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Cranial neural crest emergence and migration in the Mexican axolotl *(Ambystoma mexicanum)*

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Summary

The timing and pattern of cranial neural crest cell emergence and migration in the Mexican axolotl, *Ambystoma mexicanum*, are assessed using scanning electron microscopy (SEM). Cranial neural crest cells emerge and begin to migrate at the time of neural fold closure and soon form three distinct streams. The most anterior (mandibular) stream emerges first, at the level of the mesencephalon. Cells in this stream migrate rostroventrally around the optic vesicle. The second (hyoid) and third (branchial) streams emerge in close succession at the level of the rhombencephalon and extend ventrolaterally. Cells forming the hyoid stream migrate rostral to the otic vesicle, whereas the branchial stream divides into two parallel streams, which migrate caudal to the otic vesicle. At later stages (stage 26 onwards) the cranial neural crest cells disperse into the adjacent mesoderm and can no longer be followed by dissection and SEM. The pattern of cranial neural crest emergence and migration, and division into migratory streams is similar to that in other amphibians and in the Australian lungfish (*Neoceratodus forsteri*). Emergence of crest cells from the neural tube, relative to the time of neural tube closure, occurs relatively late in comparison to anurans, but much earlier than in the Australian lungfish. These results establish a morphological foundation for studies in progress on the further development and fate of cranial neural crest cells in the Mexican axolotl, as well as for studies of the role of cranial neural crest in cranial patterning.

Key words: head development, cell migration, pattern formation, salamander

Introduction

There is now renewed interest in comparative embryological studies, as part of broader attention to the relationship between evolution and development, or "evo-devo." Many recent textbooks give overviews of this field (Arthur, 1997; Gerhart and Kirschner, 1997; Hall, 1998; Carroll et al., 2001; Wilkins, 2002). Most contemporary work in developmental biology involves either studies of a single organism or comparisons among a small number of so-called model species, and often the main interests are early embryonic patterning and conserved genes and functions. It is also important, however, to extend comparative studies to embrace other species, as well as the variation in developmental processes and mechanisms that underlies the evolution of novelties.

This paper presents data on cranial neural crest development in the Mexican axolotl (*Ambystoma mexicanum*). This is perhaps the most well studied salamander species embryologically, and it has often been used as a model for salamanders in general. The Mexican axolotl remains an important organism for studies whose main focus is on the evolution of development. The present study is part of a larger project on the evolution of head development in lungfishes and amphibians, including both frogs and salamanders (e.g., Olsson et al., 2000). We emphasize neural crest development because the cranial neural crest has played an important role in the evolution of cranial patterning (Hunt et al., 1991;

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Hanken and Thorogood, 1993; Langille and Hall, 1993). Relatively little research has been done on the cranial neural crest in urodeles since the classic studies by Landacre (1921) and Stone (1922), and later by Sellman (1946) and Hörstadius and Sellman (1946). Indeed, a comprehensive study of cranial neural crest emergence, migration and fate, using modern techniques such as electron microscopy and fluorescent markers, does not exist for any urodele. Such a study is long overdue and would provide an important baseline for further studies of both developmental and evolutionary questions relating to the neural crest and its derivatives. The two studies containing scanning electron micrographs of the urodele cranial neural crest focus on somitomere development (Jacobson and Meier, 1984) and cranial nerve development (Northcutt and Brändle, 1995), respectively, and studies of the expression of patterning genes in the developing cranial neural crest are almost totally lacking (but see Epperlein et al., 2000). The present work provides data on the emergence and migration of the cranial neural crest in the axolotl as assessed with scanning electron microscopy. It represents the initial part of a comprehensive study of axolotl head development, which is being followed up by studies of cell differentiation and fate. We discuss our data in the context of earlier work on cranial neural crest migration in lungfishes and anurans.

Materials and methods

Embryos

Wild type and albino embryos of the Mexican axolotl, *Ambystoma mexicanum*, were obtained from the breeding colony at the Evolutionary Biology Centre, Uppsala University, Sweden, and from the Axolotl Colony, Indiana University, Bloomington, USA. Embryos were staged according to Bordzilovskaya et al. (1989) and reared using standard procedures.

Scanning electron microscopy

Early migration of cranial neural crest cells was followed by scanning electron microscopy (SEM). At least four embryos each of stages 17–25 were dejellied mechanically with forceps and fixed in modified Karnovsky fixative (1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M cacodylate buffer) overnight or longer (Karnovsky, 1965). They were then transferred to 0.1 M phosphate buffer, and the epidermis removed with tungsten needles. Embryos were postfixed in 1% OsO4 in 0.1 M cacodylate buffer for 1 h. After rinsing in 0.1 M phosphate buffer, they were dehydrated in an ethanol series (50%, 70%, 90%, 95% and absolute ethanol) and transferred into liquid CO_2 in a critical point dryer. The dried embryos were mounted, sputter-coated with gold/palladium, and examined in a Philips CM10 scanning electron microscope. Digital pictures were obtained as TIFF files. Scanning electron micrographs were assembled into montages, each containing 4–24 separate images.

Results

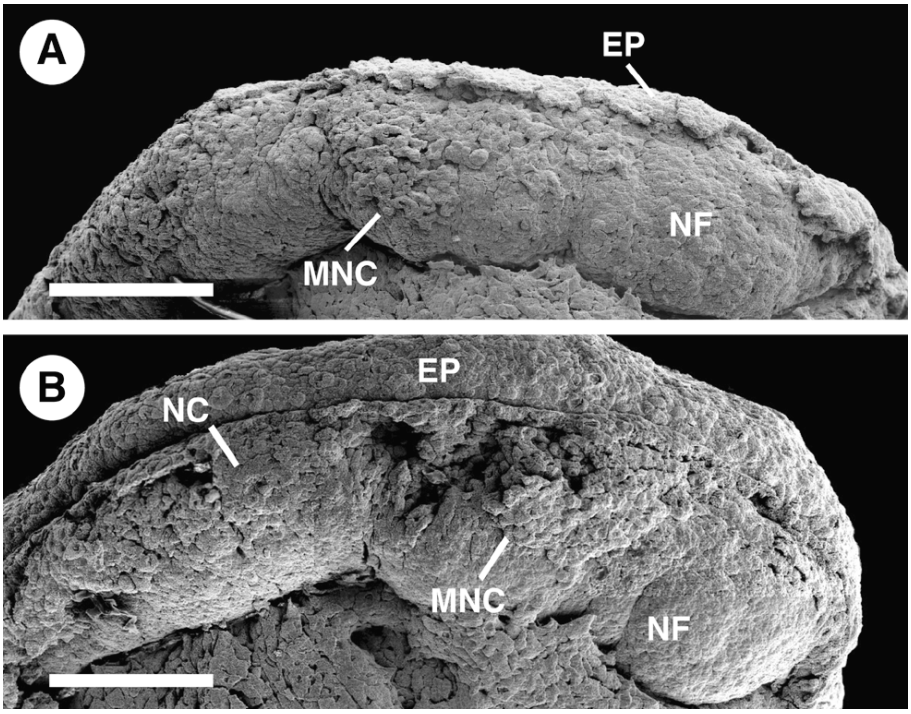
Cranial neural crest cell migration

At stage 17, cranial neural crest cells have not yet differentiated. Epidermis covers the neural fold and no crest cells are visible (data not shown). The mandibular neural crest stream appears initially at stage 18, atop the neural folds at the level of the future mesencephalon. Its cells begin to migrate rostrally and ventrolaterally between the epidermis and the neural folds (Fig. 1A). At stage 19, the mandibular neural crest stream continues to migrate rostroventrally. A second neural-crest stream appears on the neural tube, at the level of the rhombencephalon, and its cells begin to migrate ventrolaterally between epidermis and the neural folds (Fig. 1B).

Neural folds fuse at stage 20, forming the neural tube. The mandibular neural crest stream continues to migrate rostroventrally. The second neural crest stream is subdividing to form two distinct streams, an anterior hyoid stream and a posterior branchial stream, both of which migrate ventrolaterally (Fig. 2A, B). By stage 21, the mandibular neural crest stream surrounds the optic vesicle. The hyoid and branchial streams continue to migrate ventrolaterally between the epidermis and neural tube (Fig. 3A). Stage 22 resembles stage 21, although the hyoid stream has begun to extend onto the surface of the mesoderm. Crest-free zones on the neural tube between the mandibular, hyoid and branchial streams are more pronounced than in earlier stages (Fig. 3B).

By stage 23, the once thick layer of mandibular neural crest cells on the dorsal part of the neural tube is thinning (Figs. 4A, C). Hyoid and branchial neural crest streams continue to migrate ventrally between the epidermis and mesoderm. The branchial neural crest stream begins to divide into two parallel streams (Fig. 4B).

At stage 24, the once single branchial neural crest stream is clearly divided into two parallel streams (Figs. 5A, B). The hyoid neural crest stream migrates ventrally faster than either of the two branchial streams and begins to overtake them. On the dorsal part of the neural tube, the mandibular and hyoid streams remain connected by a patch of cells, although only a few cells



Figs. 1–6. Scanning electron micrographs of embryonic *Ambystoma mexicanum* showing the emergence of cranial neural crest from lateral (Figs. 1–4 and 6) and dorsal (Fig. 5) views. Anterior is to the right. Overlying epidermis (EP) has been removed.

Fig. 1. (A) Stage 18 embryo. The mandibular neural crest (MNC) has emerged from between the epidermis (EP) and the neural fold (NF) at the level of the mesencephalon. **(B)** Stage 19 embryo. Mandibular neural crest cells are migrating anteroventrally. A second neural crest stream (NC) has emerged onto the neural fold (NF) at the level of the rhombencephalon. Scale bars, 0.2 mm.

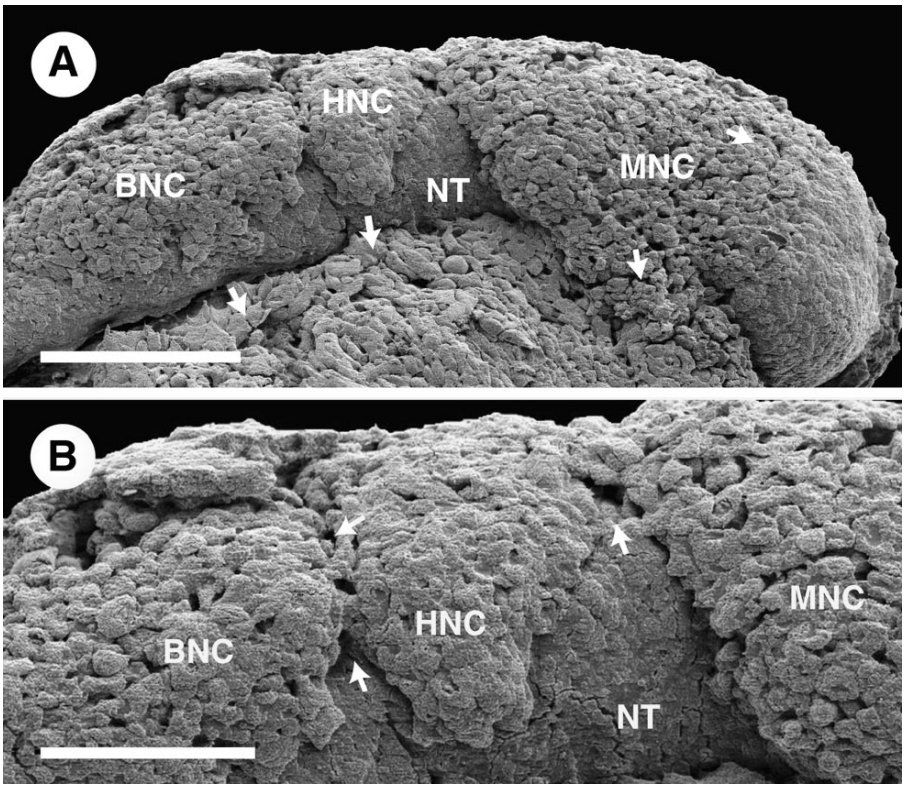


Fig. 2. (A) Stage 20. The mandibular neural crest (MNC) has migrated further anteroventrally. The second neural crest stream is subdividing to form an anterior hyoid neural crest (HNC) stream and a posterior branchial neural crest (BNC) stream. Both of these streams migrate ventrolaterally. Arrows indicate the direction of cell migration. NT, neural tube. Scale bar, 0.2 mm. **(B)** Close-up of A. Left arrows point to the few neural crest cells remaining between hyoid and branchial streams. The right arrow points to the crest-free area between mandibular and hyoid streams. Scale bar, 0.1 mm.

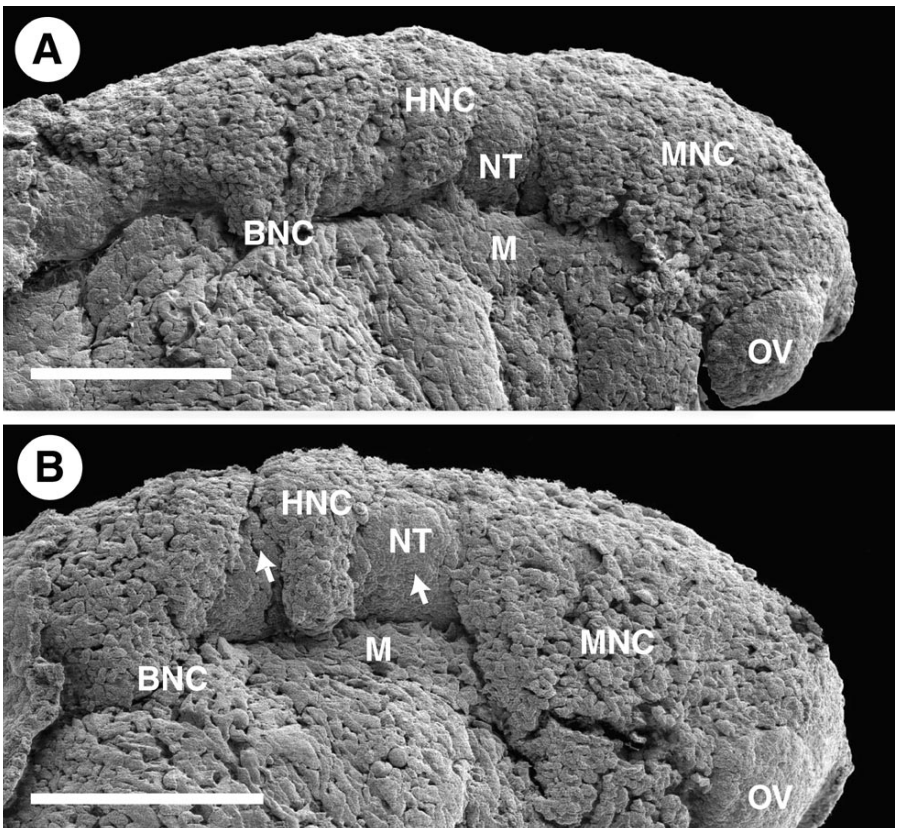


Fig. 3. (A) Stage 21. The mandibular neural crest (MNC) migrates around the optic vesicle (OV). The branchial stream (BNC) is beginning to migrate from the surface of the neural tube (NT) onto mesoderm (M). **(B)** Stage 22. The hyoid neural crest stream (HNC) has reached the mesoderm (M). Arrows point to crest-free zones on the neural tube. Scale bars, 0.2 mm.

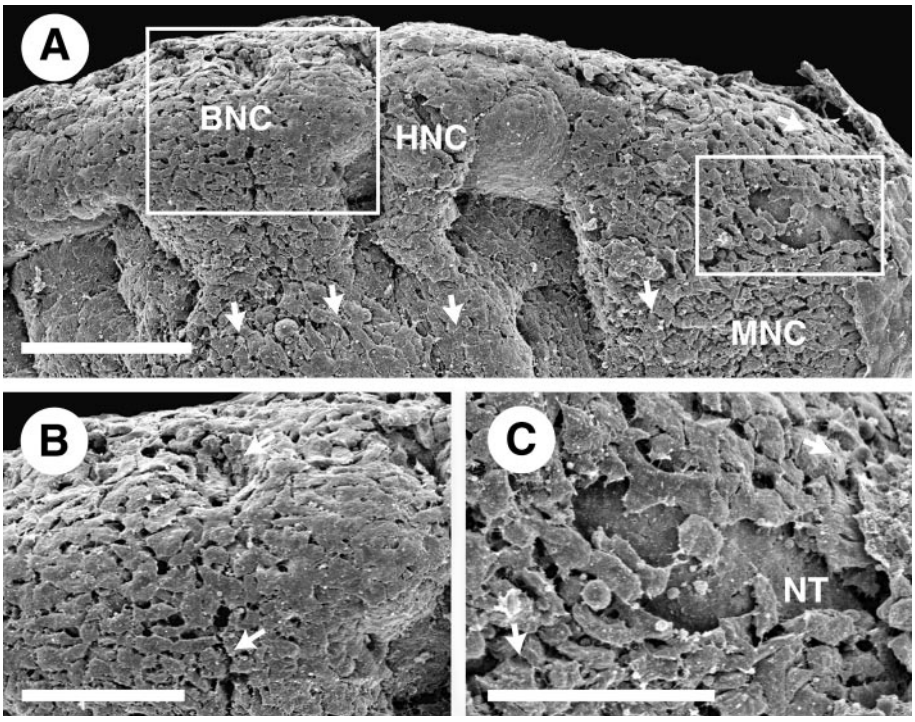
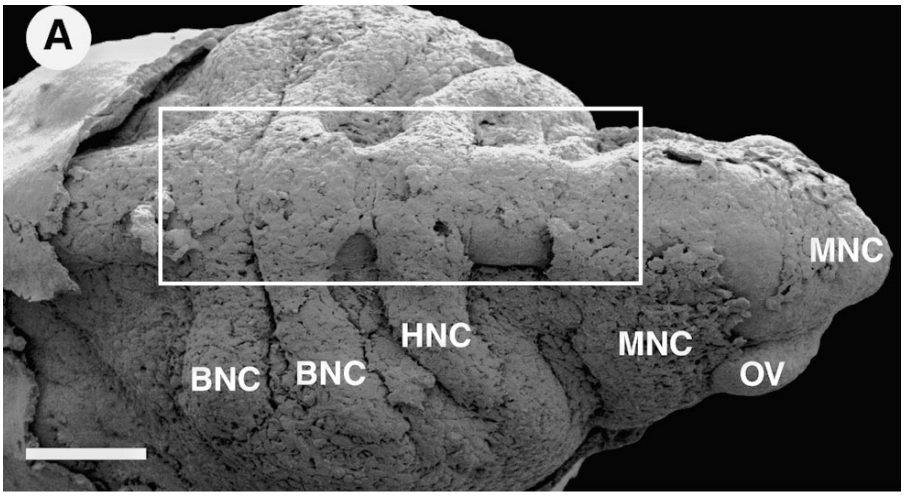


Fig. 4. (A) Stage 23. Mandibular (MNC), hyoid (HNC) and branchial (BNC) neural crest streams continue to migrate ventrally and slightly cranially, as indicated by arrows. Scale bar, 0.2 mm. **(B)** Close-up of left inset in A. The branchial neural crest stream is subdividing to form two parallel streams, which are separated by a narrow cleft (arrows). Scale bar, 0.1 mm. **(C)** Close-up of right inset in A. Continuing migration of mandibular neural crest cells begins to expose the surface of the neural tube (NT). Arrows indicate the direction of neural crest cell migration. Scale bar, 0.1 mm.



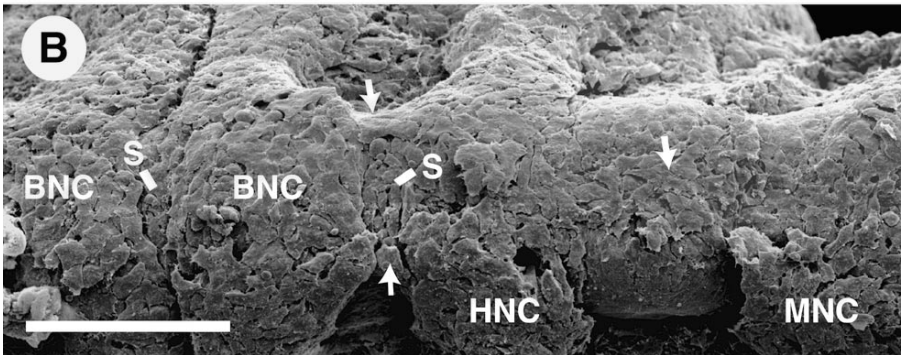


Fig. 5. Dorsal view of a stage 24 embryo. **(A)** Only a few cells from the mandibular neural crest (MNC) stream remain on the dorsal part of the neural tube. The branchial neural crest stream (BNC) is now clearly divided into two distinct, parallel streams. Scale bar, 0.2 mm. **(B)** Close-up of inset in A showing the formation of dorsal sulci (S) between the two branchial neural crest streams (BNC) and between the anterior branchial and hyoid neural crest streams (HNC). The anterior branchial, hyoid and mandibular streams nevertheless remain connected by thin zones of neural crest cells dorsally on the neural tube (arrows). OV, optic vesicle. Scale bar, 0.1 mm.

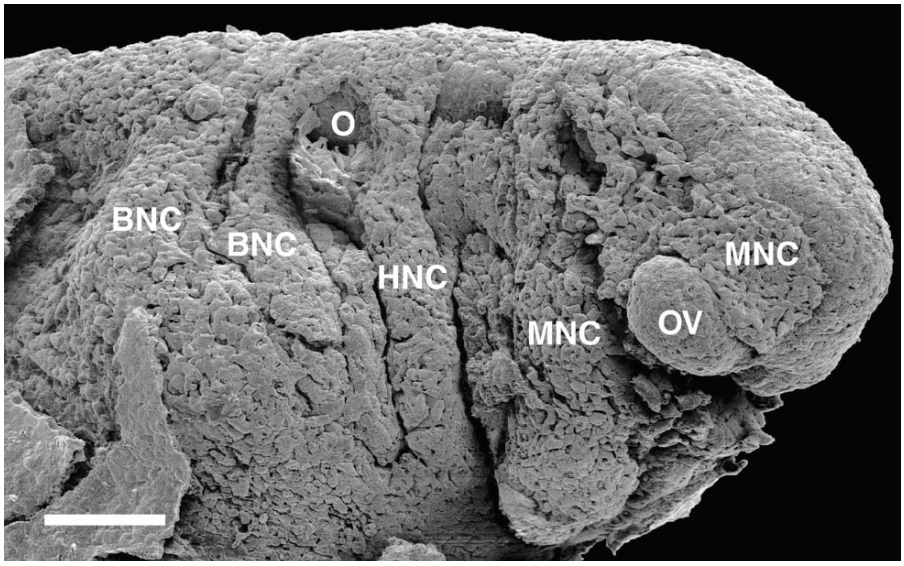


Fig. 6. Stage 25. The mandibular neural crest stream (MNC) surrounds the optic vesicle (OV). The hyoid neural crest stream (HNC), which lies rostral to the otic vesicle (O), has migrated the furthest ventrally of all the streams. Two branchial streams of cranial neural crest (BNC) are seen post-otically. Neural crest cells are beginning to disperse from their respective streams into the adjacent mesoderm. Scale bar, 0.2 mm.

remain from the mandibular neural crest stream (Fig. 5B). Two sulci have formed, beginning mid-dorsally, and appear elliptically shaped when seen from above (arrows in Fig. 5B). They separate the hyoid and two branchial streams dorsally, but a few cells still connect the hyoid and anterior branchial streams laterally.

By stage 25, the mandibular neural crest stream surrounds the optic vesicle (Fig. 6). The hyoid stream has migrated further ventrally. Subsequently, cranial neural crest cells begin to disperse from their respective streams into adjacent mesoderm. Their migration and fates can no longer be followed by dissection and SEM.

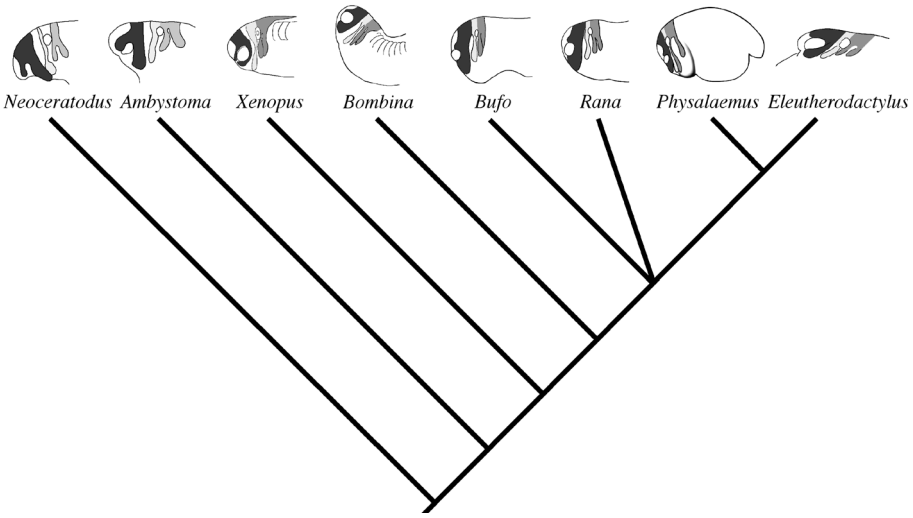


Fig. 7. Cladogram showing the conserved pattern of cranial neural crest streams at comparable stages in Australian lungfish, Mexican axolotl and several anurans. Species depicted are *Neoceratodus forsteri*, *Ambystoma mexicanum*, *Xenopus laevis*, *Bombina orientalis*, *Bufo bufo*, *Rana arvalis*, *Physalaemus pustulosus*, and *Eleutherodactylus coqui*. All are lateral views; anterior is to the left. Note that in *Neoceratodus* the migration of cranial neural crest commences much later than in amphibians, and the antero-posterior gradient is much more pronounced at early stages than at the stage depicted here.

Discussion

In the axolotl, cranial neural crest cells first emerge at stage 18, when they begin to form the largest and most anterior stream, the mandibular stream. They emerge from between the epidermis and the neural fold, at the level of the mesencephalon, and begin to migrate as the neural folds are about to close. A second stream of cranial neural crest cells begins to emerge at stage 19, at the level of the rhombencephalon, and by stage 20 it has divided into two separate streams. The more anterior and smaller of the two, the hyoid stream, migrates ventrolaterally, rostral to the otic placode (Fig. 1A, 2). The remaining, or branchial stream migrates ventrolaterally. It further divides into two distinct streams at stage 23 (Fig. 4). In earlier stages, the two branchial streams migrate faster than the hyoid stream, but by stage 24 the hyoid stream has overtaken both branchial streams, as well as the mandibular stream, and has migrated the furthest ventrally of them all (Fig. 5). The mandibular, hyoid and branchial neural crest cells form distinct streams, which generally are separated from one another by crest cell-free zones. However, adjacent streams remain connected with each other on the neural tube by small patches of crest cells (Figs. 5B, 6).

These data based on SEM are more detailed than those from earlier studies of the cranial neural crest in urodeles obtained using other methods. They both validate and extend the earlier results. Landacre (1921) and Stone (1922), working with species of *Plethodon* and *Ambystoma*, respectively, described migrating cranial neural crest cells in serial sections, taking advantage of the fact that neural crest cells can be distinguished histologically from mesoderm cells in the early stages of migration. These authors were mostly interested in ganglion formation, but their observations on cranial neural crest migration are consistent with our results. Hörstadius and Sellman (1946; reviewed in Hall and Hörstadius, 1988) applied vital dyes to prospective neural crest cells and showed migratory streams derived from those cells. Although their drawings depict only some of the stages covered in the present study, their representations of cranial crest streams are in general agreement with our results. Using SEM, however, we provide more details on how the cranial neural crest cells assemble into streams and on cell shape and orientation at different stages of migration. Short term (a few days, at most) fate mapping using a fluorescent cell label also shows marked cells in positions that correspond to the cranial crest streams directly observed with SEM (Epperlein et al., 2000). SEM observations at later stages than those used in the present study (27–35 vs. 17–25; Bordzilovskaya et al. 1989) by Northcutt and Brändle (1995), show the further development of the cranial crest streams in relation to other tissues, but neural crest cells cannot be reliably distinguished from other cells at these stages.

The assembly of emerging cranial neural crest cells into discrete migratory streams is a general phenomenon in amphibians, as well as in the Australian lungfish (Fig. 7). Indeed, the number and identity of cranial neural crest streams is highly conserved from lampreys to mammals (Kuratani et al., 1997, 2001; Hall, 1999; Horigome et al., 1999). Within classes of vertebrates, even drastic changes in embryonic cranial development and morphology, such as those associated with the evolution of direct development in amphibians (Hanken et al., 1992; 1997b), leave these basic features of cranial neural crest streams intact (Moury and Hanken, 1995; Hanken et al., 1997a). In the Mexican axolotl, as well as in the frogs we have studied, cranial neural crest

cells emerge along an anterior to posterior gradient; the mandibular stream forms first, followed by the hyoid stream, etc. The gradient, however, is relatively weak; by later stages of migration, all streams extend a similar distance from the neural tube (e.g., Figs. 5 and 6). In this respect, the Australian lungfish deviates significantly from most terrestrial vertebrates by having a very steep anteroposterior gradient in cranial neural crest development. The anteriormost (mandibular) stream, for example, is well developed before there is any sign of the branchial streams (Falck et al., 2000). The marsupial *Monodelphis domestica* is another species with a very steep anteroposterior gradient in cranial neural crest emergence (Smith, 2001).

Although cranial neural crest cell migration often begins around the time of neural tube closure, many species deviate from this pattern. Migration begins somewhat earlier in the axolotl than in newts (Jacobson and Meier, 1984), for example, and it begins even earlier – before closure of the neural folds – in most anuran amphibians (Sadaghiani and Thiébaud, 1987; Olsson and Hanken, 1996). Extremely early emergence of the cranial neural crest is seen in mammals (marsupials are a particularly drastic example) and some teleosts, where cranial crest cells can be seen migrating already at the neural plate stage. At the other extreme, cranial neural crest migration begins very late, long after the neural tube is completely closed, in the Australian lungfish (Falck et al., 2000). It An important task for future studies is to further explore these timing differences, as well as their possible causes.

Conclusions and future prospects

Cranial neural crest cell migration in the Mexican axolotl conforms to the general pattern seen in other tetrapods. Emerging cranial neural crest cells form mandibular, hyoid and branchial migratory streams, which develop in a gradient such that anterior streams develop slightly earlier than posterior ones. Emergence of crest cells from the neural tube occurs relatively late in comparison to anurans, but much earlier than in the Australian lungfish. These results provide a morphological foundation for studies currently in progress on the further development and fate of cranial neural crest cells in the Mexican axolotl, as well as for studies on the role of the cranial neural crest in cranial patterning.

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