these muscles on the muscular process (of the palatoquadrate), which is otherwise poorly represented in this section. (B) Two large levator muscles, m. levator mandibulae posterior (mlmp) and anterior (mlma), are stained with DiI where they insert onto Meckel's cartilage (MC; arrows). (C, D) DiI is present in first (mandibular) arch structures, such as the palatoquadrate (PQ) and the m. levator mandibulae (lev), but is absent from second (hyoid) arch structures, such as the m. orbitohyoideus (moh). Labeling within cartilage and not muscle in the lower right portion of (D) was confirmed by observation at higher magnification (data not shown). (E, F) Following DiI injections into the hyoid stream, the marker is present in components of second (hyoid) arch muscles, such as the connective tissue attachment of the m. orbitohyoideus (moh) to the palatoquadrate (E, arrows) and within the hyo- and quadratoangularis muscles (F, arrows). It is absent from first (mandibular) arch structures, such as the palatoquadrate cartilage and m. levator mandibulae. All scale bars equal 0.2 mm except for (C), which equals 0.5 mm.

TABLE 1

Cranial migratory stream (arch)	Muscle	Skeletal attachment	Cartilage
Mandibular (first)	m. levatores mandibulae	PQ (origin), MC (insertion)	CT, IR, MC, PQ, SR
Hyoid (second)	m. orbitohyoideus	PQ (origin), CH (insertion)	CH, TP (anterior part)
	m. suspensoriohyoideus	PQ (origin), CH (insertion)	
	m. interhyoideus	CH (origin and insertion)	
	m. hyoangularis	CH (origin), MC (insertion)	
	m. quadratoangularis	PQ (origin), MC (insertion)	
Branchial (3–6)			CB I–IV

Note. Neural crest contributions to cranial muscles and associated connective tissues in the Oriental fire-bellied toad, *B. orientalis*, as assessed by vital labeling. Muscles and skeletal attachments are based on the present study. Cartilages (right column) are based on earlier results (Olsson and Hanken, 1996). Notice that some second arch muscles have skeletal attachments on first arch cartilages: Abbreviations: first arch cartilages: CT, cornua trabecula (trabecular horn); IR, infrarostral; MC, Meckel's cartilage; PQ, palatoquadrate; SR, suprarostral. Second arch cartilages. CH, ceratohyal; TP, trabecular plate.

Although the species involved are phylogenetically and morphologically diverse, these studies have consistently revealed an extensive neural crest contribution to the cartilaginous larval skull that is highly conserved evolutionarily (Hanken, 1999). Yet, with but one exception (see below), none of the studies reports a neural crest contribution to any cranial muscle. At the same time that the neural crest's contribution to cranial tissues in amphibians was beginning to be defined, the development of cranial musculature was the subject of extensive study in these same vertebrates (reviewed in Edgeworth, 1935). Again, no role of neural crest in muscle development was reported. This important developmental relationship was missed most likely because of technical limitations of the principle methods involved—neural crest ablation and transplantation—and the lack of a stable and appropriate cell marker.

Only with the application of more reliable and effective methods of cell labeling has a neural crest contribution to cranial musculature in amphibians been demonstrated. Sadaghiani and Thiébaud's (1987) study of chimaeric *Xenopus* (neural crest transplantation from *X. borealis* to *X. laevis*) is the only previous analysis known to us that explicitly mentions (albeit in passing) a neural crest derivation of (undifferentiated) cranial muscle tissues in any amphibian. These authors describe neural crest cells from the hyoid stream contributing to several larval muscles, including the interhyoideus, orbitohyoideus, quadratoangularis, and hyoangularis (their Figs. 9a, 9c, and 10a). They also claim that neural crest cells originating within the mandibular stream, "Occasionally . . . could be seen in the undifferentiated muscle masses" (p. 99), which are not identified further. They do not state whether neural crest cells give rise to only connective tissue or to myofibers as well. Finally, mandibular stream neural crest cells are "numerous in the connective tissue on the margin of the cartilages, from which occasionally some crest cells are incorporated into the attached muscles" (p. 99). The present study uses DiI-labeling to more extensively document the neural crest contribution to cranial muscle connective tissues in a second species of anuran. Comparable experiments with the axolotl clearly indicate that a neural crest contribution to cranial muscles is present in urodeles as well (Olsson, L., Ericsson, R., and Falck, P., 2000).

Nevertheless, we strongly encourage that these claims be tested and extended by the use of alternate fate-mapping procedures, to compensate for the unavoidable shortcomings inherent in any method. For example, when applying certain fluorescent dyes such as DiI, great care has to be taken to avoid labeling other cells that migrate away from the injection site and which might be confused with neural crest, e.g., placodal epithelium. Also, because DiI is applied to a subset of cranial neural crest cells in each injected embryo, negative results may not be very informative. Neural crest-derived structures can go undetected because that particular subset of crest cells was never labeled. In future studies, we hope to be able to mark *all* neural crest cells in a stream using a recently developed protocol that labels premigratory neural crest cells with green fluorescent protein (GFP; Carl *et al.*, 1999, 2000), and which avoids many of the potential artifacts identified above. Another approach would be to use lysinated rhodamine dextrans (LRDs) to follow the fate of single cells.

Evolution of Cranial Patterning in Vertebrates

The fundamental role of the neural crest in cranial skeletal development is well established in several vertebrate species (Hall and Hörstadius, 1988). Indeed, a prominent neural crest contribution to the cranial skeleton likely represents a shared, conservative feature of vertebrates, which characterized their early evolution from nonvertebrate chordates (Gans, 1989; Gans and Northcutt 1983; Hall, 1999). The initial report—in birds—of an important role of neural crest in cranial muscle development and patterning immediately introduced the question of whether this feature also evolved early in vertebrate phylogeny, or if, instead, it is a later evolutionary innovation that is confined to a more recent and restricted clade of vertebrates, e.g.,

tetrapods. This question can only be answered definitively by additional, detailed experimental studies of neural crest fates and muscle origins in a taxonomically diverse series of taxa, especially more "basal" lineages such as fishes.

Nevertheless, data from the present study of anuran amphibians, combined with limited, initial evidence of cranial muscle development in the zebrafish (Schilling and Kimmel, 1994) and in urodeles (Olsson *et al.*, 2000), suggest that a neural crest contribution to the development of branchial-arch muscles is in fact a primitive vertebrate trait that is widespread—but largely undetected—in most living clades. Moreover, they suggest that the essential role of the neural crest in cranial musculoskeletal patterning (Noden, 1991; Graham *et al.*, 1996; Schilling, 1997; Schilling and Kimmel, 1997) may represent a fundamental, conserved feature of cranial development in vertebrates generally. Finally, the presence of similar developmental relations between musculature and skeleton in tetrapods as different as frogs and birds demonstrates how developmental processes and cell fates can be conserved even when major evolutionary innovations—such as the novel cartilages and muscles of anuran larvae—result in major anatomical differences in cranial form.

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