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Cranial neural crest-cell migration in the direct-developing frog, *Eleutherodactylus coqui:* molecular heterogeneity within and among migratory streams

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Summary

Direct development is a specialized reproductive mode that has evolved repeatedly in many different lineages of amphibians, especially anurans. A fully formed, albeit miniature adult hatches directly from the egg; there is no free-living larva. In many groups, the evolution of direct development has had profound consequences for cranial development and morphology, including many components that are derived from the embryonic neural crest. Yet, the developmental bases of these effects remain poorly known. In order to more fully characterize these changes, we used three molecular markers to analyze cranial neural crest-cell emergence and migration in the direct-developing frog, *Eleutherodactylus coqui:* HNK-1 immunoreactivity, Dlx protein expression, and cholinesterase activity. Our study validates and extends earlier results showing that the comprehensive changes in embryonic cranial patterning, differentiation, and developmental timing that are associated with direct development in *Eleutherodactylus* have not affected gross features of cranial neural crest biology: the relative timing of crest emergence and the number, configuration and identity of the principal migratory streams closely resemble those seen in metamorphic anurans. The three markers are variably expressed within and among neural crest-cell populations. This variation suggests that determination of cranial neural crest-cells may already have begun at or soon after the onset of migration, when the cells emerge from the neural tube. It is not known how or even if this variation correlates with differential cell lineage or fate. Finally, although HNK-1 expression is widely used to study neural crest migration in teleost fishes and amniotes, *E. coqui* is the only amphibian known in which it effectively labels migrating neural crest-cells. There are not enough comparative data to determine whether this feature is functionally associated with direct development or is instead unrelated to reproductive mode.

Key words: Anura, cell migration, head development, HNK-1, Dlx, cholinesterase

Introduction

The typical and phylogenetically ancestral reproductive mode in recent amphibians involves a complex, biphasic life history. Embryogenesis culminates in a free-living, aquatic larva; a subsequent, posthatching metamorphosis transforms the larva into a terrestrial adult (Duellman and Trueb, 1994; Hanken, 1999). Yet, phylogenetically derived, alternate modes have evolved in all three living orders (Duellman, 1989; Wake, 1989). Among the most extreme evolutionary modifications of the ancestral, metamorphic ontogeny is direct development: eggs typically are laid on land, and the young emerge at hatching as fully formed, albeit miniature adults; there is no free-living larva. Direct development has evolved many times, at least 10 times in anurans alone (Duellman and Trueb, 1994), and is the predominant reproductive mode in some clades, e.g., the lungless salamanders (Plethodontidae; Wake and Hanken, 1996). Evolution of direct development may promote

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evolutionary success by eliminating larval constraints on adult morphology (Wake and Roth, 1989). However, despite the seeming importance of this evolutionary shift in reproductive mode to the biology of amphibians, many basic features of direct development, as well as their underlying genetic and developmental mechanisms, remain poorly understood.

We study a species of direct-developing frog, *Eleutherodactylus coqui*, whose pattern of development is drastically different to that seen in most anurans. Many adult characters that form after hatching in species with a biphasic life history, e.g., limbs, instead form during embryogenesis in *E. coqui*, which at the same time lacks many typical larval features (Elinson, 1990, 1994, 2001; Fang and Elinson, 1996, 1999; Richardson et al., 1998; Schlosser et al., 1999; Callery and Elinson, 2000; Callery et al., 2001; Hanken et al., 2001; Ninomiya et al., 2001). Consequences of the evolution of direct development are especially conspicuous in the head; derived features include embryonic development of bone (which does not form until well after hatching in metamorphic species), and altered patterning of cranial cartilages and musculature (Hanken et al., 1992; 1997b). These and other changes in both developmental timing and pattern formation constitute a comprehensive modification of embryonic cranial ontogeny (Hanken et al., 1997a).

The locus of many of these evolutionary changes in cranial development is likely to be the neural crest (Hanken and Thorogood, 1993). Neural crest-cells contribute to most of the cranial cartilages in vertebrates, including frogs (Stone, 1929; LeDouarin, 1982; Sadaghiani and Thiébaud, 1987; Hall and Hörstadius, 1988; Olsson and Hanken, 1996; Hall, 1999; LeDouarin and Kalcheim, 1999). The neural crest also mediates the patterning of cranial cartilage, bone and muscle, thereby contributing to their integrated development (Hall, 1950; Noden, 1983; Köntges and Lumsden, 1996). Cranial neural crest development in *E. coqui* is thus receiving increased attention, including features such as emergence and early migration (Moury and Hanken, 1995), gene expression (Fang and Elinson, 1996; 1999), and fate-mapping.

In this study, we use three different molecular markers to characterize migrating cranial neural crest-cells in *E. coqui*: HNK-1 immunoreactivity, Dlx protein expression, and cholinesterase activity. All three molecular markers have been used to study neural crest biology in other vertebrates (see Materials and Methods). We use the data to address three principal aims. The first is to validate patterns of early neural crest emergence and migration defined initially by using scanning electron microscopy (Moury and Hanken, 1995) and to extend the study of crest migration to later stages. The second aim is to assess cellular heterogeneity both within and among neural crest-cell populations, which may correlate with cell lineage or fate. Finally, we use the markers to screen for interspecific differences in molecular expression that might correlate with reproductive mode and which might underlie associated differences in cranial patterning.

Materials and methods

Acquisition and maintenance of embryos

Embryos of *Eleutherodactylus coqui* were obtained from spontaneous matings among wild-caught adults maintained as a laboratory breeding colony at the University of Colorado (CU). Animal collection and care followed standard procedures (Elinson et al., 1990) and were in accordance with the regulations of the Puerto Rico Department of Natural Resources and CU Boulder. Fertilization is internal; following oviposition, eggs are guarded by the male parent. Egg clutches were removed from the brooding male within 24 h of deposition and incubated at 25º C in Petri dishes on filter paper moistened with 10% Holtfreter solution (Hamburger, 1960). Embryos were staged according to the table of Townsend and Stewart (1985), which defines 15 stages from fertilization (TS 1) to hatching (TS 15). Embryonic jelly layers were removed from living embryos by using a chemical solution (0.63 g cysteine HCl, 0.12 g NaCl, and 24 ml water, adjusted to pH 7.9–8.1 with 5 N NaOH) and watchmaker's forceps. Dejellied embryos that were not used immediately were maintained in 10% Holtfreter solution at 25°C (Elinson, 1987). Vitelline membranes were removed manually prior to fixation.

Immunostaining

HNK-1 is a monoclonal antibody that recognizes an acidic sulfated glycosphingolipid (Mailly et al., 1989). Because migrating neural crest-cells of many vertebrates express the HNK-1 epitope soon after emerging from the neural tube, HNK-1 immunoreactivity has been used to document pathways of neural crest-cell migration in several species, including lamprey (Hirata et al., 1997), teleost fish (Sadaghiani and Vielkind, 1990; Hirata et al., 1997), softshell turtle (Hou and Takeuchi, 1994), chicken (Bronner-Fraser, 1986; Tucker et al., 1986; Newgreen et al., 1990; 1996), quail (Heath et al., 1992), and rat (Erickson et al., 1989). HNK-1 has not been used to study neural crest migration in amphibians because early migrating crest-cells are not immunoreactive in the few species tested to date (e.g., *Xenopus laevis*, M. Bronner-Fraser, pers. comm.; *Bombina orientalis*, Olsson and Hanken, unpublished observations; *Gastrotheca riobombae*, del Pino and Medina, 1998). We used two different monoclonal antibodies against HNK-1: American Type Culture Collec-

tion TIB 200 (Dr. Ruth Nordlander, Ohio State University, Columbus), and anti-human Leu-7 (CD 57, clone HNK-1; Becton Dickinson, San Jose, CA).

The gene *distal-less* (*Dll*) was originally described from the fruit fly, *Drosophila*. It is involved in several different aspects of arthropod development, including development of appendages (legs, wings, and mouthparts) and specification of color pattern (O'Hara et al., 1993; Carroll et al., 1994; Panganiban et al., 1994; 1995). Several homologous, so-called *Dlx* genes are expressed during vertebrate embryogenesis, including in migrating cranial neural crest-cells in zebrafish (Akimenko et al., 1994), *Xenopus* (Dirksen et al., 1993; Papalopulu and Kintner, 1993), chicken (Ferrari et al., 1995), rat (Zhao et al., 1994), and mouse (Robinson et al., 1991; Dolle et al., 1992; Bulfone et al., 1993; Robinson and Mahon, 1994; Weiss et al., 1994; Morasso et al., 1995). The severe head abnormalities seen in gene knockout mice produced for *Dlx-2* (Qiu et al., 1995) also implicate *Dll* homologs as important players in vertebrate cranial development. Two *Dlx* genes have been cloned in *E. coqui* (Fang and Elinson, 1996). We used a polyclonal antibody against a conserved region of the distal-less protein in arthropods (Panganiban et al., 1995; Dr. Grace Panganiban, University of Wisconsin, Madison). This antibody binds Dlx protein in *E. coqui* (Fang and Elinson, 1996).

Immunostained embryos were prepared according to the technique of Klymkowsky and Hanken (1991), with slight modifications to minimize non-specific background staining. Embryos were fixed for 1–2 h in either 10% neutral-buffered formalin (NBF) or 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline, run through a 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 bleaching in Dent bleach (1 part 30% hydrogen peroxide: 2 parts Dent fixative) overnight or longer (as long as 3 d for older, pigmented embryos), specimens were dehydrated in three changes of 100% methanol and preserved in 100% methanol at –20 ºC.

After rehydration in a methanol series, preserved specimens were washed for 3 × 5 min in „saline cocktail" (0.1 M Niu-Twitty saline (Hamburger, 1960), 0.1 M phosphate buffer [K/Na; pH 7.4], 0.4% Triton X-100), pre-incubated 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 in primary antibody diluted with serum cocktail (1:100 for the HNK-1 antibodies; 1:50 for the *Dlx* antibody). 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 [HNK-1; Bio-Rad], or HRP-conjugated goat anti-rabbit IgG [*Dlx*; Bio-Rad]) diluted with serum cocktail at 1:1000 and 1:400, respectively. Specimens were washed 2 x 30 min and then overnight in serum cocktail, further washed for 3 × 1 h in saline cocktail, and reacted for 1–2 h with 0.5 mg/ml diaminobenzidine (DAB) in saline cocktail containing 0.02% hydrogen peroxide. At least five embryos each of stages TS 3–8 were stained with each antibody. Most specimens were finally prepared as cleared whole-mounts by immersion in BABB (1 part benzyl alcohol: 2 parts benzyl benzoate). One-to-three specimens in each series were sectioned. They were dehydrated in ethanol, cleared in Hemo-De (Fisher Scientific, Pittsburgh, USA), embedded in paraplast, and sectioned at 7 µm with a Leitz 1512 microtome.

Chicken embryos (3- and 4-d) were used as positive controls for the specificity of each antibody. Negative controls included omission of the primary antibody or replacement of the primary antibody with pre-immune immunoglobulins.

Cholinesterase staining

Cholinesterase activity has been used as a marker for early migrating neural crest-cells in amniotes (Cochard and Coltey, 1983; Martins-Green and Erickson, 1988; Layer and Kaulich, 1991), but it has never before been employed to study neural crest migration in amphibians. We modified the sections-based technique of Karnovsky and Roots (1964) to stain whole-mount embryos for cholinesterase using acetylthiocholine iodide as the cholinesterase substrate. At least five embryos each of stages TS 3–7 were fixed briefly (5–30 min) in 4% PFA in 0.1 M phosphate buffer (K/Na; pH 7.4) and then immersed for 10 min in 0.1 M phosphate buffer (K/Na; pH 6.0). They were stained for 12–24 h, fixed overnight in 10% NBF, dehydrated in an ethanol series, and prepared either as whole-mounts or sections as described above. The concentration of each reagent except the buffer was doubled to intensify staining in whole-mounts.

Microscopy and photomicrography

Specimens were viewed with Wild M8 dissecting (whole mounts) and Leitz Dialux 20 compound (serial sections) microscopes. Digital images were either recorded directly with an Optronix DEI-470 video camera and processed using Adobe Photoshop on Apple Macintosh computers; or specimens were first photographed using a Wild MPS55 photoautomat and Kodak Ektachrome 160T or 320T (Wild) or Kodachrome 25 (Leitz) film, and subsequently scanned electronically. Final figures were prepared by using Adobe Photoshop and Adobe Illustrator on Apple Macintosh computers.

Results

In embryos of *E. coqui*, cranial neural crest-cells begin to emerge from the neural folds at stage TS 3 and continue to emerge at least until early in stage TS 5 (Moury and Hanken, 1995). Emerging cells soon constitute three principal migratory streams: mandibular (rostral), hyoid (rostral otic), and branchial (caudal otic). Staining patterns obtained with each marker are described below by stage, beginning at neural crest emergence and continuing to beyond the time when cells have dispersed from the migratory streams. Each account begins with brief notes that describe the characteristic features of embryos of the corresponding stage, based on Moury and Hanken (1995).

Table 1. Cranial neural crest-cell heterogeneity in *Eleutherodactylus coqui*. Plus and minus signs denote the presence and relative staining intensity of three different molecular markers within the transverse neural fold and the three main cranial neural crest migratory streams.

Molecular-marker	Transverse neural fold	Neural crest stream			
		Mandibular		Hyoid	Branchial
		(lateral)	(medial)		
Cholinesterase	++	++	+	+	+
Dlx protein	+	+	+	+	+
HNK-1	–	+	++	–	–

Stage 3

Stage 3 comprises three substages. Stage 3.1 begins when neural folds are first visible around the thickened neural plate. Stage 3.2 is characterized by the paired neural folds moving towards the midline. At stage 3.3 the neural folds meet but are not yet fused. Cranial neural crest-cells that will constitute the mandibular stream begin to migrate ventrolaterally over the neural folds and anteroventrally onto the endoderm at stage 3.2. Cells that form the hyoid stream migrate parallel, but posterior, to the mandibular stream beginning at stage 3.3. Posterior neural crest-cells do not emerge to form the branchial stream until stage 4.

Positive staining for HNK-1 is seen in the mandibular stream but not the hyoid stream. All cells within the mandibular stream appear to be stained as early as stage 3.2, although staining is most intense in the anteromedial portion and fainter laterally (Figs. 1A, B; Table 1). Cells within the transverse neural fold are unstained.

Both mandibular and hyoid streams stain positively for Dlx protein and cholinesterases. Staining for Dlx is relatively uniform throughout both streams, and all cells appear to be stained (Fig. 1C). Dlx protein also is expressed within adjacent portions of the lateral neural folds and along the rostral edge of the transverse neural fold. The pattern of cholinesterase staining in the mandibular stream is the opposite of that for HNK-1; the lateral portion stains strongly and the medial portion weakly (Fig. 1D). Additional staining is present within the lateral and transverse neural folds. Indeed, the most intense staining for cholinesterases is present within a transverse band of neural-fold cells that lies at the approximate level of the midbrain-hindbrain boundary and between emerging crest-cells of the mandibular and hyoid streams (Fig. 1D).

Stage 4

Midline fusion of the cranial neural folds marks the beginning of stage 4. Neural crest-cells in the massive mandibular stream migrate rostrally around the developing eye; most cells migrate ventral to the optic vesicle, whereas a smaller number take a dorsal route. The hyoid stream is long and thin and parallels medial portions of the mandibular stream. Posterior cranial neural crest-cells emerge to form the broad, sheet-like branchial stream, which initially comprises at least two parallel cell masses.

As seen at stage 3, only neural crest-cells within the mandibular stream stain for HNK-1 (Fig. 2A). Staining is most intense at the leading (rostral) edges of the stream, both dorsal and ventral to the optic vesicle, and within cells in the ventral migratory route that are closest to the optic vesicle. Cells within the lateral portion of the mandibular stream stain only lightly, if at all. Staining is absent from crest-cells within both the hyoid and branchial streams, but positive staining begins to appear in motoneurons developing within the presumptive brain and spinal cord (Fig. 2A).

The pattern of cholinesterase staining in the mandibular stream is similar to that at stage 3. The most intense staining, however, is now seen within the hyoid stream and the transverse neural fold, along with the lateral neural folds at the midbrain-hindbrain boundary, which began staining intensely at stage 3 (Fig. 2B). Cholinesterase staining is difficult to assess in the branchial stream because of extensive, non-specific background staining; positive staining in this stream is either absent or, at most, weak.

Dlx protein expression is strong within distal parts of all three cranial neural crest streams, including both parallel cell masses that comprise the branchial stream, but it is absent from proximal parts (Figs. 2C, D). As with the cholinesterase assay but unlike HNK-1 (Figs. 2A, C), Dlx protein also is expressed within the transverse neural fold. In addition, Dlx antibody stains the otic vesicle, which separates hyoid and branchial crest streams as they emerge from the neural fold. Otic vesicles are not labelled with either HNK-1 or cholinesterase.

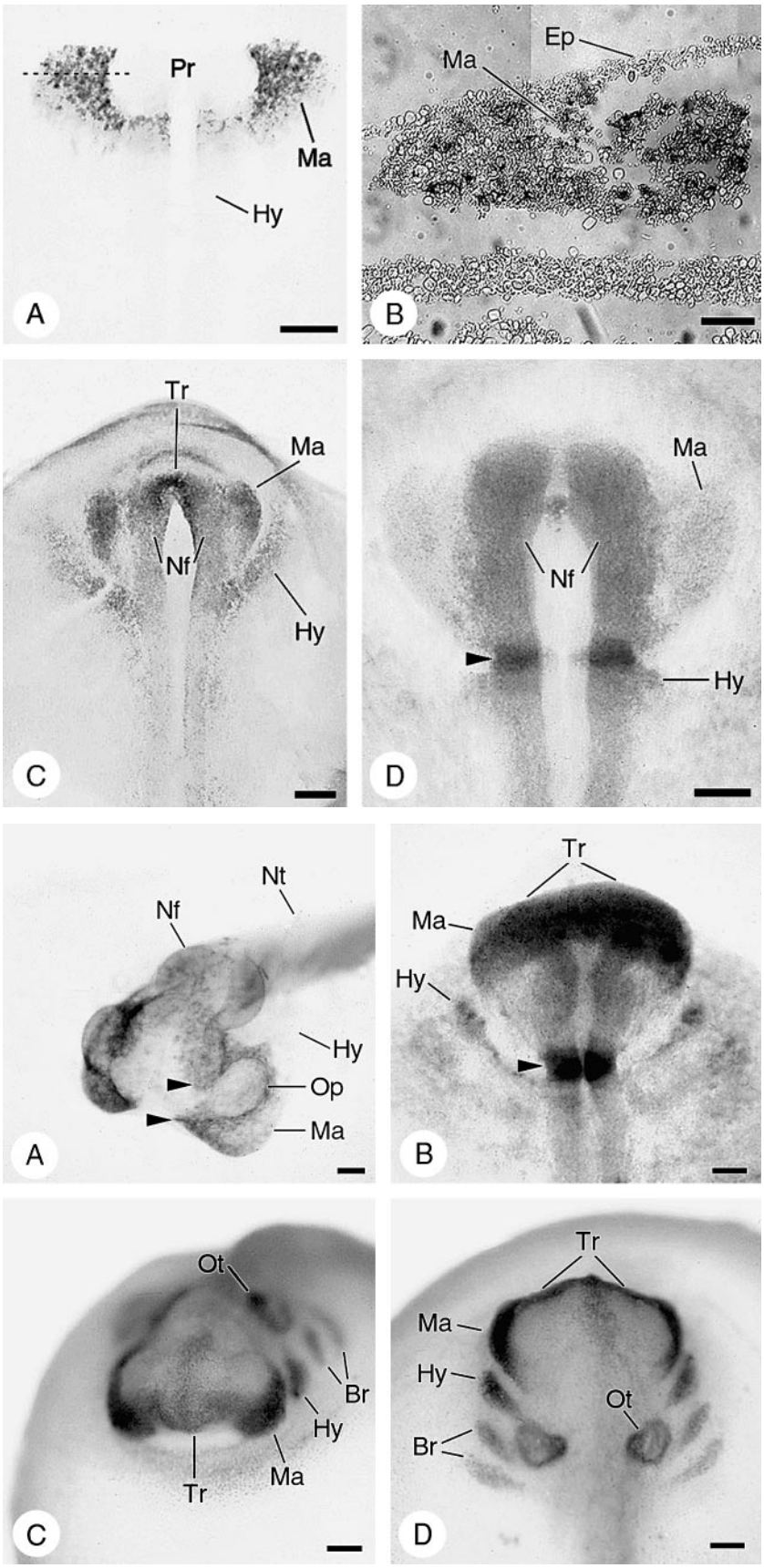

Fig. 1. Stained embryos of *Eleutherodactylus coqui*, stage TS 3. A, C, and D are whole-mounts seen in dorsal view, anterior is at the top. **(A).** TS 3.2. Positive staining for HNK-1 is most intense in neural crest-cells within the anteromedial portions of the mandibular stream (Ma) and is fainter laterally. Staining is absent from the hyoid stream (Hy). Dashed line denotes the approximate plane of section in B. **(B).** TS 3.2. Transverse section through the left mandibular stream; dorsal is at the top, lateral is to the left. HNK-1 staining is present in neural crest-cells but not in neighboring tissues. **(C).** TS 3.3. Dlx protein is expressed by neural crest-cells within the mandibular and hyoid streams. Lateral cranial (Nf) and transverse (Tr) neural folds also stain positively for Dlx. **(D).** TS 3.3. Positive staining for cholinesterases labels mandibular and hyoid neural crest streams, as well as lateral cranial and transverse neural folds. The transverse band of intense staining (arrowhead) lies anterior to the emerging hyoid stream (which emerges approximately at the level of rhombomere 4) and immediately posterior to the midbrain-hindbrain boundary. Additional abbreviations: Ep, epidermis; Pr, prosencephalon. Scale bars: A, C, D 0.2 mm, B 0.005mm.

Fig. 2. Stained, whole-mount embryos of *Eleutherodactylus coqui*, stage TS 4. A and C are anterolateral views, anterior is to the left. B and D are dorsal views, anterior is at the top. **(A).** HNK-1–positive cells of the mandibular neural crest stream (Ma) are migrating anteriorly around the optic vesicle (Op) via both dorsal and ventral pathways (arrowheads). Staining is absent from the hyoid stream (Hy) but is present in the lateral cranial neural folds (Nf) and the postcranial neural tube (Nt). **(B).** Positive staining for cholinesterases within the head is especially strong in the hyoid neural crest stream, in the transverse neural fold (Tf), and in the lateral neural folds at the level of the midbrain-hindbrain boundary (in front of approximately rhombomere 4, arrowhead; cf. Fig. 1D). **(C, D)** Dlx protein is expressed by cells within all three cranial neural crest streams, as well as the transverse neural fold and the otic vesicle (Ot). The branchial stream (Br) has subdivided to form two distinct, parallel cell masses. Scale bars: 0.2 mm.

Stage 5

Limb and tail buds, which first appear during stage 4, are elongating. All three primary cranial neural crest streams are larger and extend further ventrally. Individual neural crest-cells begin to disperse among other cranial cell types and cannot be followed reliably with scanning electron microscopy.

HNK-1 staining is now widespread, both in the head and in neural tissues along the body axis (Fig. 3A). In addition to peripheral nervous system components that are wholly or partly derived from the neural crest, such as dorsal root and cranial ganglia, much of the central nervous system is stained. The latter includes neuroepithelium within both the developing spinal cord and the retina of the eye.

Cholinesterase activity is no longer an effective marker for neural crest-cells; non-specific background staining is more intense and widespread, and enzyme activity has begun within non-neural crest-cells both in the head and postcranially (Figs. 3B, D). There is strong activity, for example, in myogenic cells within the differentiating somites and in retinal neuroepithelium of the developing eye. Olfactory and otic placodes also stain intensely. Nevertheless, neural crest-cells are stained within all three cranial migratory streams (Fig. 3D).

Dlx protein expression is confined to distal parts of each cranial neural crest stream and to the dorsal portion of the otic vesicle (Fig. 3C)**.** The broad, rostral band of Dlx protein expression seen within the transverse neural fold at stage 4 has disappeared.

Stage 6 and later

All four limbs and the tail continue to elongate at stage 6 (Figs. 4A, B). Tiny external gills extend laterally from the branchial arches (Fig. 4B), and prechondrogenic condensations begin to form within the head, e.g., otic capsules (Hanken et al., 1992). Cartilage is present for the first time at stage 7, both in the head and postcranially (Hanken et al., 1992). Cranial neural crest-cell emergence ceases at stage 6, shortly before the onset of chondrogenesis.

At stage 6, HNK-1 staining is widespread in cranial and spinal nerves (Fig. 4A). Especially strong staining is seen in the trigeminal, facial, glossopharyngeal and vagal nerves (Fig. 4C). Dlx staining remains strong in distal portions of all three cranial neural crest streams and in the otic vesicle (Figs. 4B, D). Protein is also expressed in the olfactory placode, in the apical ectoderm of each limb bud, and along the dorsal edge of the developing tail. Cholinesterase activity is not a useful marker for neural crest-cells because of extensive non-specific background staining (not illustrated).

By the beginning of stage 7, none of the three markers differentially labels neural crest-cells or their derivatives. Dlx protein expression disappears entirely, and HNK-1 and cholinesterase staining becomes even more widespread than at stages 5 and 6.

Discussion

Patterns of early neural crest emergence and migration in *E. coqui*

HNK-1 immunoreactivity, Dlx protein expression, and cholinesterase activity provide convenient and reliable markers for emerging cranial neural crest-cells in *Eleutherodactylus coqui*. They also allow crest-cell migration to be followed further, later, along deeper pathways, and with greater resolution than can be obtained by using scanning electron microscopy (SEM). Using these molecular markers, we confirm and extend the following earlier findings based on SEM and analyses of gene expression (Moury and Hanken, 1995; Fang and Elinson, 1996; Hanken et al., 1997a): cranial neural crest emergence begins at stage 3.2, before neural tube closure; emerging crest-cells assemble into three principal migratory streams: mandibular (rostral), hyoid (rostral otic) and branchial (caudal otic). The branchial stream comprises two parallel cell masses, which form separate streams at stage 4. These studies reinforce the earlier finding that the order in which cranial neural crest streams emerge in direct-developing *E. coqui*, as well as the number, configuration, and identities of the principal migratory streams, are the same as those in anurans with biphasic development (Moury and Hanken, 1995; Fang and Elinson, 1996; Olsson and Hanken, 1996). Indeed, many basic features of cranial neural crest emergence and early migration seem to be highly conserved among tetrapods generally (Anderson and Meier, 1981; Tosney, 1982; Jacobson and Meier, 1984; Meier and Packard, 1984; Tan and Morriss-Kay, 1986; Serbedzija et al., 1992; Hou and Takeuchi, 1994).

One difference noted previously between *E. coqui* and metamorphosing anurans is the relative size of the mandibular neural crest stream vis-à-vis the other cranial streams. This difference may correspond to the variable development of particular larval components among species (Del Pino and Medina, 1998). Nevertheless, the pervasive changes in embryonic cranial patterning, differentiation, and developmental timing that are correlated with the evolution of direct development in *Eleutherodactylus* (Hanken et al., 1992; 1997b; Fang and Elinson, 1996; 1999; Schlosser et al., 1999) have not affected many gross features of cranial neural crest biology. To the extent that the neural crest is the primary locus of these changes in embryonic cranial ontogeny and form, these changes instead must involve more subtle aspects of neural crest biology, such as

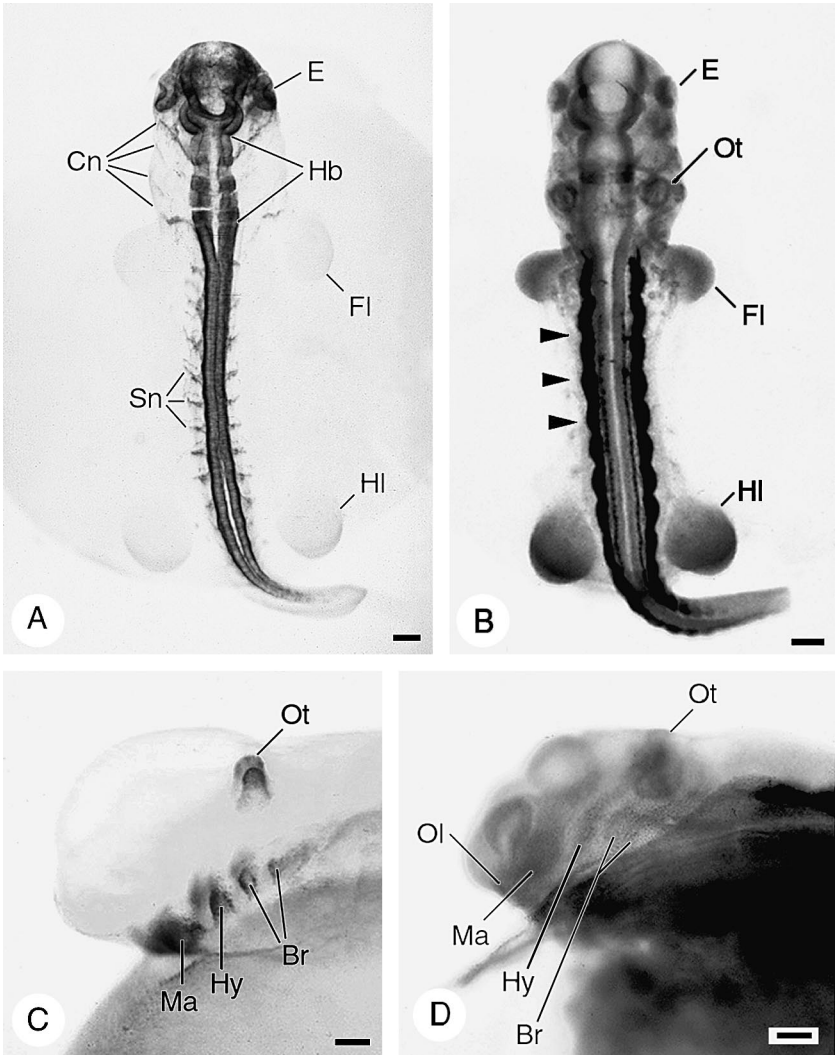

Fig. 3. Stained, whole-mount embryos of *Eleutherodactylus coqui*, stage TS 5. A and B are dorsal views of entire embryos, anterior is at the top; C and D are lateral views of the head, anterior is to the left. **(A).** Most of the central nervous system stains with HNK-1, including the neural tube and retinal neuroepithelium within the developing eye (E). Note the segmental staining pattern within the hindbrian (Hb). Additional staining is seen in cranial (Cn) and spinal (Sn) nerves. **(B).** The most intense cholinesterase staining is seen in postcranial axial musculature (arrowheads). The otic vesicle (Ot) and the eye are stained, as is neuroepithelium in the brain. **(D).** In lateral view, cholinesterase staining is apparent in the mandibular (Ma), hyoid (Hy) and branchial (Br) neural crest streams, as well as in the olfactory placode (Ol) and otic vesicle. **(C).** Dlx staining is confined to distal parts of all three cranial neural crest streams and to the otic vesicle. Additional abbreviations: Fl, forelimb; Hl, hind limb. Scale bars: 0.2 mm

gene expression and cell determination or fate. Conversely, such changes may be mediated by modifications principally affecting adjacent tissues with which the neural crest interacts, such as cranial ectoderm. Changes in ectodermal competence have already been shown to underlie the evolutionary loss of the larval hatching gland and lateral line placodes in *E. coqui* (Fang and Elinson, 1996, 1999; Schlosser et al., 1999).

Variation within and among neural crest-cell populations

Differential staining patterns obtained with the three markers constitute evidence for neural crest-cell heterogeneity both within and among cranial migratory streams (Table 1). The HNK-1 antibody stains only the mandibular stream, making it very useful as a marker for first arch cranial neural crest. The staining is more pronounced in the medial portion and weaker laterally. Cholinesterase stains all three streams, but the lateral portion of the mandibular stream is stained more heavily than the medial portion. The antibody recognising Dlx proteins stains all three streams; staining is initially widespread throughout each stream, but it gradually becomes restricted to distal portions at later stages. Viewed by stream, the mandibular stream is the only one to express all three markers, and there are discrete differences in relative intensity between medial and lateral portions. The hyoid stream displays the same staining pattern as both the branchial stream and, interestingly, the transverse neural fold, which lacks neural crest-cells.

It is possible that differential staining within premigratory and early migrating neural crest-cells correlates with variable cell lineages or fates. Some neural crest-cells are multipotent before migration and differentiate later according to environmental signals (Bronner-Fraser and Fraser, 1988; Hall, 1999; LeDouarin and Kalcheim, 1999; Dorsky et al., 2000). Others, however, are already determined before migration and will differ-

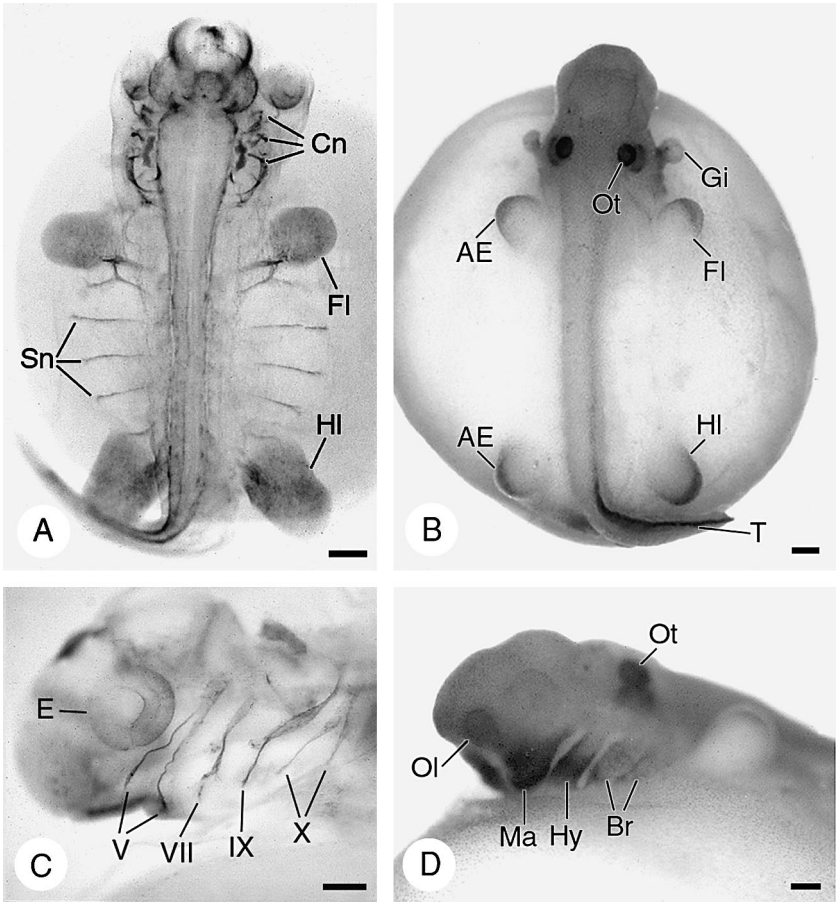

Fig. 4. Stained embryos of *Eleutherodactylus coqui*, stage TS 6. A and B are dorsal views of entire embryos, anterior is at the top; C and D are lateral views of the head, anterior is to the left. (**A, C).** HNK-1 is expressed in cranial (Cn) and spinal (Sn) nerves. In the head, staining is seen in neuroepithelia of the brain and the retina of the eye (E), and in the trigeminal (V), facial (VII), glossopharyngeal (IX), and vagal (X) nerves. (**B, D).** Dlx protein is expressed in the otic vesicle (Ot), in the apical ectoderm (AE) of each limb bud, and in the tail (T). In lateral view, strong staining is seen in distal parts of the mandibular (Ma), hyoid (Hy) and branchial (Br) neural crest streams, and in the otic vesicle and olfactory placode (Ol). Additional abbreviations: Fl, forelimb; Gi, external gills; Hl, hind limb. Scale bars: 0.2 mm.

entiate in a donor-specific manner when transplanted to an ectopic position (Hörstadius and Sellman, 1946; Wagner, 1949,1959; Noden, 1983). In zebrafish, premigratory cranial neural crest-cells display "cell type restriction," in which a given crest-cell will yield only a single type of differentiated derivative, and progenitors of different cell types are spatially segregated within the premigratory crest population (Schilling and Kimmel, 1994). Molecular heterogeneity in premigratory neural crest has already been described in birds. Heath et al. (1992), using monoclonal antibodies raised against quail premigratory neural crest, showed that (unspecified) molecular heterogeneity appears in cultured chicken and quail neural crest-cells before morphological differentiation. Other examples of molecular heterogeneity are provided by the numerous reports of differential expression of several genes, especially Hox genes, among cranial neural crest-cell populations in several different taxa (Graham et al., 1996; Schilling, 1997; Couly et al., 1998; Hunt et al., 1998). Our data provide another example of this general phenomenon. They also suggest that, in *E. coqui*, determination of cranial neural crest-cells may have already begun by the time or soon after the cells emerge from the neural tube and begin to migrate. Whether in *E. coqui* determination is complete before migration is unknown at present, although this could be tested by transplanting neural crest-cells between streams. Similarly, the specific relation between any of the three molecular markers or overall staining pattern and any particular differentiated cell or tissue type is unknown at this time.

del Pino and Medina (1998) report the expression patterns of several molecular markers within migrating cranial neural crest in the marsupial frog, *Gastrotheca riobombae*. Five markers yield discrete, positive staining patterns, which provide evidence of molecular heterogeneity both within and among the three principal migratory streams. All five markers, however, differ from the three that we employ in our study of *E. coqui*, and the specific pattern of molecular heterogeneity obtained in *Gastrotheca* shows both similarities and differences relative to the pattern in *Eleutherodactylus*. Both studies reveal a molecular difference between mandibular and hyoid neural crest streams. Only in *Gastrotheca*, however, do the same markers that reveal this difference also differentiate between anterior and posterior portions of the branchial stream. No molecular differences within the branchial stream are observed

in *Eleutherodactylus*. A second difference concerns HNK-1, which is expressed by migrating neural crest-cells in *E. coqui* but is not expressed in *G. riobombae* (see below). Finally, a difference in expression between the medial and lateral portions of the mandibular stream is seen in *E. coqui* (both HNK-1 and cholinesterase; Table 1), but not in *G. riobombae*.

Interspecific differences in molecular expression

Our final reason for assessing the expression of the above markers in *E. coqui* was to see if we could identify molecular features of cranial neural crest that correlate with reproductive mode and which might underlie associated differences in embryonic cranial patterning and developmental anatomy between direct-developing and metamorphosing anurans. The paucity of comparable data from other species, however, precludes any definitive discussion of this topic at this time, and the following conclusions are tentative.

Of the three potential molecular markers for cranial neural crest that we examined, only one, HNK-1 immunoreactivity, appears to be variably expressed among anuran species. We detected no obvious differences in the patterns of cholinesterase staining or Dlx protein expression between *E. coqui* and two metamorphosing species, the fire-bellied toad, *Bombina orientalis*, and the clawed frog, *Xenopus laevis* (Olsson and Hanken, unpublished observations). Indeed, even though HNK-1 immunoreactivity is routinely used as a marker for early migrating neural crest-cells in both teleost fishes and amniotes (references above), *E. coqui*, a direct developer, is the only species of amphibian in which this marker is known to be effective (see Materials and Methods). Other species that represent phylogenetically independent derivations of direct development (Wake and Hanken, 1996; Hanken, 1999) need to be assessed to see if the correlation between HNK-1 expression and reproductive mode is a general phenomenon or one that is limited to *Eleutherodactylus* (or even only *E. coqui*). Variation among closely related mammals, HNK-1 is expressed in cranial neural crest in the rat (*Rattus*; Erickson et al., 1989) but not in the mouse (Kubota et al., 1996), indicates that evolutionary change in HNK-1 immunoreactivity may occur independent of any fundamental change in life history or cranial patterning. The reason for HNK-1 expression in cranial the neural crest in *E. coqui*, and, conversely, for its absence in at least some other frogs, is unknown. As noted in Materials and methods, the HNK-1 antibody used specifically recognizes an acidic sulfated glycosphingolipid, so apparent differences in HNK-1 immunoreactivity among species does not necessarily mean that the underlying HNK-1 proteins are expressed differentially in the species investigated.

The general pattern of cranial neural crest emergence and migration in the direct developing frog *E. coqui* is similar in gross aspects (viz., relative timing and the number and configuration of migratory streams) to that seen in metamorphosing anurans. Patterns of expression for three different molecular markers reveal heterogeneity among neural crest-cells belonging to different streams, as well as molecular differences between cells in different portions of the same stream. Uniquely among those anuran species sampled to date, *E. coqui* expresses the HNK-1 epitope in migrating cranial neural crest, but only within the mandibular stream. A causal relation between HNK-1 expression and evolutionary change in reproductive mode remains to be established.

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