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Embryonic origin of some evolutionarily significant viscerocranial structures in amphibians

PhD. thesis

by

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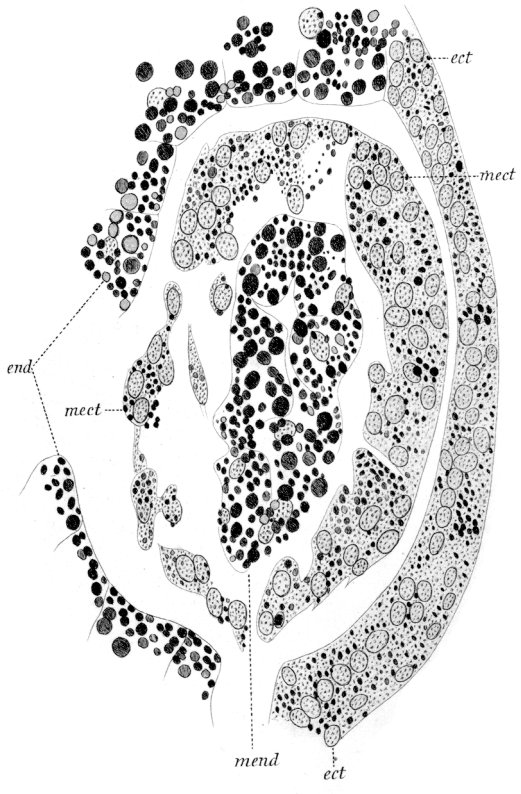
From being considered as merely a source for cranial and spinal ganglia to being a major player in the evolution of vertebrate head, the road [of neural crest cells] was long and winding (**Ericsson, 2003**)



Hyomandibularspalte; Taf. 40.

Julia B. Platt: **Ontogenetische Differenzirung des Ektoderms in *Necturus*.**

Archiv für Microsk. Anat. Entw. Mech. (Platt, 1894)

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ABOUT THIS THESIS

This thesis entitled "Embryonic origin of some evolutionarily significant viscerocranial structures in amphibians" is intended as a doctoral dissertation for the degree of Doctor of Philosophy (PhD.) in the Faculty of Science, Charles University, Prague. This thesis contains an introductory part covering some conceptual definitions and basic approaches in the field as well as briefly summarizing the novelties and outcomes of my published papers in the context of head patterning processes. However, the main part of this thesis is represented by three papers added in the end.

The purpose of this study was to contribute to the solution of a suite of long-standing questions about head development and evolution known in continental biology as "das Kopfproblem". In fact, the inquiries regarding head origin and morphogenesis, especially whether the vertebrate head is segmented or not, was the origin of morphology itself (Kuratani, 2003). The vertebrate cranium is a very complicated structure being both anatomically complex and phylogenetically diverse, and to reveal the nature of head morphogenesis has been a key goal for generations of comparative anatomists and embryologists (see Goodrich, 1930; de Beer, 1937; Jarvik, 1980). However, recent advantages in the field of developmental biology that elucidate the cellular properties and molecular mechanisms of cranial tissue development offer unique opportunities to understand and to directly test cranial tissue interactions and hierarchies, as well as to examine the role of developmental processes in evolutionary change. The classical anatomical-embryological issue of head morphogenesis might thus be re-interpreted in a much more subtle way; we can now reveal the developmental mechanisms which solve questions like: "How do the cranial structures form in the proper location, with their proper sizes and shapes?" or "How are cranial elements orchestrated together to finally form one structural unit, the cranium?".

The evolution of morphological characters is undoubtedly mediated by the evolution of hierarchically organized developmental programmes. To pin down the segmentation of the vertebrate head we analysed how the pattern of "segmental structures" is actually generated during embryogenesis. In the first paper (Cerny et al., 2004b) we focused on the development and morphogenesis of the pharynx and neural crest streams. We followed migration of cranial neural crest cells in detail (as the key tissue for building the vertebrate head) in the Mexican axolotl (*Ambystoma mexicanum*) and described the proximate mechanisms underlying tissue regionalization. We revealed intrinsic as well as extrinsic properties as important for neural crest cell migration, and described a novel mechanism, an "outside-in movement". This study, the most comprehensive analysis of cranial neural crest migratory pathways in any vertebrate, suggests a dual process for patterning the cranial neural crest. Together with an intrinsic tendency to form separate streams, neural crest cells are further constrained into channels by close tissue apposition and sorting out from neighboring tissues. Therefore, aside from the fact that cranial neural crest streams are commonly considered as segmental structures showing a clear metamerization, they are in fact only a secondarily regionalized tissue depending on the segmentation of neural and pharyngeal generative constraints.

In the second paper (Ericsson et al., 2004), the role of cranial neural crest cells in the formation of visceral arch musculature was investigated, again in the Mexican axolotl. We showed that neural crest cells contribute to the connective tissues, but not the myofibers, of developing visceral arch muscles. These do, however, initially develop in their proper location also in the absence of neural crest cells. Our experimental data indicate, that the initial formation of visceral arch musculature is controlled by mesodermal factors. However, the cranial neural crest-derived connective tissues provide directional guidance, which is important for the proper extension of the cranial muscles and their subsequent attachment to the insertion on the correct cartilage. In a comparative context, these data support the view that the cranial neural crest

plays a fundamental role not only in the development of the skeleton of the vertebrate head but also in the anatomical patterning of the cranial muscles, and that this might be a primitive feature of cranial development in vertebrates.

In the last published paper (Cerny et al., 2004a) we studied the morphogenetic processes by which cranial neural crest cells within the first arch build the primordia for jaw cartilages and the anterior part of the cranium in both the Mexican axolotl and chick embryo. We showed that the common textbook opinion about a contribution from a dorsal ("maxillary") primordium to the upper jaw structures and from a ventral ("mandibulary") primordium to the lower jaw structures is not true. The upper primordium was found to contribute to the trabecula cranii and both jaw cartilages were found to be formed entirely from the ventral primordium. The data might indicate that the so-called first arch of vertebrates is a much more complex structure than previously assumed, however, in the context of head patterning processes I tend to conclude that there are no needs to consider the trabecula as an element of a premandibular arch true. In the end of the paper we proposed a hypothesis regarding subtle developmental modification that provides a mechanism through which changes may have arisen in jaw evolution.

Clearly, the vertebrate cranium shows a dissociation of segmental processes: dorsal "neural" and ventral "pharyngeal" regions are under the influence of very different patterning mechanisms, and the trunk body plan is being characterized by still another kind of patterning. Interestingly, these mechanisms do not seem to be derived from a single one ancestor; vertebrate head and trunk are therefore dissimilar in their metamerization. This new view on the body plan of vertebrates (see citations in the following chapters) leads to a new concept of serial homology, for instance, and clearly disproves classical imaginations about the ancestral pattern of vertebrate craniogenesis.

Cerny, R., Meulemans, D., Berger, J., Wilsch-Brauninger, M., Kurth, T., Bronner-Fraser, M. and Epperlein, H.-H.: **Combined intrinsic and extrinsic influences pattern cranial neural crest migration and pharyngeal arch morphogenesis in axolotl.**
Developmental Biology; 2004, 266: 252-269.
(IF 2003 (ISI®) = 5,4)

Ericsson, R., **Cerny, R.**, Falck, P. and Olsson, L.: **The role of cranial neural crest cells in visceral arch muscle positioning and patterning in the Mexican axolotl, *Ambystoma mexicanum*.**
Developmental Dynamics; 2004, 231: 237-247.
(IF 2003 (ISI®) = 3,2)

Cerny, R., Lwigale, P., Ericsson, R., Meulemans, D., Epperlein, H.-H. and Bronner-Fraser, M.: **Developmental origins and evolution of jaws: new interpretation of "maxillary" and "mandibular".**
Developmental Biology; 2004, 276: 225-236.
(IF 2003 (ISI®) = 5,4)

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ON THE EMBRYONIC ORIGIN OF SOME EVOLUTIONARILY SIGNIFICANT VISCEROCRANIAL STRUCTURES

The cranium as a composite structure

Although merged into a harmonious unit, the vertebrate skull is a composite structure comprising three distinct parts with dissimilar phylogenetic origins (e.g., Kardong, 1995; Liem et al., 2001). The *viscerocranium* (or *splanchnocranium*) is the most ancient cranial part that – according to a common belief (see citations above) - evolved from pharyngeal arch elements supporting gill slits of cephalochordate-like animals. Viscerocranial components support the gills and/or contribute to the jaw and hyoid apparatus in gnathostomes; however, they probably form parts of anteriormost cranium as well. The second part, the *neurocranium*, consists of endochondral bone or cartilage elements that surround and protect the brain and sensory organs. This part of the skull was originally supposed to have evolved from fused vertebrae; however, we are still very far from a consensus about of how many vertebrae the neurocranium is comprised or whether it consists of vertebrae at all. Finally, the outer dermal skeleton or *dermatocranium* is composed exclusively of dermal (membrane) bones that, phylogenetically, arise from the bony armor of the integument of early fishes and is added to the neuro- and splanchnocranium. The vertebrate skull elements can also be seen as belonging to either an *exoskeleton*, which is formed from dermis within the integument (dermatocranium), or to an *endoskeleton*, which originates from mesoderm or mesenchyme deeper in the body (neuro- and viscerocranium).

Regarding the germ layer origin of cranial elements, the viscerocranium seems to be derived exclusively from neural crest cells (for a review and more species-specific quotations, see Hall and Hörstadius, 1988; Hall, 1999; Le Douarin and Kalcheim, 1999). Interestingly, however, the name viscerocranium stems from a mistaken view saying that these cells originate from the same embryological source as the wall of the digestive tract, i.e., from the endoderm

(Kardong, 1995). The neurocranium, according to a common opinion, evolved from fused vertebrae and thus, *per definitionem*, these components are of mesodermal (somitic) origin. Dermatocranial bones, on the other hand, develop as condensations of the dermis, which in the head originates either from neural crest or mesodermal cells, depending on the position alongside the antero-posterior axis (e.g., Larsen, 1993; Liem et al., 2001).

Does the embryological origin of cranial elements matter?

All these cranial components of diverse phylogenetic and developmental origin arise during ontogenetic development as a result of complicated cell movements and tissue interactions. Finally, however, all these elements become integrated into one structural and functional unit – the cranium. Knowing how single skeletal elements originate in development might provide crucial evidence about their evolutionary origin as well; recall that similarity of development is one of the key tests we use for deducing evolutionary homology. („Community of embryonic structure reveals community of descent" (Darwin, 1859)).

For example, it would be considered strange if a bone of mesodermal origin in one vertebrate is a direct homologue of a bone of neural crest origin in another species. However, the parietal bone seems to illustrate just such a case: in the mouse it originates from cephalic paraxial mesoderm (Morriss-Kay, 2001; Jiang et al., 2002), whereas in chicken the parietal bone was traced back to a neural crest origin (Couly et al., 1992; Couly et al., 1993) (Fig. 1). In the clawed frog (*Xenopus laevis*), the only other animal for which the embryonic origin of a membrane bone is currently known (Gross and Hanken, 2004b), a single fronto-parietal bone does originate from neural crest cells as well. These differences suggest that either the pattern of neural crest contribution to the vertebrate skull has changed significantly during evolution, or that widely accepted skull bone homologies across major clades may be incorrect (e.g., Gross and Hanken, 2004b).

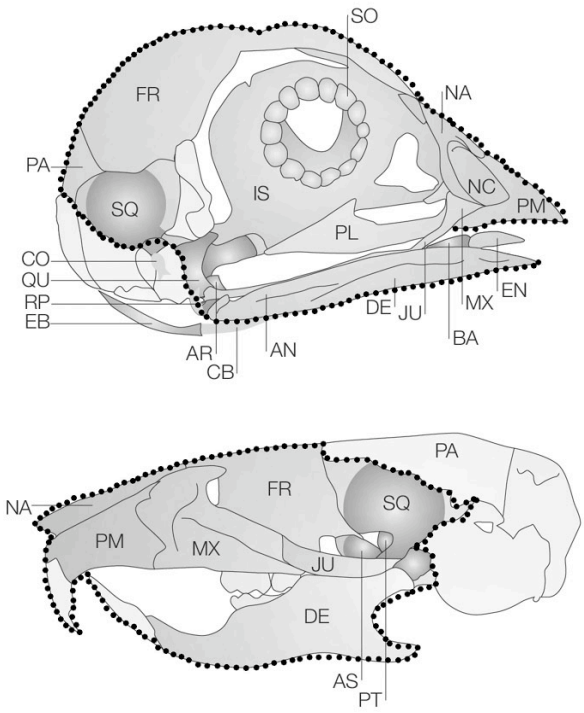

FIG. 1: CONTRIBUTION OF NEURAL CREST CELLS (encircled by dark dotted lines) TO THE SKULL OF CHICK (upper panel) AND MOUSE (lower panel). Modified from (Santagati and Rijli, 2003); according to (Le Douarin and Kalcheim, 1999; Morriss-Kay, 2001). Notice, that the parietal bone (PA) originates from neural crest in chick whereas from mesoderm in mouse.

That the same final tissue, either cartilage or bone, can develop from independent embryonic cell lineages tells us something important about the flexibility of developmental programs and regulatory pathways which underlies evolutionary changes. Head evolution - as recently seen - was clearly largely driven by functional co-option and elaboration of different embryonic cell lineages by neural crest cells (Meulemans et al., 2003; Rudel and Sommer, 2003). Interestingly, cranial neural crest cells appear to be a highly plastic population (e.g., Trainor and Krumlauf, 2000b; Santagati and Rijli, 2003, for recent reviews). For example, avian neural crest cells, when experimentally transplanted into the mesodermal mesenchyme, do actually participate in the formation of bones and some neighboring elements normally derived from

mesoderm (Schneider, 1999). Neural crest cells are therefore able to respond to environmental cues that otherwise promote mesodermal skeletogenesis. This would have been seen as nearly unimaginable just a few years ago.

Admittedly, the embryonic origin of skull elements does matter; however, homology of these components does not equate simple constancy of embryonic origin of single elements, tissues or organs. The whole issue about defining the "sameness" in the vertebrate head is therefore much more complicated than previously thought, and we have to be able to determine e.g. the constancy of cell lineages, cell fates and gene regulation on many levels of biological organization to solve this question (for reviews, Raff, 1996; Hall, 1998a; Arthur, 2000; Hall, 2003; Wake, 2003).

Neural crest cells and building the vertebrate head

Clearly, in the head of all vertebrate species examined in some detail, neural crest cells create or at least contribute to most of the cranial cell types and tissues (Couly et al., 1992; Couly et al., 1993; Hall, 1998a; Le Douarin and Kalcheim, 1999). Neural crest cells thus in fact "build the vertebrate head" (Santagati and Rijli, 2003).

The neural crest is an embryonic cell population unique to vertebrates, and a key vertebrate character (Gans and Northcutt, 1983; Benton, 1998; Hall, 1998a; Hall, 1998b; Ruffins et al., 1998; Selleck et al., 1998; Studer et al., 1998; Le Douarin and Kalcheim, 1999; Shimeld and Holland, 2000; Le Douarin and Dupin, 2003), but see (Jeffery et al., 2004). From an evolutionary point of view it has been proposed that the evolution of vertebrates from a cephalochordate-like ancestor was driven largely by the evolution of neural crest and neurogenic placodes (Gans and Northcutt, 1983; Northcutt and Gans, 1983). This assertion reflects the fact that neural crest and placodes give rise to adult structures that define the vertebrate clade (Shimeld and Holland, 2000), and thus the neural crest can be considered a fourth germ layer (Hall, 1998b; Hall, 2000).

TAB. 1: MESENCHYMAL DERIVATIVES OF THE NEURAL CREST

CEPHALIC NEURAL CREST:

Dermatocranium:

Frontal, Parietal, Squamosal, Sphenoid (basipre-), Otic capsule (partly), Nasal, Vomer, Maxilla, Jugal, Quadratojugal, Palatine, Pterygoid, Dentary, Opercular, Angular, Supraangular.

Chondrocranium:

Nasal capsule, Interorbital septum, Scleral ossicles, Meckel's cartilage, Quadrate, Articular, Hyoid, Columella, Entoglossum.

Odontoblasts and tooth papillae

Other tissues:

Dermis, smooth muscles, adipose tissue of the skin over the calvarium and in the face and ventral part of the neck; Musculo-connective wall of the conotroncus and all arteries derived from aortic arches (except endothelial cells); Pericytes and musculo-connective wall of the forebrain blood vessels and all of the face and ventral neck region; Meninges of the forebrain; Connective component and tendons of ocular and masticatory muscles; Connective component of the pituitary, lacrymal, salivary, thyroid, parathyroid glands and thymus.

TRUNK NEURAL CREST:

Dorsal fins in lower vertebrates

Based mostly on data from avian embryos (Le Douarin et al., 2004)

Cranial neural crest cells differentiate into a wide variety of derivatives as different as myofibroblasts, fibroblasts, cartilage, bones, melanocytes, endocrine tissues and various types of neurons and glial cells in the peripheral nervous system (Tab. 1) (for a review, Couly et al., 1993; Etchevers et al., 2001; Le

Douarin and Dupin, 2003; Le Douarin et al., 2004). Connective and supportive tissues are generated only by cranial neural crest cells (e.g., Noden, 1991; Couly et al., 1993), although a hidden capacity of trunk neural crest to yield skeletal derivatives (Epperlein et al., 2000; McGonnell and Graham, 2002) can be revealed by appropriate environmental cues (Le Douarin et al., 2004).

Problematic identification of neural crest cells

Neural crest cells seem to be a predominant component of the vertebrate head, as seen from the table above. However, our knowledge of this issue is very limited and in fact relies on just a few of model animals. These results are often generalized for all other vertebrate species. How is it possible that something as common as the embryological origin of main skeletal structures is not precisely known? The main problem is associated with the transient nature of the neural crest, the invasiveness of these cells and their problematic identification.

Neural crest cells were discovered by Wilhelm His (His, 1868) and named "Zwischenstrang" (intermediate cord). The participation of neural crest cells in the facial and visceral skeleton was first recognized at the end of the 19th century by the pioneering work of Kastschenko on selachian embryos (Kastschenko, 1888) and of Julia Platt on *Necturus* (Platt, 1893). However, there had been great controversies about the importance and even existence of these cells for a very long time (see, e.g., Hall, 1998a; Hall, 2000). As nicely pointed out by others: "One hundred years ago, claiming that an ectodermal derivative such as the neural crest was in any way involved with the formation of skeletal structures was the embryological and evolutionary equivalent of nailing an additional thesis to the cathedral door. That skeletal structures were mesodermal in origin was dogma, known and accepted by all; an ectodermal origin was heresy" (Langille and Hall, 1993). Establishing the neural crest as not only a source of cranial and spinal ganglia, but actually a major player in

skeletogenesis and the evolution of the vertebrate head itself was a difficult process.

After the recognition that neural crest cells do exist, the central issue in this controversy was to distinguish cranial mesenchyme of neural crest origin (ectomesenchyme or mes-ectoderm) from mesenchyme of mesodermal origin (mes-endoderm). Classical studies attempted to analyze neural crest cell migration and differentiation by means of simple histological techniques (e.g., Platt, 1897; Landacre, 1921; de Beer, 1947), extirpation and transplantation experiments (e.g., Stone, 1929; Ichikawa, 1937; Hörstadius and Sellman, 1946), chromatic dyes (e.g., Hirano and Shirai, 1984) or radiographic labeling (e.g., Johnston, 1966; Chibon, 1967). However, the main problem has persisted: mesenchymal cells of very different embryonic origin actually do not contain any specific morphological, histological, biochemical or molecular components which can unequivocally be distinguished in adult tissue. Yet, despite the fact that for amphibians, the neural crest has long been attributed a direct role in cranial bone development (for amphibians, e.g., Hörstadius and Sellman, 1946; de Beer, 1947), direct evidence for neural crest contributions to the bony skull has remained elusive (but see Carl et al., 2000; Gross and Hanken, 2004b; Gross and Hanken, 2004a).

The only way of a reliable detection of neural crest cells is to perform lineage fate-maps (Fig. 2), i.e. to trace single cells or small cell clusters from their origin to the places of final differentiation of descendants of these cells (Clarke and Tickle, 1999; Stern and Fraser, 2001). Such reliable cell markings are mostly carried out using fluorescent dyes such as DiI or DiO (e.g., Lumsden et al., 1991; Serbedzija et al., 1992; Osumi-Yamashita et al., 1994; Epperlein et al., 2000; McCauley and Bronner-Fraser, 2003), or by injecting GFP mRNA or FITC-conjugated dextrans followed by experimental embryological or laser un-caging techniques (e.g., Carl et al., 2000; Sato and Yost, 2003). Recently transgenic Cre/lox recombinated mice where for instance the *Wnt-1* gene expression indelibly marks neural crest cells have also been used (e.g., Chai et al., 2000;

Jiang et al., 2000). Another possibility is to create chimaeric animal systems where neural crest cells from a donor species (like *Xenopus borealis*) are transplanted to a closely related host species (*X. laevis*) in which donor neural crest cells can be recognized by some specific feature (Sadaghiani and Thiebaud, 1987; Krotoski et al., 1988). Undoubtedly, the "modern era" of neural crest research started after the introduction of quail-chick chimaeras by N. Le Douarin in the late 1960s (see Le Douarin and Kalcheim, 1999, for a review). However, our direct and reliable knowledge about cranial neural crest cell contributions to skeletal (and especially bony) tissues is based almost exclusively on research on avian embryos (Noden, 1983; Couly et al., 1992; Couly et al., 1993), with some minor and very recent contributions based on mice (Jiang et al., 2002) and frogs (Gross and Hanken, 2004b; Gross and Hanken, 2004a) .

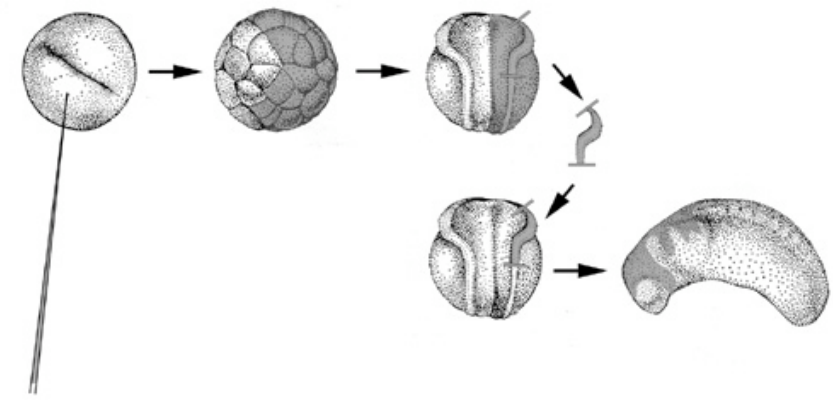

FIG. 2: LINEAGE NEURAL CREST FATE-MAPPING IN AXOLOTL. GFP mRNA is injected into one blastomere where it incorporates into DNA; thus all descendant cells are GFP-positive (turn green after illumination of UV light). When GFP-positive neural fold (a precursor population of neural crest, NC) is homotopically grafted into a host embryo, all NC cells emigrating from the graft are GFP-positive and thus green. In this way, the population of NC cells can be certainly traced for very long time.

Interestingly, data from these different tracing approaches tend to show that amphibians, birds and mammals possess unique patterns of neural crest contribution to the skull (Gross and Hanken, 2005). At least in some major groups of tetrapods the patterns of neural crest derivation of the skull seems to be evolutionarily labile and have not been rigidly conserved during vertebrate history.

The viscerocranium and the nature of the trabecula cranii

Of all skeletal structures formed during embryogenesis, the viscerocranium and especially the facial skeleton is frequently considered to be the most intriguing and intricate (Richman and Lee, 2003; Clouthier and Schilling, 2004). The viscerocranium comprises the parts thought to be derived from pharyngeal arch elements; thus, interestingly, the only pragmatic definition of viscerocranial elements is their neural crest origin (e.g., Kardong, 1995; Le Douarin and Kalcheim, 1999). Consequently, the anterior internal cranium (roughly until the otic level, see Fig. 3), being neural crest-derived, is considered to belong to the viscerocranium, although it is topographically placed within the neurocranium.

A predominant component of this "neurocranial" viscerocranium is a paired cartilage known as *trabecula cranii*. The trabeculae (or praechordalia) were discovered first by Rathke in the grass-snake (de Beer, 1931) and later recognized as the essential elements of the cartilaginous anterior cranial base in all vertebrates, just as parachordal cartilages are the basis from which the posterior region of the skull arose (e.g., de Beer, 1931; Bertmar, 1959; Kuratani et al., 1997; Liem et al., 2001). Whereas parachordal cartilages and thus elements of the posterior skull are mesoderm-derived, the opinion that trabeculae belong in fact to visceral structures was validated only by their neural crest origin (e.g., de Beer, 1931; de Beer, 1937; Kuratani et al., 2001). Therefore, according to an accepted evolutionary story (see Fig. 3), the trabecula represents a viscerocranial component of the neurocranium being metamerically organized in register with cranial nerves and pharyngeal arches. From a radical

segmentalist point of view (e.g., Bjerring, 1977) the trabecula might be considered as an original cartilaginous component of a hypothetical pre-oral arch (Allis, 1923; Jarvik, 1980) - but this view became highly controversial recently (Janvier, 1996; Shigetani et al., 2005).

It is difficult to find a developmental support for segmental theories. For example, in the Mexican axolotl (*A. mexicanum*) the trabecula originates among the neural crest cells on the dorsal side of the embryonic mandibular arch, in the maxillary portion (de Beer, 1931; Cerny et al., 2004a). This fact can be interpreted such that the maxillary part of the first arch is in fact the ancient pre-mandibular arch that fused with the mandibular arch, as mentioned for instance by deBeer (de Beer, 1931). Therefore, the trabecula could be regarded as a true element of the pre-mandibular arch (Allis, 1923; Allis, 1924). Alternatively the trabecula, with its origin in the maxillary portion, might be explained as the ancient dorsal element of the mandibular arch. One cannot find any strict developmental support for segmental theories, everything depends on interpretatios and unfortunately, there is no useful fossil evidence in favour of any of these theories (for a recent review, see Janvier, 1996).

In the context of the viscerocranium and its neural crest origin, it is interesting to note that a condensation of the main anterior cranial component in the lamprey (called here trabecula as in all jawed vertebrates) could be traced back to the first arch mesoderm instead (Kuratani et al., 2004). The trabecula of the lamprey - according to these authors – is most likely an equivalent to the parachordal, a mesodermally derived element in gnathostomes. A true homologue of the vertebrate trabecula in lamprey, i.e., the premandibular and prechordal part of the neurocranium might be found in the ectomesenchyme of the upper lip.

The other interesting case, where truly viscerocranial elements do not seem to be derived from neural crest cells, is the basihyal and the second basibranchial cartilage in frogs. These elements are traditionally considered to arise from mesoderm (Hall, 1999). Moreover, many structures on the border between neural crest- and mesoderm-derived areas (e.g., the posterior

trabeculae or *pila antotica*) seem to be of mixed origin (e.g., Olsson and Hanken, 1996).

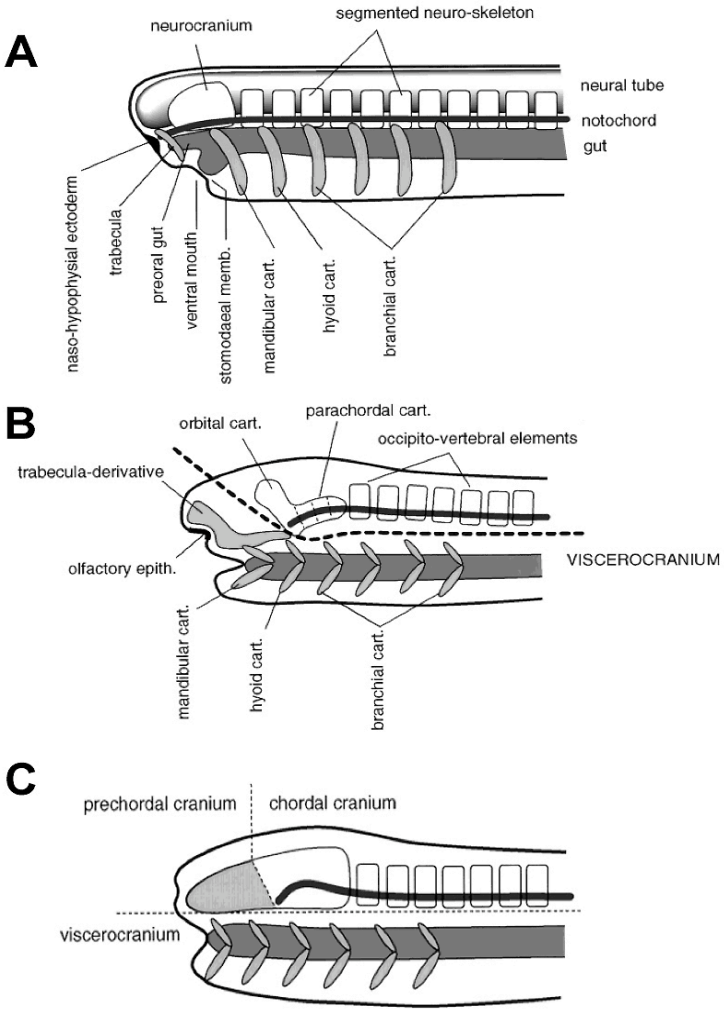

FIG. 3: EVOLUTIONARY HYPOTHESIS EXPLAINING THE TRABECULAR CARTILAGE AS A VISCEROCRANIAL ELEMENT. A: As an ancestral state of a hypothetical early vertebrate animal, the trabecula comprises an anterior visceral arch structure being topographically in a pre-oral and dorso-anterior position. **B:** During the course of evolution, trabecula anlarged to protect anterior brain and subsequently become the secondary (chondrocranial) viscerocranium. Thick dotted line indicates the border between neural crest-derived viscerocranium (dark) and mesoderm-derived chondrocranium (white). **C:** Consequent morphological plan of vertebrate cranium consists of dorsal chondrocranium and ventral viscerocranium; however, the prechordal cranium is neural crest-derived whereas chordal cranium is mesoderm-derived. Based on (Kuratani et al., 1997).

We therefore ask whether traditionally regarded viscerocranial elements, like the basibranchial and basihyal catilages of frogs, as well as the trabecula of lamprey, really belong to the viscerocranium, even if they do not seem to be derived from the neural crest? Clearly we have no answer yet, however, any purely embryological definition of the viscerocranium is very problematic. First we have to take the surprising plasticity of neural crest cells (mentioned above) into account. Cranial neural crest cells are capable to condense in response to the signals which normally initialize mesodermal skeletogenesis (Schneider, 1999). Dermal bones, for example, might develop either from neural crest-derived dermis in the anterior head or from mesoderm-derived dermis as is the case in more posterior head regions (for a review, see Le Douarin and Kalcheim, 1999), depending on the type of skeletogenic tissue available. Therefore, it is easily conceivable that in contrast to the classical theory described above (Fig. 3), an anterior cranial element like the trabecula might develop *de novo* in order to support the enlarging brain. As only prerequisite an existing informative signal would be needed for condensation that meets pluripotent neural crest cells. Interestingly, that trabeculae are structures *sui generis* was also assumed by Goodrich (Goodrich, 1930), among others. However, in his opinion trabeculae are non-segmented structures of mesodermal origin, as he never accepted other than neuronal derivatives of neural crest.

Neither the embryological derivation from neural crest cells nor evolutionary way of definition can be used here as a definitive proof. Any conceivable definitions of viscerocranial elements we can propose are very problematic, therefore, we do not know whether the trabecula cranii really belongs to the viscerocranium.

MECHANISMS OF CRANIAL PATTERNING AND SEGMENTATION

„The study of segmentation is comparable to the study of Apocalypse – that way leads to madness – A.S. Romer"

As mentioned above, asking whether the vertebrate head is segmented or not was the origin of morphology itself (Kuratani, 2003). According to a common past opinion, the vertebrate head is metamerical, meaning that the head develops from serially homologous segments or metameres with identical potency to develop homological structures within each metamere. From the strictest segmentalist point of view, "a single metameric pattern" can be observed within the head of vertebrates (Bjerring, 1977). More recently, however, the vertebrate head is defined as „a new head" (Gans and Northcutt, 1983; Northcutt and Gans, 1983), a *de novo* developed structure being in fact the main invention of vertebrates. Below I analyse segmentation mechanisms during ontogenetic development of different cranial tissues and germ layers in order to understand their role in establishing the pattern of cranial structures. Segmentation mechanisms apparently bias or canalize development of cranial tissues according to ancestral patterns. On the other hand, an orchestral balance must exist between original segmentation and an obvious plasticity in cranial neural crest cells (especially in the most anterior, the trigeminal area) that is largely responsible for the endless variation of vertebrate craniofacial features.

Cranial neural crest streams are patterned, not segmented

After delamination from the neural folds, neural crest cells migrate extensively along defined pathways. Although all neuromeres - except the most anterior ones - generate neural crest cells, cranial neural crest migration is strictly portioned into three common streams which are distinct and often separated by clear neural crest-free zones (for reviews, Hall, 1999; Le Douarin and Kalcheim, 1999). Thus the first neural crest stream, the trigeminal or mandibular stream, is separated from the second one, the hyoid, by the crest-

free zone alongside rhombomere 3 (the rhombencephalon, or hindbrain, is transiently separated into neuroepithelial compartments, rhombomeres). In a similar way, a neural crest-free zone at rhombomere 5 and the adjacent otic placode separates the hyoid from the third, or common branchial neural crest stream. This later one divides into several sub-streams according to the number of branchial arches in the organism (e.g., Sadaghiani and Thiebaud, 1987; Lumsden et al., 1991; Horigome et al., 1999; Epperlein et al., 2000; Falck et al., 2000; Meulemans and Bronner-Fraser, 2002; Trainor et al., 2002). Interestingly, the general pattern of cranial neural crest cell migration is highly conserved across vertebrate species (Fig. 4). However, mechanisms might differ that keep cranial neural crest streams separate and guide neural crest cells along correct pathways during craniofacial development (for discussion, see Cerny et al., 2004b).

FIG. 4: PATTERN CONSERVATION OF CRANIAL NEURAL CREST STREAMS IN VERTEBRATES. Embryos going from lungfish (*Neoceratodus*) to many different kind of amphibians (*Ambystoma* = axoltol; *Xenopus-Physalaemus* = frogs with very different larvae; *Eleutherodacrylus* = frog species with a direct development) displey very constant pattern of early cranial neural crest stream migration. From (Falck et al., 2002). Basically, this stable pattern is seen in all known vertebrate embryos from lamprey to mammals.

At first, patterning of cranial neural crest cells into streams is intrinsic and depends on a molecular signaling from the rhombencephalon (e.g., Guthrie and Lumsden, 1991; Birgbauer and Fraser, 1994; Trainor and Krumlauf, 2001). Neural crest cell clusters originating from different rhombomeres adopt a distinct set of molecular and cellular properties (like the transcription factors *kreisler* and *Krox20*, and *ephrin/Eph* receptors or ordered domains of *Hox* gene

expression) to maintain their own positional identity, segmental characteristics and to prevent mixing between streams (for reviews, see Trainor and Krumlauf, 2000b; Trainor and Krumlauf, 2001). However, recently it has been shown that this molecular pre-patterning of cranial neural crest cells is not definitive but rather plastic, because, for instance, *Hox*-genes can be regulated independently in neural tube and emigrating neural crest cells (especially in the case of the anterior-most gene, *Hoxa-2*) (e.g., Prince and Lumsden, 1994; Trainor and Krumlauf, 2000a; Schilling et al., 2001).

Moreover, molecular signals or mechanical constraints from other cranial tissues help to maintain cranial neural crest streams separate (see Cerny et al., 2004b, for discussion). For example, in the zebrafish *van gogh* (*vgo*) mutant, segmentation of pharyngeal arches is specifically affected (Piotrowski et al., 1996). Thus the endoderm does not form pouches and consequently neural crest streams fuse at a ventral (pharyngeal) but not dorsal (paraxial) level where neural crest cells migrate in normal streams (Piotrowski and Nusslein-Volhard, 2000). We found in the Mexican axolotl that ablating the cranial epidermis causes fusion of neural crest streams and finally cessation of neural crest migration (Cerny et al., 2004b).

All these experiments suggest that interactions of neural crest both with ectoderm and endoderm are critical for a proper migration of cranial neural crest cells in streams. Therefore initial intrinsic patterning information within cranial neural crest cells is insufficient for maintaining later neural crest streams (Cerny et al., 2004b). Clearly, cranial neural crest cannot be considered as a segmented structure *per se*; aside from the fact that these cells bring some positional information to the periphery. Cranial neural crest cells are only secondarily constrained or regionalized to move in streams. It is therefore rather questionable whether any kind of information about segmental origin and identity of viscerocraial elements can be found among cranial neural crest cells themselves.

Dual segmentation of the vertebrate head

The neural tube, especially the hindbrain (rhombencephalon) is clearly a segmented structure as rhombomeres are true cell lineage restricted compartments, i.e., clones of cells that do not cross rhombomere boundaries (Fraser et al., 1990; Lumsden, 1990; see also Figdor and Stern, 1993; Larsen et al., 2001 for the case of diencephalon). Migrating neural crest cells then carry positional identity and patterning information from rhombomeres (like *Hox* or *Otx* gene expression codes) to the cranial environment (e.g., Hunt et al., 1991; Rijli et al., 1998; Trainor and Krumlauf, 2001). Neuromeres are certainly primary metameres of the vertebrate head (Neal, 1918).

However, the segmentation of the pharyngeal region occurs independently of that of the hindbrain as shown clearly by the *vgo* zebrafish mutant mentioned above (Piotrowski and Nusslein-Volhard, 2000). Moreover, endodermal pharyngeal pouches are formed, patterned and have a sense of their own identity even in the absence of the neural crest (Veitch et al., 1999; Graham and Smith, 2001).

According to a modern consensus, there are only two segmented structures in the head region of vertebrates, the hindbrain and pharyngeal pouches (e.g., Kuratani et al., 1997; Piotrowski and Nusslein-Volhard, 2000; Kuratani, 2003). "Segmentation" does not simply mean the compartmentalization or epithelialization of tissues, or the restriction of cell lineages associated with these phenomena. It also implies that primary segments serve as generative constraints (see Wagner, 1994) that impose pattern to other tissues (Kuratani, 2003, for a review). As a result, various structures that display evident metameric patterns are in fact non-segmented tissues. I regard this as a clear outcome of modern developmental studies that was undetectable with classical morphological approaches.

For example, early migrating cranial neural crest cells selectively adhere to even-numbered rhombomeres such that rhombomeres 2, 4 and 6 create exit points for neural crest streams (e.g., Lumsden et al., 1991; Köntges and Lumsden, 1996). Also, the patterning of cranial nerves is not independent but in

fact built upon the presence or absence of either even- or odd-numbered rhombomeres, respectively (Kuratani and Eichele, 1993). Moreover, not only neural crest streams and cranial nerves are patterned according to their rhombomeric origin, but the entire ectodermal layer including the presumptive epidermis seems to form genetically defined units, so called ectomeres, according to their neuromeric origin (Couly and Le Douarin, 1990).

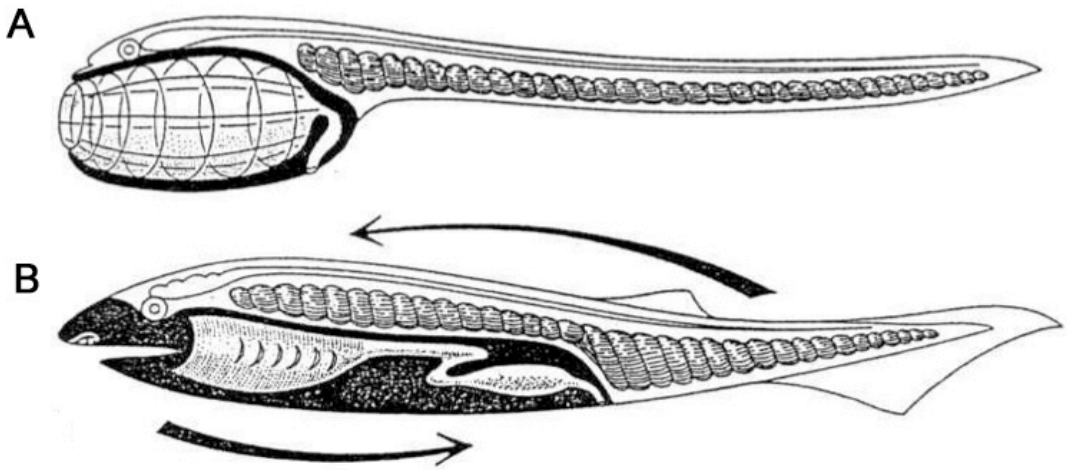

FIG. 5: VERTEBRATE BODY PLAN AS A DUAL METAMERIC PATTERN. Comparing a tunicate larva-like ancestor and the vertebrate body, A. S. Romer pointed out that there are two independent metameric patterns in vertebrates. Vertebrate animals thus can be seen as "visceral" and "somatic" (Romer, 1972). Recent studies added another clear primary segmentation mechanism in the head, the neuromerism, that pattern the anteriormost head (Kuratani, 2003). This complex segmentation of the vertebrate head is seen as the apomorphic feature of vertebrates and therefore an invariant body plan of cephalochordate Amphioxus (e.g., Horder et al., 1993) cannot be directly compared to vertebrates.

The ventral part of the vertebrate head develops, on the other hand, depending on the generative constraints of the pharyngeal pouches (Fig. 5). The endodermal layer of the pharynx forms segment boundaries between arches which limit neural crest migration (Schilling and Kimmel, 1994) and has a profound and direct impact on pharyngeal arch mesoderm. In fact, the initially homogeneous lateral mesodermal layer on both sides of the pharynx is broken

up by reiterative outpocketings of the pharyngeal endodermal pouches into single arch portions (e.g., Kingsbury, 1926; Cerny et al., 2004b). Epibranchial placodes, on the other hand, seem to be induced by the endodermal pouches (Begbie et al., 1999) and experimental elimination of the pharyngeal pouches causes absence of pharyngeal arch and pouch derivatives and also loss of inferior ganglia and distal portions of cranial nerves (Kuratani and Bockman, 1992).

In the vertebrate head, distinctly different developmental mechanisms are therefore used for metamerization of different cranial components; nothing like „a single metamerical pattern" does exist. Neuromerism on the dorsal part and branchiomerism on the ventral part of the embryonic head are primary segmental systems that control patterning of other cranial tissues. From an evolutionary point of view, this proximate understanding of how reiterative elements or structures of the vertebrate head develop is an essential step towards an explanation of how the body plan of pre-vertebrates was modified and how the key vertebrate synapomorphy - a complex vertebrate head – evolved.

Head vs. trunk segmentation: the vertebrate head is not a continuation of the trunk

"… within the last few years it has been more or less generally accepted that the head is, in part at least… merely a modified portion of the trunk and composed, like that, of a series of homodynamous segments." (Balfour, 1878)

The classical accounts, emanating from J. W. von Goethe and L. Oken hold that the metamerism of the vertebrate body extends throughout its length (Fig. 6). Head and trunk segmentation was seen comparable or identical; head and trunk just became specialized in divergent directions, therefore they look so different now (for bibliography see Goodrich, 1930; de Beer, 1937; Bertmar, 1959; Bjerring, 1977; Jarvik, 1980; Horder et al., 1993); but see also (e.g., Froriep, 1902; Kingsbury, 1926; Starck, 1963). Head metameres were regarded as once

having all the essential components of typical trunk metameres, namely myotome, sclerotome, neuromere, somatic motor and somatic sensory nerves, as well as sympathetic anlagen (Neal, 1914; Bjerring, 1977).

Most of the evidence for similarities between head and trunk metamerism was observed in the mesoderm. Classical morphologists were sure that a true somitic segmentation occurs in the pre-otic and post-otic regions alike (e.g., Van Wijhe, 1883; Platt, 1897; Koltzoff, 1899). However, in fact, pre-otic cranial mesoderm is never separated up to clear somites, even at very early developmental stages. Instead, histological descriptions of Balfour and others drew attention to mesodermal cranial cavities that were thought to be serially homologous to trunk somites. Interestingly enough, segmentalists were unable to find a clear conclusion about how many segments or metameres actually exist or once existed in the vertebrate head. For the braincase alone, Oken, Owen, Huxley and Goette saw four segments, Goethe six, Balfour eight and van Wijhe nine. Moreover, when the braincase and visceral skeleton are combined, Jollie considered five and a half metameres, Bjerring nine, Gegenbaur nine or ten and Kingsbury eight to fourteen (Janvier, 1996).

However, considerable progress made within the last decades in developmental biology has clarified that there are profound differences between head and trunk, concerning organization and patterning mechanisms and even cell and tissue behaviour. For example, in the head of all vertebrates neural crest cells migrate in three common streams (see the chapter above), whereas in the trunk neural crest cells migrate singly. Moreover, a number of studies have shown that trunk neural crest cell patterning depends upon the metamerical organization of somites because only the anterior portion of each somite (of each sclerotome) is permissive for neural crest cell migration (Bronner-Fraser, 2000; Krull, 2001 for reviews). In strong contrast, cranial neural crest patterning depends primarily on the metamerical organization of neuromeres (Trainor and Krumlauf, 2001; Kulesa et al., 2004 for reviews). Also, for example, cranial and trunk neural crest cells utilize different mechanisms for

attachment to the extracellular matrix components in which they migrate (Lallier et al., 1992).

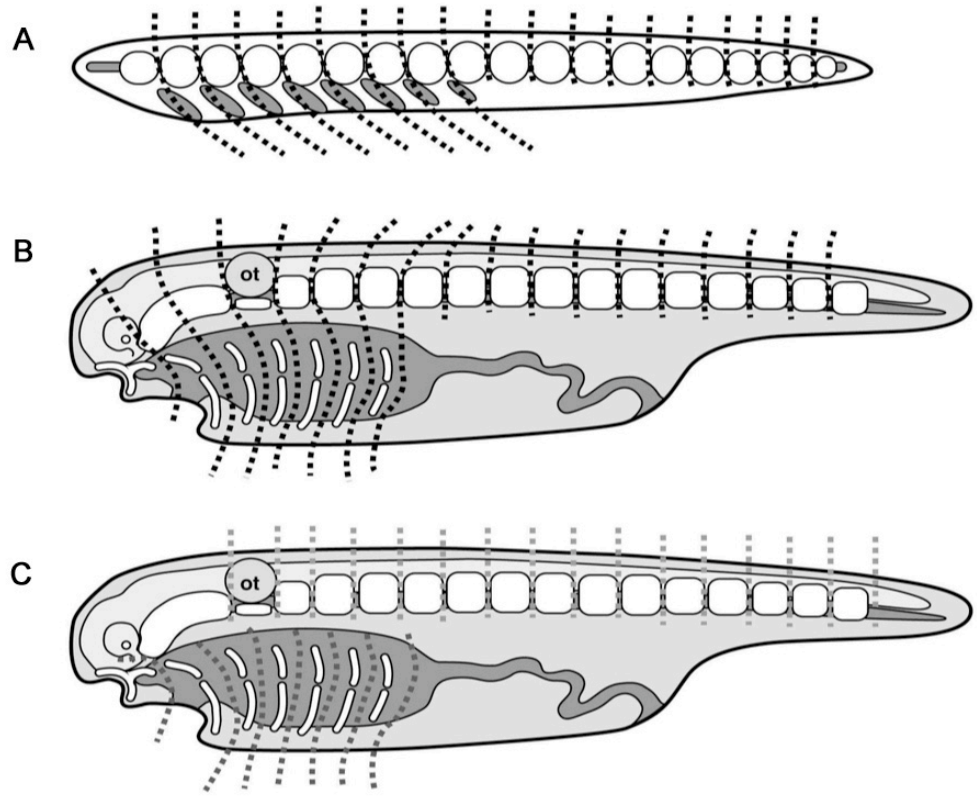

FIG. 6: VERTEBRATE HEAD SEGMENTATION. A: cephalochordate amphioxus as an animal possessing a common body plan. B: segmentalists view based on amphioxus body plan – the vertebrate head is seen to comprise a serial array of homodynamous elements equivalent to somites or vertebrae. In this extreme extrapolation of somitomerism, pharyngeal arches are reiterating with the same intervals as somites. C: a non-segmental view of the vertebrate head: somitomerism is seen in the trunk and more posterior head, whereas branchiomerism and neuromerism are recognized as primary generative constraints in the head. (Kuratani, 2003).

The metameric pattern of trunk spinal nerves seems to depend solely on the arrangement of preexisting somites (which are mesodermal). Rearrangement of trunk somites results in repatterning of the spinal nerves long after neurogenesis (e.g., Keynes and Stern, 1984; Bronner-Fraser, 1986;

Stern et al., 1991). Myelomeres, the neuromeres in the trunk spinal cord, are also generated by the presence of somites (Lim et al., 1987; Lim et al., 1991). However, the pattern and organization of cranial nerves depends on neuromeres.

Clearly, the vertebrate body plan is characterized by the presence of somite-derived constraints in the trunk and rhombomere- and pharyngeal pouch-derived constraints in the pre-otic head (Kingsbury, 1926; Romer, 1972; Kuratani, 2003). Distinctly different patterning mechanisms of cranial vs. trunk cells and tissues strongly contradict the long-standing segmentalist's view that the vertebrate head is (just) a differently specialized part of the trunk (see citations above). Craniates clearly do not pass ontogenetically through an acraniate stage, comparable to the adult form of *Amphioxus,* as initially thought (Neal, 1914). The vertebrate head, with its full array of sensory organs involved in an active lifestyle, is in fact the main invention of vertebrates; this complex organ was added to the body plan of pre-vertebrate animals (Gans and Northcutt, 1983; Northcutt and Gans, 1983). Most of the apomorphic features of vertebrates are embryologically derived from neural crest cells (Hall, 2000) and it is the interplay between patterning and plasticity of the neural crest that is largely responsible for the endless variation of vertebrate craniofacial features. The construction of the vertebrate head may therefore be seen as a result of complex cell migrations and interactions, rather than of almost geometrical anamorphies of independent segments. As pointed out by Janvier (Janvier, 1996), the statement that "this or that organ belongs to the mandibular metamere, which often occurs in segmental writings", does not seem to have much explanatory power anymore.

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Combined intrinsic and extrinsic influences pattern cranial neural crest migration and pharyngeal arch morphogenesis in axolotl

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Abstract

Cranial neural crest cells migrate in a precisely segmented manner to form cranial ganglia, facial skeleton and other derivatives. Here, we investigate the mechanisms underlying this patterning in the axolotl embryo using a combination of tissue culture, molecular markers, scanning electron microscopy and vital dye analysis. In vitro experiments reveal an intrinsic component to segmental migration; neural crest cells from the hindbrain segregate into distinct streams even in the absence of neighboring tissue. In vivo, separation between neural crest streams is further reinforced by tight juxtapositions that arise during early migration between epidermis and neural tube, mesoderm and endoderm. The neural crest streams are dense and compact, with the cells migrating under the epidermis and outside the paraxial and branchial arch mesoderm with which they do not mix. After entering the branchial arches, neural crest cells conduct an "outside-in" movement, which subsequently brings them medially around the arch core such that they gradually ensheath the arch mesoderm in a manner that has been hypothesized but not proven in zebrafish. This study, which represents the most comprehensive analysis of cranial neural crest migratory pathways in any vertebrate, suggests a dual process for patterning the cranial neural crest. Together with an intrinsic tendency to form separate streams, neural crest cells are further constrained into channels by close tissue apposition and sorting out from neighboring tissues.

Keywords: Axolotl; Head; Cranial neural crest; Migration; Branchial arches; AP-2; Snail

Introduction

The neural crest is a transient population of cells that arises from the dorsal neural tube of vertebrate embryos. These cells migrate extensively along defined pathways to give rise to a wide variety of derivatives including pigment cells, neurons and glia (Hall and Hörstadius, 1988; Le Douarin and Kalcheim, 1999). Although both head and trunk neural crest cells share many common derivatives, connective and supportive tissues are predominantly generated by cranial neural crest (Couly et al., 1993; Jiang et al., 2002; Noden, 1988; but see Epperlein et al., 2000; McGonnell and Graham, 2002).

An important question is how cranial neural crest cells are directed to their ultimate destinations during embryogenesis. Fate mapping and transplantation experiments have demonstrated that inherent information in the hindbrain (Lumsden and Krumlauf, 1996; Trainor and Krumlauf, 2000b) contributes to cranial neural crest migratory pattern (Lumsden et al., 1991) and cell fate decisions. However, cell and tissue interactions are also critical (Trainor and Krumlauf, 2000a; Trainor et al., 2002), emphasizing the importance of understanding interactions between neural crest cells and their surrounding environment both at the neural tube and branchial arch level (Farlie et al., 1999; Golding et al., 2002; Trainor et al., 2002).

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Because it is a uniquely vertebrate structure, evolution of the neural crest is intimately interlinked with vertebrate origins (Shimeld and Holland, 2000). To date, neural crest migration is best understood in the avian embryo, though mouse and zebrafish mutants have also improved our understanding of the molecular mechanisms guiding neural crest migration and branchial arch patterning. Much less is known about neural crest migration in other vertebrates. Comparative analysis of neural crest migration in a number of vertebrate species is critical because it can elucidate fundamental vertebrate features as well as reveal mechanisms for generating distinct cranial morphologies. The axolotl possesses several advantages for analyzing pathways of neural crest migration. The slow development of axolotl embryos and their relatively large size allows a gradual tracing of neural crest migration. Furthermore, the premigratory neural crest in axolotl is morphologically distinguishable from the rest of the neural tube, facilitating cell lineage tracing with vital dyes. In the present study, we use the axolotl to investigate whether cranial neural crest migration and patterning into streams is regulated autonomously and/or influenced by adjacent tissues. We identify neural crest cells and the surrounding tissues with a combination of molecular markers, scanning electron microscopy and vital dye analysis in vivo and study cranial neural crest migration from isolated neural tube explants in vitro.

Our results reveal that neural crest cells initially migrate from the neural tube in an autonomous manner, forming separate streams emanating from the hindbrain. Later, these streams are further constrained by ectodermal infoldings that create channels for neural crest migration. At the pharyngeal level, we demonstrate for the first time that cranial neural crest cells migrate initially superficial to the branchial arch mesoderm with which they do not intermix. Then they conduct an ''outside-in'' movement that brings them medial to the arch core and in contact with pharyngeal endoderm. Such a shift in migration is a new developmental mechanism that has been hypothesized in zebrafish (Kimmel et al., 2001) but not previously documented in any vertebrate. The results demonstrate that patterning of the axolotl neural crest involves a combination of intrinsic and extrinsic influences.

Materials and methods

Embryos

Embryos of the Mexican axolotl (*Ambystoma mexicanum*) were obtained, reared and staged as previously described (Epperlein et al., 2000). Before operating on the embryos, they were decapsulated manually.

Injections of DiI and GFP

DiI injection was performed as described previously (Epperlein et al., 2000). For injection, an IM 300 microinjector with fine glass micropipettes that were attached to a needle holder were used (Oxford instruments).

For ectopic expression of GFP (green fluorescent protein) about 1–5 ng GFP mRNA (in 20 nl solution) was injected with a picospritzer into one blastomere of axolotl embryos at a two- to four-cell stage. For injection, the embryos were placed in an agar dish containing 5% Ficoll in normal strength Steinberg solution with antibiotics. After 1 day, the embryos were transferred into 20% saline and after gastrulation into normal strength saline. Embryos were allowed to develop to the neurula stage (15–16) and then used for grafting.

Grafting experiments

Using tungsten needles, green fluorescent head neural fold segments (region 2–7; see Epperlein et al., 2000) from neurulae (stages 16–18) injected with GFP mRNA at the two-cell stage were grafted orthotopically into uninjected hosts (n = 13) and used for the analysis of cranial neural crest migration.

For culturing early cranial neural tubes, the head epidermis was removed from embryos at stages 20–22 (n = 11). Neural tubes reaching from the level of the prosencephalon till the end of the rhombencephalon were carefully excised and placed on a permissive substrate (a polylysine-coated glass disk incubated with fibronectin (100 μg/ml; Sigma). Small pieces of glass were used to press the neural tube against the substrate. Cultures were grown in small plastic dishes at room temperature containing Steinberg solution with antibiotics.

For another experiment, the head epidermis was removed from embryos at stages 23–26 in regions of prospective hyoid and branchial arch neural crest streams. Operations were carried out at room temperature in agar dishes (2% agar in Steinberg solution) containing distilled water and antibiotics. After the operations, embryos were reared in Steinberg solution and allowed to develop to a larval stage.

Histology and immunostaining

Embryos were anaesthetized in a solution of tricaine methane-sulfonate (MS-222, Sandoz) and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PFA/PBS) overnight. After washing in PBS, specimens were dehydrated through a graded series of ethanol and embedded in JB4 (Polysciences, Inc.). Sections (5 μm) were cut with a Reichert-Jung microtome (Biocut 2035), stained with Azure B–Eosin (SERVA) and mounted in DePeX (SERVA). Embryos processed through in situ hybridization were stored in 100% methanol and cut (100 μm) using a Vibratome Series 1000 sectioning system (Ted Pella, Inc.). Sections were counterstained with a primary polyclonal anti-fibronectin antibody (1:100, Dako) followed by a goat anti-rabbit Cy3 secondary antibody (1:100, Dako) to visualize tissue borders. Afterwards, these sections were stained with DAPI (0.1–1 μg/ml PBS) to mark cell nuclei.

Whole-mount double-staining of embryos fixed in DMSO/methanol was performed using mouse monoclonal anti-actin antibodies (CLT 9001, Cedarlane) and polyclonal rabbit anti-β-catenin antibodies (P14L; Schneider et al., 1996). These primary antibodies were detected by goat anti-mouse Alexa 488 (1/50, Molecular Probes) and goat anti-rabbit Cy3 (1/500, Dianova). Incubation times for primary and secondary antibodies were 2–3 days at room temperature to allow complete antibody penetration. Following washing in PBS and postfixation in PFA/PBS, specimens were dehydrated and embedded in Technovit 7100 (Kulzer). Sections (3–5 μm) were counterstained with DAPI to visualize cell nuclei.

Cartilage staining

Larvae (13–17 mm long) were anaesthetized with MS-222, fixed in PFA/PBS overnight, rinsed and transferred into 80% ethanol. Cartilage was visualized with alcian blue using a modified protocol of Kimmel et al. (1998). Staining lasted for about 20 h (at room temperature). After destaining in acid–alcohol and rinsing in PBS, specimens were treated for about 15 h with a mixture of trypsin (0.05%) and EDTA (0.02%) in PBS. Then cartilages were dissected free from surrounding tissue, cleared in a mixture of KOH (1%) and H_2O_2 (1%) under intense illumination and transferred to glycerol/ethanol (1:1).

Scanning electron microscopy

For a direct visualization of cranial neural crest streams, the head epidermis was removed from embryos (stages 18–30) prefixed in Karnovsky's fixative using tungsten needles. After rinsing in PBS, embryos were postfixed with 4% osmium tetroxide. Following another rinse, embryos were dehydrated through a graded series of ethanol, critical point-dried (liquid CO_2), sputter-coated with gold-palladium and examined under scanning electron microscopes LEO 430 or Hitachi S-800.

In living embryos at stage 23–24, the cranial epidermis was folded back on the left side to expose the epidermal invaginations at r3 and r5. The operations were carried out in ice-cold Steinberg solution (fourfold strength) using tungsten needles. Operated embryos were processed further for SEM as indicated above.

Apoptosis

Embryos (stages 17–28) were fixed in MEMFA and stored in 100% methanol at −20°C. After gradual rehydration, embryos were labeled using TUNEL (In Situ Cell Death Detection Kit, Roche). For a negative control, the embryos were incubated in the labeling buffer omitting the enzyme component. No phosphatase positive cells were observed under these conditions, indicating that no endogenous phosphatases were labeled in the experimental embryos. For a positive control, chick embryonic limb buds (7–8 days) and *Xenopus* embryos (stages 24–35) were stained. NBT/BCIP was used for a substrate to visualize the alkaline phosphatase signal.

Cell division

Embryos (stages 20–29) were fixed in PFA/PBS and postfixed in DMSO/methanol. After rehydration, embryos were stained with anti-Phosphohistone-3 (1 μg/ml; Upstate Biotechnology; Hendzel et al., 1997; Saka and Smith, 2001) that detects cells at late G2 to telophase. Antibody incubation was performed overnight at room temperature. Bound anti-Phosphohistone-3 was detected with secondary biotinylated antibodies and a tertiary Avidin–Peroxidase complex. DAB was used as a substrate for the peroxidase (Vectastain Elite ABC Kit). To block endogenous peroxidase, embryos were treated with 1% H_2O_2/PBS before antibody incubation. DAB-stained embryos were washed, postfixed in PFA/PBS and dehydrated in a graded series of methanol. The pigment of embryos was bleached in methanol/H_2O_2. Embryos were cleared in a mixture of benzyl alcohol and benzyl-benzoate (1:2). As a negative control, albino embryos processed in parallel but without the antibody incubation displayed no DAB staining (not shown), ensuring that the observed signals are not due to the activity of endogenous peroxidases.

In situ hybridization of AP-2, Snail and Fgf-8

cDNA was synthesized from *Ambystoma* embryo total RNA (stages 22–35) by poly-T-primed reverse transcription and used as a template for PCR. The following degenerate primers were designed against conserved regions of vertebrate Snail genes and used to amplify an approximately 400 bp fragment of the *Ambystoma* Snail homolog; 5′ primer, AAGACCTAYTCYACKTTCTCTGGG, 3′ primer, CAGCARCCAGAYTCCTCATG. The fragment was cloned, sequenced and compared to known Snail genes using the Megalign program. The conceptual translation product of the axolotl *snail*-like PCR fragment is 84% identical to *Xenopus* Snail and 82% identical to *Xenopus* Slug. Due to high conservation in the amplified zinc-finger region, we cannot conclusively assign this gene to either the Snail or Slug subfamilies, and thus label it simply axolotl *snail*. Preparation of AP-2 riboprobe and in situ hybridization was as previously described (Epperlein et al., 2000). Axolotl Fgf8 cDNA was a gift from R. Christensen and R. Tassava (Christensen et al., 2001).

Image analysis

Whole-mount embryos were photographed under a stereomicroscope (Zeiss) with a Coolpix 950 camera. Sections were investigated under an epifluorescence microscope (Olympus BH 2). Separate brightfield and fluorescence images were captured with a SPOT RT

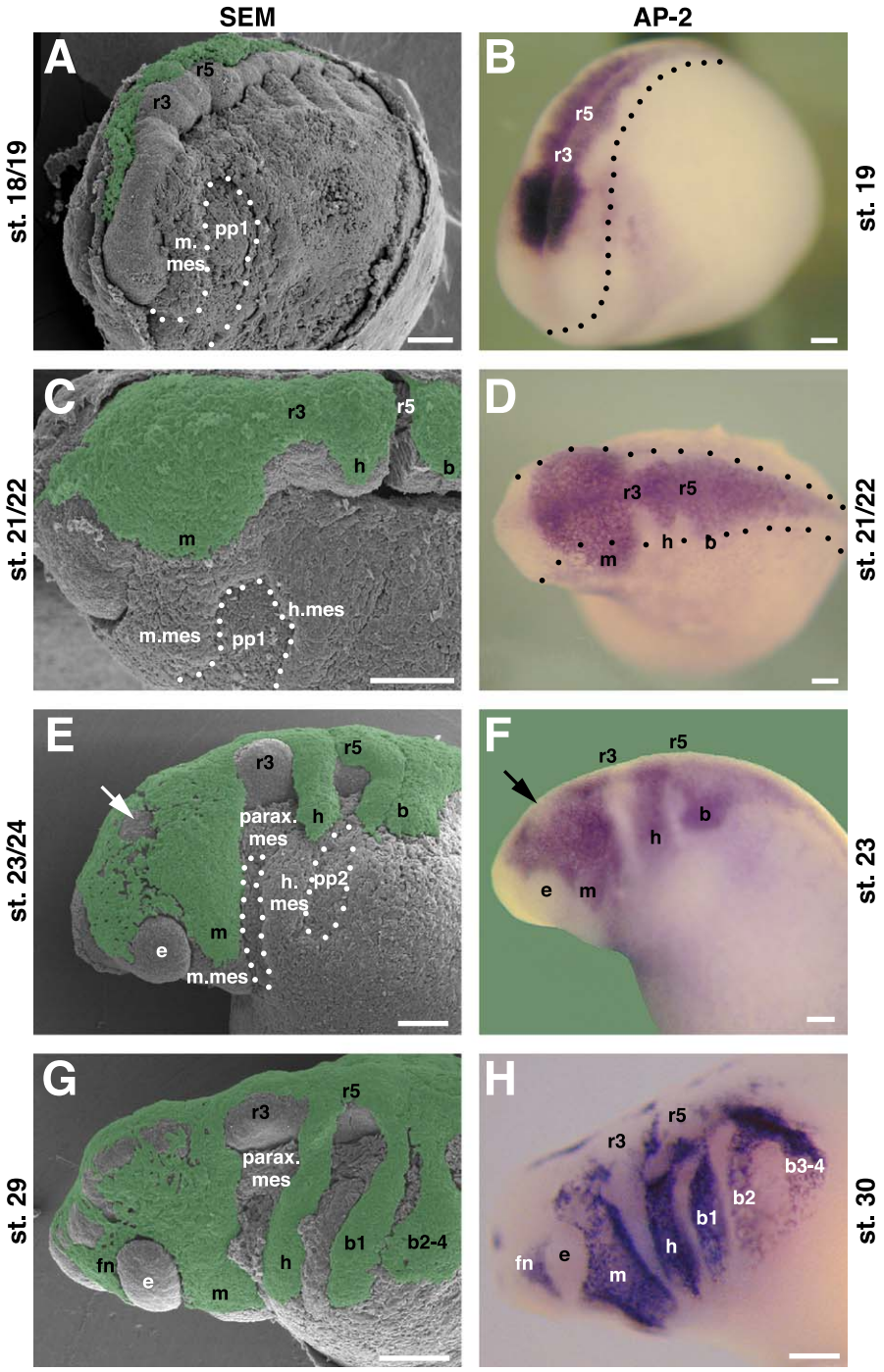


Fig. 1. Morphogenesis of cranial neural crest streams in the Mexican axolotl. (A, C, E, G) SEM micrographs of embryos after removal of the epidermis. The neural crest is colored green. (B, D, F, H) Embryos after hybridization with the AP-2 riboprobe. Head to the left in all cases. (A, B) Premigratory neural crest (stage 18/19). At prospective anterior brain levels, the neural crest is visible between the open neural folds; at the hindbrain, it forms the top of the fused folds. The dotted line in (A) outlines the first pharyngeal pouch (pp1) and in (B) the lateral border of the neural tube. Three heterogeneities are visible along the premigratory neural crest from which the future streams bulge out laterally. Fewer neural crest cells are found at rhombomere (r) r3 and r5. (C, D) Neural crest spreading (stage 21/22). The three initial neural crest bulges have spread laterally. Only the mandibular crest (m) has approached the mandibular mesoderm (m.mes). h, hyoid and b, common branchial neural crest stream, respectively. The dotted line in (C) delineates the dorsal part of the first pharyngeal pouch and in (D) the lateral border of the neural tube. (E, F) Formation of early neural crest streams (stage 23/24). The mandibular stream has migrated onto the mandibular mesoderm, the hyoid and common branchial stream over the paraxial mesoderm (parax.mes). Within the mesencephalon, another crest-free area is visible (arrows). In (E), the contours of the first two pharyngeal pouches are clearly visible (dotted lines). (G, H) Late neural crest migration (stage 29/30). The mandibular stream reaches its ventral-most position; the hyoid and branchial streams have reached a less ventral position. By comparing (G) with (E), the hyoid stream obviously follows the course of the hyoid mesoderm. The branchial stream shows subdivision. fn, frontonasal neural crest mass; b1, first branchial neural crest stream; b2–4, branchial neural crest streams 2–4; h.mes, hyoid arch mesoderm; pp2, pharyngeal pouch 2; e, eye. Scale bars, 200 μm.

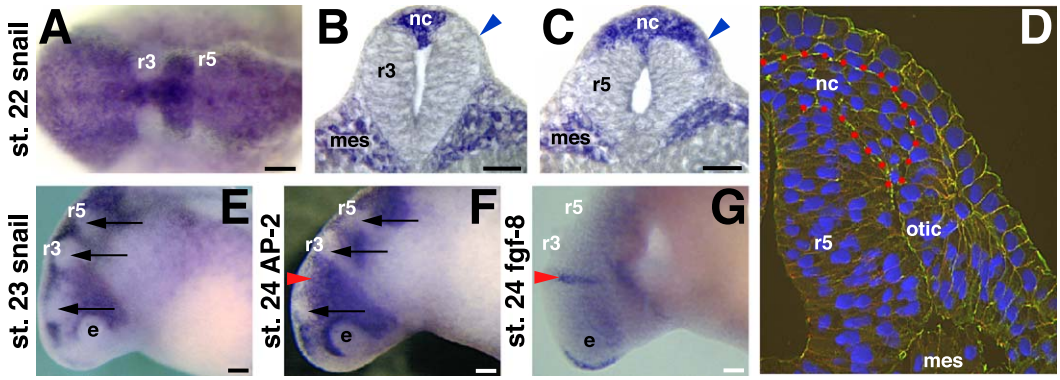


Fig. 2. Cranial crest-free spaces pattern neural crest streams. (A) Dorsal view of an embryo at stage 22 after hybridization with the Snail riboprobe showing early neural crest patterning. At r3, the neural crest string is very narrow; at r5, it is wider. (B, C) Transverse sections through the same embryo at r3 (B) and r5 (C). In contrast to r3, at r5 neural crest cells have migrated to a midventral position along the dorsoventral neural tube. The otic placode has not yet developed. Snail positive paraxial mesoderm (mes) is situated far away from early neural crest. (D) Soon after (at stage 24), the invaginated sensory layer of the otic placode (otic) inhibits neural crest migration from r5. Transverse section stained with anti-actin (red) and anti-β-catenin (green) antibodies (yellow in regions of overlay) to demonstrate tissue borders. DAPI (blue) visualizes cell nuclei. Red dots delineate neural crest. (E, F) From stage 23 onwards, another crest-free space arises rostral to r3 and r5 as observed with both neural crest markers (E, Snail; F, AP-2). Black arrows point to all three crest-free zones. (G) The position of the FGF-8 transcript (indicating the mid-hindbrain border) revealed that the new crest-free area is situated within the mesencephalon. The red arrowhead in (E and F) indicates the mid-hindbrain border. Scale bars, 100 μm.

camera, merged and optimized in Spot, MetaView and Adobe Photoshop software.

Results

Morphogenesis of cranial neural crest streams in the Mexican axolotl

We used morphological criteria in combination with molecular markers to determine neural crest migratory pathways (Fig. 1). By removing the epidermis, it is possible to identify neural crest cells by their morphology and arrangement under the scanning electron microscope (SEM). As molecular markers, we used AP-2 (Shen et al., 1997) and Snail (Essex et al., 1993), both of which exhibit a nearly identical expression in the axolotl neural crest. This represents the first molecular description of Snail expression in axolotl. In addition to the neural crest, Snail is expressed in the paraxial mesoderm (data not shown).

Premigratory neural crest

Around stage 18/19, the cranial neural crest first becomes morphologically recognizable (Figs. 1A,B) along the dorsal neural tube with the exception of the anteriormost portion, which remains neural crest-free throughout development. The width of the neural crest string varies at

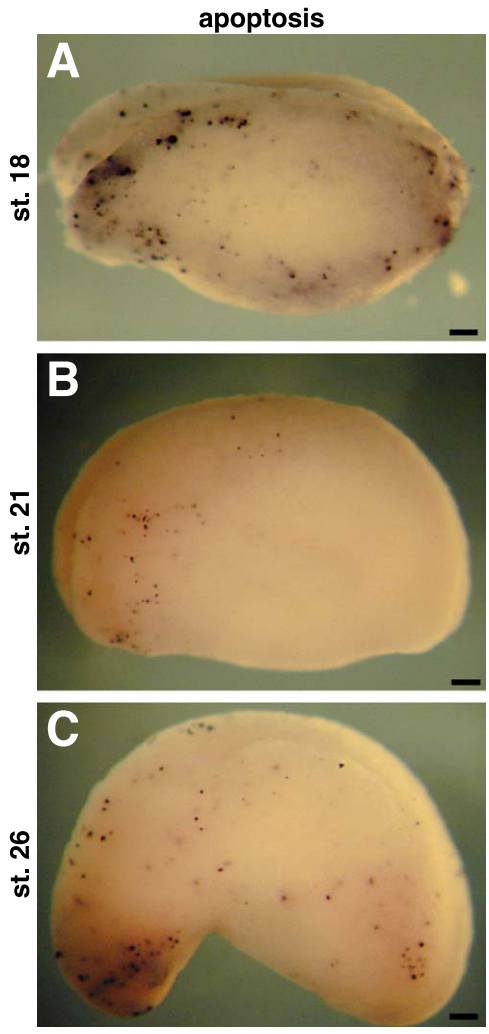


Fig. 3. Apoptosis has no influence on cranial neural crest patterning. TUNEL staining in axolotl embryos. Stages 18 (A), 21 (B) and 26 (C). Apoptotic cells are distributed randomly and do not display any relationship to rhombomeres or neural crest streams. Scale bars, 200 μm.

different axial levels. It is narrow at the future sites of rhombomeres (r) 3 and 5 but wider rostral to r3, within r4 and caudal to r5. The three wider areas develop into the mandibular, hyoid and common branchial neural crest streams.

Before the onset of neural crest migration, the cranial mesoderm spans from an area lateral to the neural tube ventrally to the prospective pharynx with the exception of the first endodermal pouch where endoderm contacts ectoderm. The first pouch divides the future pharyngeal part of the cranial mesoderm into an anterior and posterior area (Fig. 1A). The paraxial (dorsal) mesoderm is unsegmented anterior to r5, but develops into regular somites posterior to r5 (Fig. 1A).

Early stages of neural crest migration

The three presumptive neural crest streams spread laterally over the dorsolateral neural tube at stage 21/22 (Figs. 1C,D). The first bulge forms the future mandibular stream which moves over the paraxial mesoderm and approaches the arch mesoderm. The tongues of the hyoid and branchial neural crest streams have migrated only as far as to the lateral aspect of the neural tube and do not contact the paraxial mesoderm at this stage (Figs. 1C,D).

Around stage 23/24, early cranial neural crest streams have formed. The wide mandibular stream has migrated ventrally onto the arch mesoderm, whereas the hyoid stream is much shorter and narrower as it migrates over the paraxial mesoderm. It is similar in length but only one third the width of the common branchial stream (Fig. 1E).

By stage 23, the dorsolateral aspect of the neural tube becomes devoid of neural crest cells (Figs. 1E,F, arrow) anterior to the mid/hindbrain border (for details, see Fig. 2). Concomitantly, the second pharyngeal pouch evaginates (Fig. 1E) and contacts the ectoderm, whereby the hyoid arch mesoderm becomes separated from the posterior part of the branchial mesoderm.

Late neural crest migration

At stage 29–30, the wide mandibular stream has reached its ventral-most extent, whereas the narrow hyoid and individual branchial neural crest streams are still progressing ventrally (Figs. 1G,H). The anterior part of the mandibular stream migrates into the frontonasal process, whereas the posterior, postoptic neural crest contributes to the first pharyngeal arch. The common branchial stream and branchial mesoderm gradually become subdivided (Figs. 1G,H) by local outgrowth of the endoderm and ingrowth of the epidermis. At stage 29/30, AP-2 (Figs. 1H) and Snail (not shown) appear to be decreased in the dorsal portions of the neural crest streams but stain strongly in the ventral portions. This may be due to down-regulation of neural crest markers (Epperlein et al., 2000) and/or a depletion of cranial neural crest cells which aggregate into the primordia of sensory ganglia (Northcutt and Brändle, 1995).

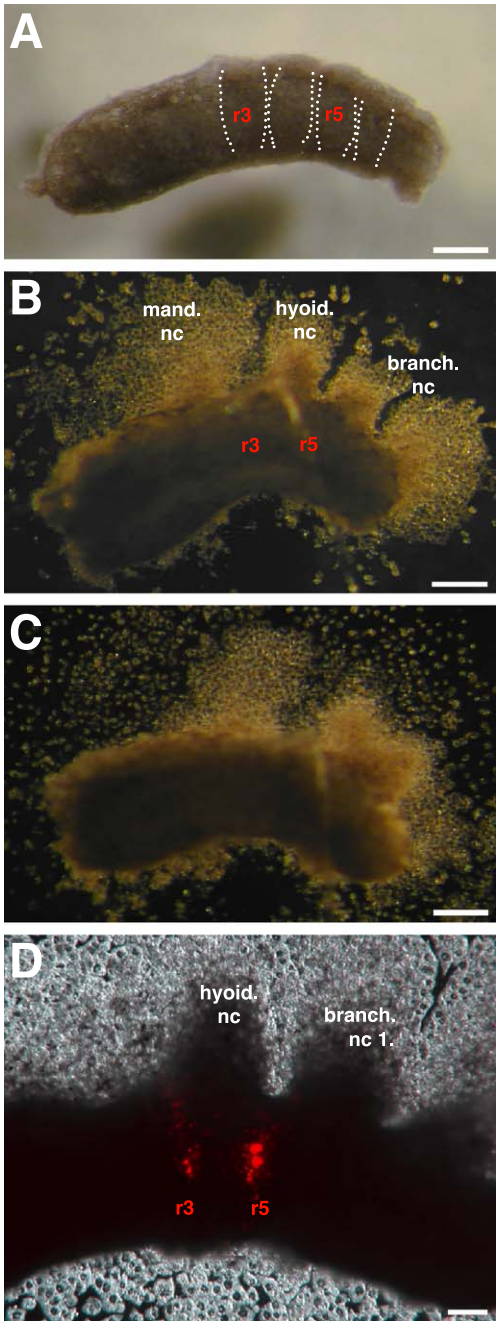


Fig. 4. Cranial neural tube explants can produce distinct neural crest streams. (A) Excised cranial neural tube, anterior to the left, stage 21. White dots outline the borders of rhombomeres. (B) The same tube 20 h later on a fibronectin-coated substrate. Neural crest cells migrate out laterally in well-defined streams without being influenced by other tissues. (C) The same specimen about 35 h later. Streams are now fused. (D) Enlarged view of a different culture 15 h after the rhombencephalon was labeled with DiI at the position of r3 and r5. Some neural crest cells migrate from r3 and r5 but join streams from all even numbered rhombomeres. mand.nc; hyoid.nc; branch.nc; branch.nc.1., mandibular, hyoid, branchial and the first branchial neural crest stream, respectively. Scale bars: 200 μm in (A–C), 100 μm in (D).

Neural crest-free regions along the head neural tube pattern early neural crest streams

There are two principal neural crest-free areas adjacent to r3 and r5 that pattern early neural crest streams (Fig. 2A). At r3, neural crest cells form only the middle part of the neural tube roof and do not migrate laterally (Fig. 2B). In contrast, they cover the entire roof at r5 and have migrated half way down the neural tube (Fig. 2C). Subsequently, the sensory layer of the otic placode starts to invaginate at r5 and limits neural crest migration (Fig. 2D). Detailed cellular analysis of sectioned embryos hybridized with AP-2 and Snail (not shown) as well as of embryos stained for actin and β-catenin (Fig. 2D) reveal that no neural crest cells migrate between the otic placode and r5.

Using SEM (Fig. 1E; $n = 4$) as well as Snail (Fig. 2E; $n = 5$) and AP-2 in situ hybridization (Fig. 2F; $n = 11$), we noted another neural crest-free space in the dorsal cranial neural crest from stage 23 onwards. To accurately determine the rostrocaudal level of this space, we compared embryos stained for AP-2 and FGF-8 transcripts to indicate the position of the mid-hindbrain border (Figs. 2F,G; Han et al., 2001; Riou et al., 1998). It is likely that this neural crest-free space is situated within the mesencephalon, anterior to the mid-hindbrain border and may contribute to patterning of the mandibular neural crest.

Apoptosis and local cell proliferation have no apparent influence on early cranial neural crest patterning

In avian embryos, it has been proposed that localized cell death eliminates cranial neural crest precursors at r3 and r5 and thus patterns neural crest cells into streams

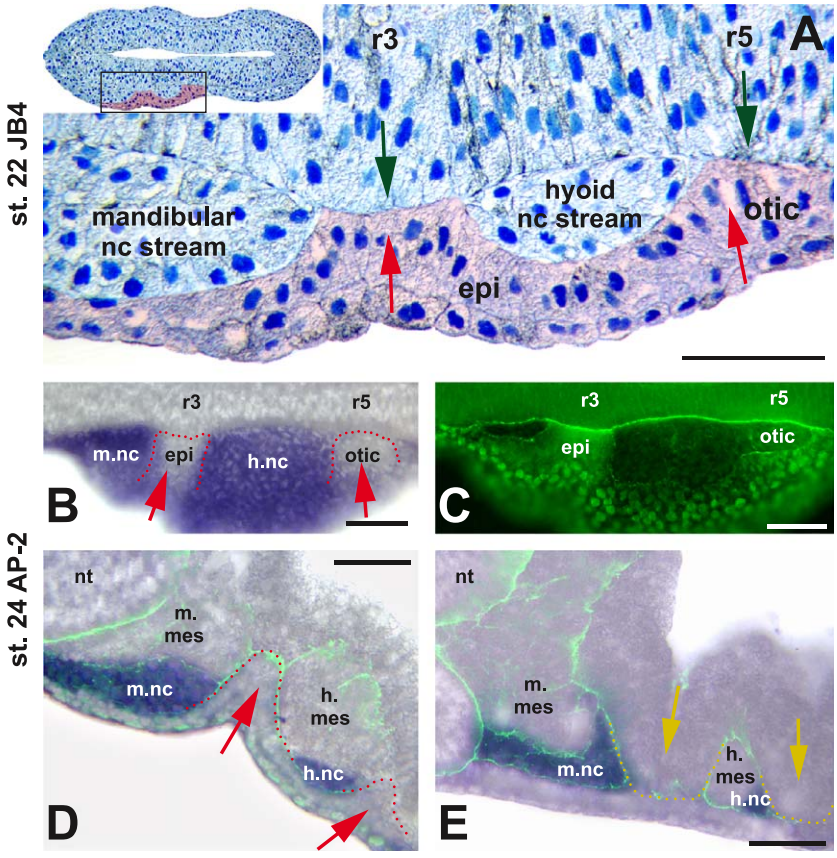


Fig. 5. Neural crest streams are constrained in tissue channels (anterior to the left, only left side shown in all cases). (A) Horizontal plastic section through the neural tube of an embryo at stage 22. The frame within the small inset indicates the position of the enlarged image. The epidermis (epi, colored red) invaginates and contacts the neural tube epithelium intimately at r3 and r5. Thus, channels are formed through which neural crest streams are directed. Red arrows show epidermal invaginations; green arrows show the bulging neural tube at r3 and r5. (B, C) Horizontal vibratome section through the neural tube at stage 24 after in situ hybridization with the AP-2 riboprobe. The mandibular neural crest (m.nc) and hyoid neural crest (h.nc) streams are stained. (C) shows the same section after counterstaining with anti-fibronectin to reveal tissue borders. Images in (A–C) demonstrate identical epidermal invaginations (red arrows and dotted lines in B) in two different embryos constraining neural crest migration. (D, E) Horizontal sections through the dorsal (D) and ventral (E) pharynx (the same embryo as B and C). The sections with the AP-2 riboprobe were counterstained with anti-fibronectin (green) to reveal tissue borders. Dorsally, at a paraxial level (D), the epidermis invaginates in register with r3 and r5 (red arrows and dotted lines) and divides mandibular from hyoid crest. Ventrally (E), the first and the second endodermal pouches (yellow arrows and dotted lines) evaginate in register with r3 and r5 and keep neural crest streams separate. otic, otic placode; nt, neural tube; m.nc, mandibular neural crest; h.nc, hyoid neural crest; m.mes, mandibular arch mesoderm; h.mes, hyoid arch mesoderm. Scale bars, 5 μm in (B and C), rest 100 μm.

(Graham et al., 1993). To investigate whether programmed cell death contributes to neural crest patterning in the axolotl, we used TUNEL to determine the distribution of apoptotic cells in albino embryos. Randomly distributed dying cells were observed through development (Fig. 3), with no obvious increase of apoptosis adjacent to r3 or r5. The same was observed on sections (not shown). As positive controls (not shown), similar staining in chick limb buds revealed cell death as expected in the interdigital zone (Zou and Niswander, 1996) and in *Xenopus* embryos comparable to that described in previous publications (Hensey and Gautier, 1998).

To examine whether regionally different rates of neural crest proliferation are involved in the control of cranial neural crest migration, proliferating cells were identified using an anti-Phosphohistone-3 antibody (data not shown). Many positive epidermal cells were observed, with no obvious regional differences. After removal of the head epidermis, positive cells (very likely neural crest) were found randomly scattered along the dorsal neural tube. Within the pharyngeal neural crest streams, the density of mitotic cells was higher than in adjacent mesodermal tissue. Therefore, there is extensive proliferation in the neural crest population but no obvious regional specificity to the proliferation.

Cranial neural tube explants produce distinct neural crest streams in vitro

Cranial neural crest cells move in a segmental fashion through adjacent tissues. This segmentation may be inherent to the migrating cells themselves and/or imposed by neighboring tissues. To investigate neural crest migratory patterns in the absence of other tissues, cranial neural

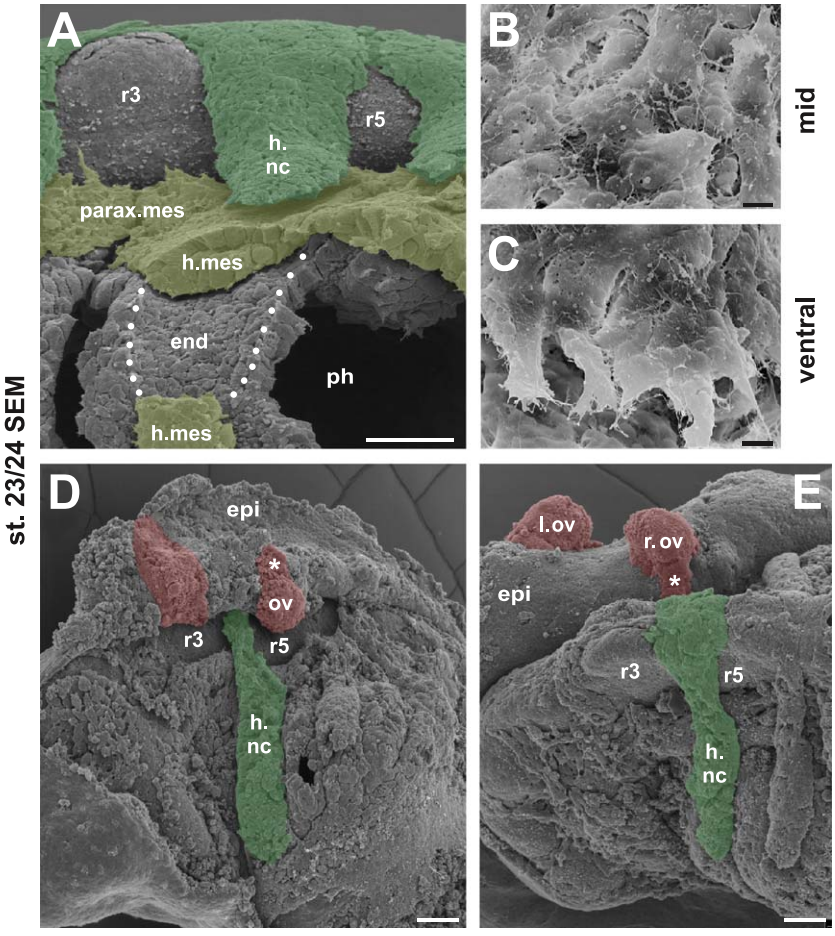


Fig. 6. Shape and constraints of cranial neural crest streams. (A) Pseudo-colored SEM micrograph of an epidermis-ablated embryo at stage 23/24 (head to the left outside); neural crest cells are colored green and mesoderm yellow. Notice the compactness of the hyoid stream (h.nc). The dotted lines delineate part of the hyoid arch mesoderm (h.mes) which was removed to expose the endoderm (end). (B, C) Details of neural crest cell shape in the middle (B) and at the leading edge (C) of the migrating hyoid stream. Only those cells at the leading edge show an obvious dorsoventral orientation that suggests an active ventral cell movement. (D, E) SEM micrographs of embryos in which the epidermis (epi) was unfolded to show the strong ectodermal invagination of the otic vesicle (ov) with its ventral extension (asterisk) at r5 (colored red) and of another dorsoventral invagination of the epidermis at r3 (colored red). These epidermal barriers keep neural crest streams apart. For clarity, the hyoid neural crest stream is colored green. l.ov, left otic vesicle; r.ov, right otic vesicle; parax.mes, paraxial mesoderm. Scale bars, 5 μm in B and C, rest 100 μm.

tube explants were grown in tissue culture on a permissive substrate. The cranial ectoderm was removed from embryos at the earliest stages of neural crest migration (stages 20–22) and the cranial neural tube was carefully excised and placed on a fibronectin-coated substrate (Fig. 4A). Surprisingly, even in isolation, neural crest cells migrated in distinct streams (Fig. 4B) and maintained this separation for at least 30 h. However, neural crest cells became nearly confluent with little or no obvious separation at later times (Fig. 4C). DiI labeling of r3 and r5 reveal that the gaps in migration are across from the odd numbered rhombomeres (Fig. 4D). Some labeled neural crest cells emerged from r3 and r5 and joined migratory streams from even numbered rhombomeres (Fig. 4D), as

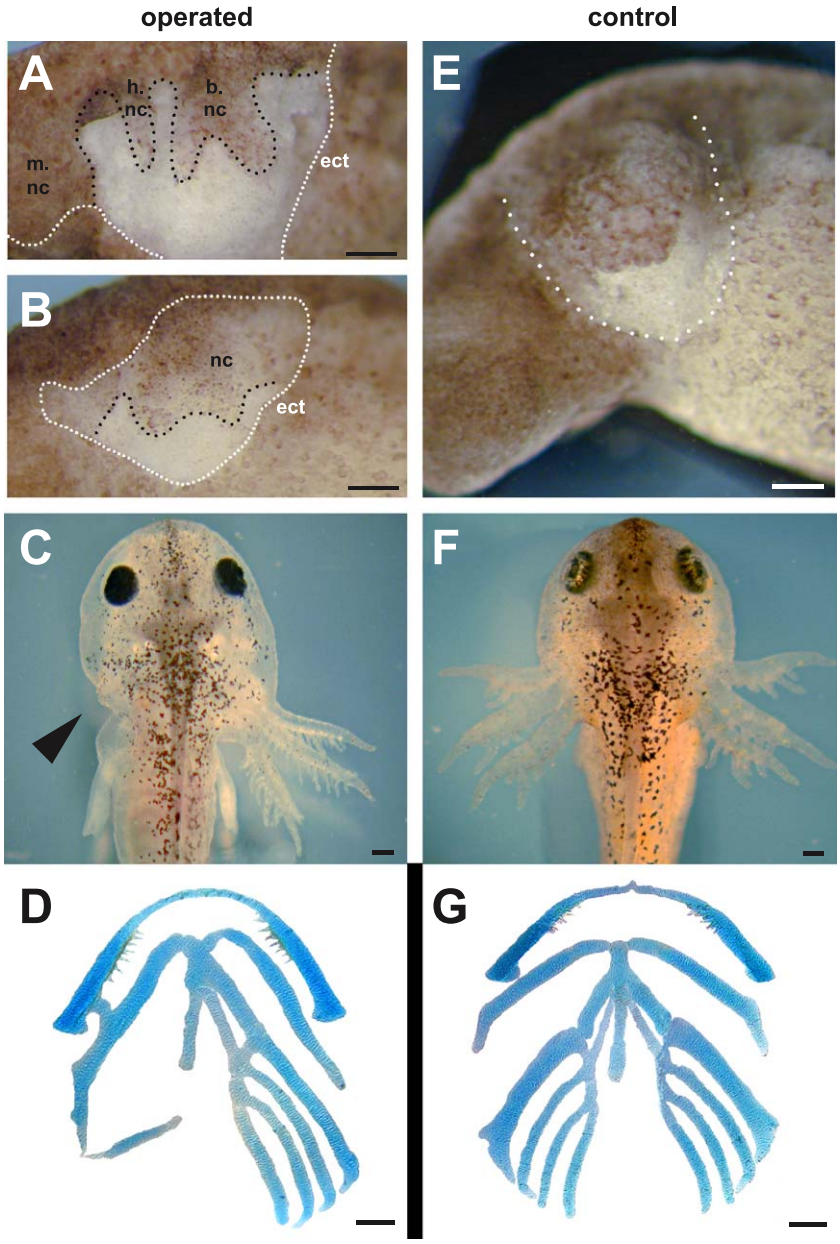


Fig. 7. Removal of the head epidermis stops neural crest migration and perturbs normal patterning of the visceral skeleton. (A, B) Lateral views of an embryo in which the dorsolateral part of the head epidermis was removed at stage 24 (A). Black dotted line delineates neural crest, which contrasts against the white mesoderm because of pigment granules. The white dotted line delineates part of the ectodermal area (ect) removed. (B) Seven hours later, the white dotted line delineates ectoderm-free area. Without the epidermal covering, neural crest streams fuse. (C) Four days later, the epidermis regenerated and covered the wound but did not develop into outer gills (arrowhead). (D) Alcian blue staining of the specimen's visceroskeleton 10 days later; lack of cartilages on the operated side. (E) Control embryo at stage 26, lateral view. The epidermis was lifted but replaced immediately. White dots delineate the operated area. (F) Four days later, the outer gills of the larva are developed in the same way on the operated side as on the control side. (G) Alcian blue staining of the control specimen, 10 days later showing normal development of viscerocranial skeleton on both sides. m.nc, mandibular; h.nc, hyoid; b.nc, branchial neural crest streams. Scale bars, 200 μm.

has been previously reported in vivo (Sechrist et al., 1993).

Tissue channels reinforce distinct cranial neural crest streams

The fact that cranial neural crest cells have an inherently segmental migratory pattern raises the interesting question of whether or not the tissue environment also influences migration. To examine this question, we carefully analyzed the relationship between neural crest cells and surrounding tissue using a combination of high-resolution light microscopy and SEM. As soon as neural crest cells initiate their migration from the dorsal neural tube, they appear in condensed streams (Figs. 1C,D) interposed between regions where the neural tube is tightly apposed to the ectoderm at the levels of r3 and r5 (Fig. 5A). This continues along the dorsoventral axis such that the outer epidermis and internal tissues (neural tube, mesoderm and endoderm) are locally closely apposed. This produces several dorsoventrally oriented apposition zones that create adjacent channel-like spaces through which neural crest streams migrate. These tissue barriers are visible from the beginning of neural crest cell migration (stage 22, Fig. 5A), becoming progressively more evident at later stages (stage 24, Figs. 5B–E). For example, at a dorsal level, the epidermis and the dorsolateral neural tube are closely apposed at the neural crest-free regions of r3 and r5 (Figs. 5B,C). Further, ventral to these spaces, intimate contact exists between the thickened and invaginated epidermis and the paraxial mesoderm (Fig. 5D). Still more ventrally, the first and second endodermal pouches appose the epidermis (Fig. 5E). The first pouch forms in register with the neural crest-free space at r3, the second pouch in register with r5.

Scanning electron microscopy was used to visualize the tissue appositions and channels in a three-dimensional manner (Figs. 6A,D,E). These images reveal the presence of continuous dorsoventral apposition zones first between ectoderm and neural tube and then between ectoderm, mesoderm and endoderm as they progress ventrally. Neural crest streams are present extending from dorsal to ventral between these apposition zones. Within the streams, individual migrating neural crest cells appear polygonal in shape (Fig. 6B), with no obvious orientation. In contrast, neural crest cells at the leading edge of the streams appear oriented dorsoventrally and have numerous filopodia (Fig. 6C).

Following removal of the epidermis, cranial neural crest streams fuse, and the pattern of the visceral skeleton is perturbed

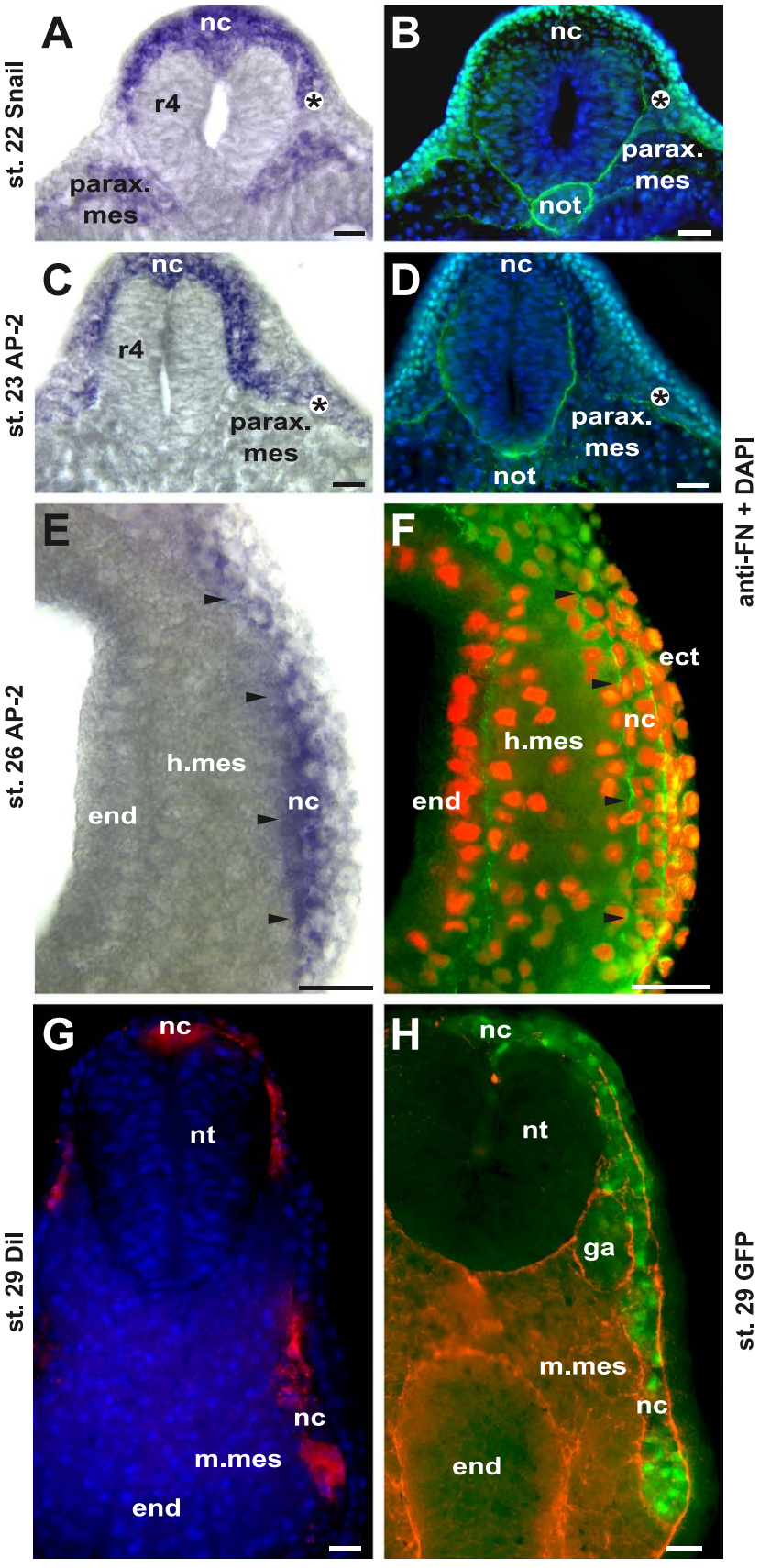
The above results suggest that formation and maintenance of neural crest streams involve intrinsic patterning information together with a structural importance for neighboring tissues. To test a possible role for the epidermis in formation or maintenance of neural crest streams, we removed the dorsolateral region of the head epidermis at stages 23–26 (Fig. 7A; n = 15). Following such an operation, neural crest streams stop their lateral movement, lose their compact configuration, and become confluent after 3–8 h (Fig. 7B). About 4 days later, regenerated epidermis has grown over the ablated area but fails to support the formation of outer gills (Fig. 7C) and proper viscerocranial cartilage (Fig. 7D). To assure that the development of these structures did not fail because of a lack of neural crest cells that were detached during epidermis removal, the epidermis was detached and then repositioned after a brief lifting (Fig. 7E). In these control embryos (n = 7), outer gills (7/7) and visceral cartilage (5/7) developed normally (Figs. 7F,G). These results suggest that neural crest–epidermal interactions are necessary for maintenance of cranial neural crest cell migration in streams and for their continuous migration.

Cranial neural crest cells only migrate laterally and do not mix with mesoderm

SEM analysis suggests that neural crest streams leaving the neural tube continue to spread on the outside of the paraxial and arch mesoderm (Fig. 6A). However, the precise relationship between migratory neural crest and mesoderm cannot be determined by SEM. Therefore, we analyzed sections of embryos hybridized with neural crest markers AP-2 or Snail. The latter also stains paraxial, but not branchial arch, mesoderm. The axolotl Snail expression pattern is highly reminiscent of Snail and Slug in *Xenopus* (Mayor et al., 1995).

We focused on neural crest migration at the hyoid (r4) level because of its optimal visibility. At stage 22, (Figs. 8A,B), neural crest cells cover the dorsolateral surface of the neural tube. By stage 23 (Figs. 8C,D), the hyoid stream reaches the paraxial mesoderm where it turns laterally but not medially (between the mesoderm and neural tube). Later, at the pharyngeal level, the hyoid stream migrates on the outside of the arch mesoderm (stage 26, Figs. 8E,F). No mixing of neural crest cells with mesoderm was observed at either paraxial or pharyngeal levels.

Because molecular markers are not definitive lineage markers, we directly labeled cranial neural crest cells with lineage tracers to visualize their relationship with surrounding tissue. First, focal injections of DiI were performed into the cranial neural folds. Second, two- to four-cell stage embryos were injected with GFP mRNA, grown to the neurula stage and then GFP-labeled head neural folds were transplanted to unlabeled host embryos. Both these techniques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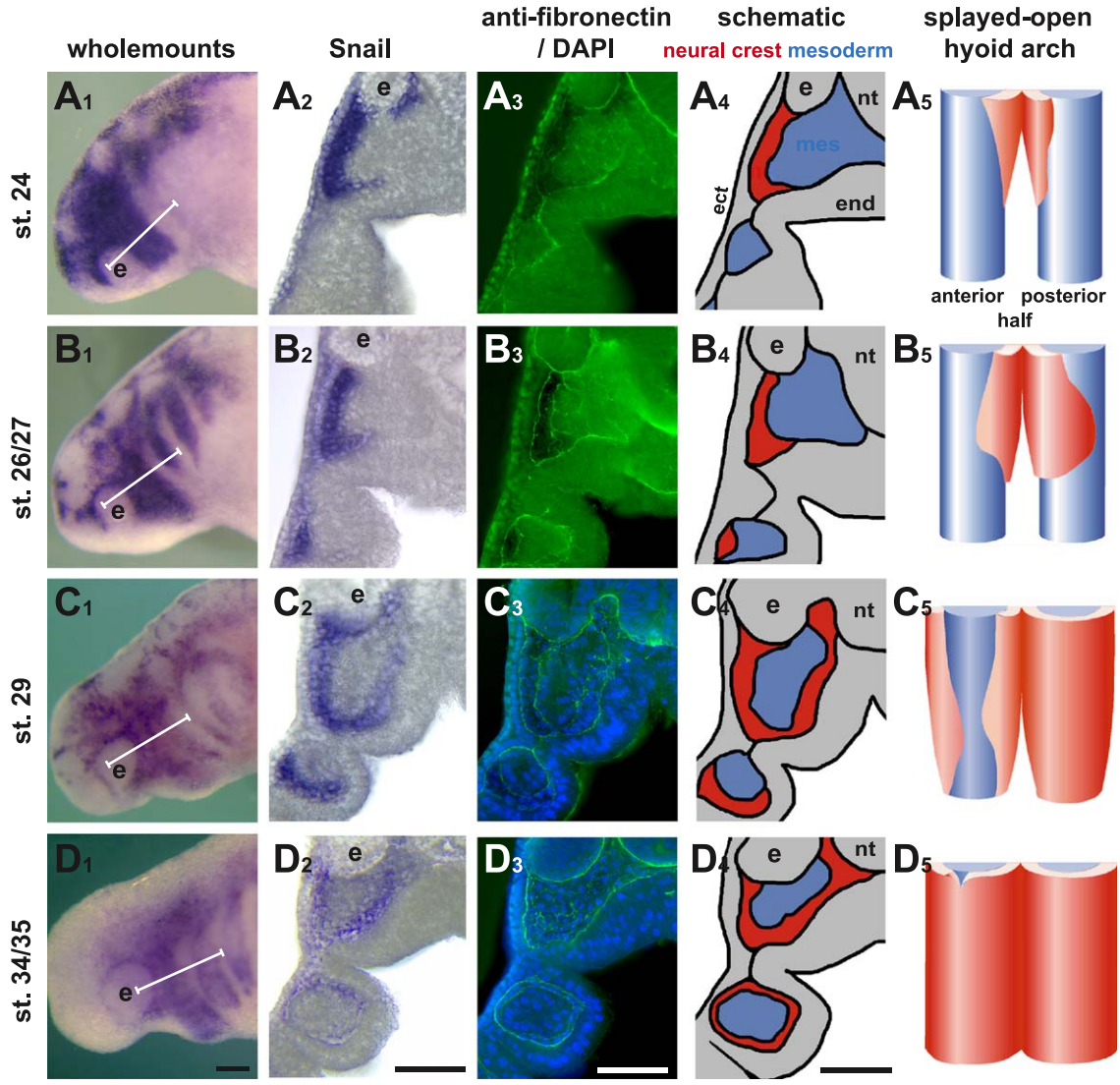

Fig. 9. Outside-in movement of neural crest cells during pharyngeal arch morphogenesis. First column shows embryos after hybridizing with the Snail riboprobe at stages 24 (A), 26/27 (B), 29 (C) and 34/35 (D). White lines indicate the position of horizontal sections shown in column 2 (left half and mandibular and hyoid areas shown only). Sections were made at six to eight different horizontal planes along the dorsoventral axis; of these always a middle one is shown at the level of the eye vesicle (e). Column 3 shows the same sections after counterstaining with anti-fibronectin (anti-FN) and DAPI; column 4 summarizes the previous information and column 5 offers a 3D view of the ensheathment of left hyoid mesoderm (blue) with neural crest (red). In column 5, the anterior part of the arch was unfolded and turned to the left; the posterior part stayed in place. From their subepidermal position, neural crest cells migrate deep into the arches around the mesodermal core but do not mix with it. Neural crest cells start to migrate from a posterolateral position around the arch mesoderm both anteriorly (shown on the left half of the arch) and posteriorly (right half). The anterior encirclement is delayed. ect, ectoderm; end, endoderm; nt, neural tube; e, eye. Scale bars, 200 μm.

demonstrated a definite segregation between labeled neural crest cells and adjacent mesoderm (Figs. 8G,H). Thus, molecular markers as well as direct cell marking demonstrate that axolotl neural crest cells migrate over but do not intermix with mesodermal tissues.

Fig. 8. Neural crest cells migrate only laterally and do not mix with mesoderm. (A–D) Transverse sections through r4 showing early migration of the hyoid neural crest stream at stages 22 (A, B) and 23 (C, D). In (A), both neural crest and paraxial mesoderm (parax.mes) are Snail positive. The lateral front of neural crest cells (asterisk) has not yet reached the paraxial mesoderm. (B) Counterstaining of the same section with anti-fibronectin (green) and DAPI (blue). At stage 23 (C), AP-2 positive neural crest cells (asterisk) have moved laterally but not medially toward the notochord (not). (D) Counterstaining of the same section as in C. (E, F) Transverse section (detail) through a hyoid arch at stage 26. (E) Neural crest cells are hybridized with the AP-2 riboprobe. (F) Counterstaining of the same section as in (E), but DAPI was converted into red. Neural crest cells migrate only on the outside of the hyoid arch mesoderm (h.mes) and do not invade it (black arrowheads demarcate the border between neural crest and mesoderm). (G) Transverse section through a DiI-injected embryo (stage 29) through the mandibular arch. Neural crest cells (red) migrate only along the lateral pathway. Counterstained with DAPI (blue). (H) Same situation as in (G), all neural crest is stained by GFP (green). Counterstained with anti-fibronectin (red). m.mes, mandibular mesoderm; end, endoderm; ect, ectoderm; nt, neural tube; ga, ganglion. Scale bars: 100 μm.

Cranial neural crest streams conduct an outside-in movement during pharyngeal arch morphogenesis

We have shown that initially, cranial neural crest cells migrate subepidermally, neither mixing with mesoderm nor migrating along a medial pathway (Fig. 8). Later, neural crest cells are found deep within the pharyngeal arches. Our analysis at the level of the mandibular and hyoid arch shows that they reach this location by migrating inward around the mesodermal arch cores in an ''outside-in'' movement (Fig. 9) during which no mixing occurs between neural crest cells and mesoderm. More posterior arches develop in essentially the same manner, though the number of cells in individual arches is lower and therefore this process is not as clearly visible.

Initially, neural crest cells occupy a lateral position on the outer mesodermal surface (Fig. 9A, stage 24) as can be envisioned by the Snail signal (Fig. 9A1,2). After reaching their ventral-most position within the arch, the neural crest cells turn medially and gradually ensheath the arch mesoderm (Figs. 9B–D). The inward movement of neural crest cells starts from a posterolateral level (Figs. 9B,C) before lateral migration is completed. Finally (Fig. 9D, stage 34/35), the entire central mesoderm is homogeneously enveloped by neural crest cells. As they migrate, they increase their contact area with pharyngeal endoderm. The ensheathment process of a single hyoid arch is illustrated in a 3D reconstruction (Fig. 9). A splayed-open view of the ventral portion of the hyoid arch illustrates how neural crest cells (red) migrate around but do not mix with the mesodermal arch core (blue).

Because molecular markers are not true lineage markers, we next confirmed the outside-in movement of neural crest cells using cell marking techniques. Embryos were labeled either with rhodamine dextran or GFP mRNA at the two-cell stage. Embryos were allowed to develop until the neurula stage at which time one of the neural folds was grafted isotopically into an unlabeled host. For both types of lineage label, similar results were observed (data not shown). Labeled neural crest cells were initially seen along the lateral pathway. At subsequent times, they appeared to ensheath the mesoderm in an outside-in manner identical to that observed with molecular markers for neural crest, AP-2 and Snail.

Discussion

The global pattern of cranial neural crest migration in axolotl closely resembles that of other vertebrates (Lumsden et al., 1991; Meulemans and Bronner-Fraser, 2002; Olsson et al., 2002; Sadaghiani and Thiébaud, 1987; Trainor et al., 2002) and might suggest a broad conservation of basic patterning mechanisms. However, numerous specific properties of cranial neural crest cell migration are distinctly different among vertebrates (e.g., Serbedzija et al., 1992; Trainor et al., 2002). Therefore, it is essential to perform analyses across divergent species to uncover the fine regulation of patterning, and to determine which mechanisms are controlled intrinsically versus extrinsically by the environment.

Patterns of premigratory and early migrating cranial neural crest

In axolotl, the premigratory cranial neural crest appears as a ''string'' of variable thickness on the dorsal side of the neural tube in the late neurula (Figs. 1A,B). The wider portions of the string give rise to the three principal neural crest streams: the mandibular, hyoid and common branchial stream (Falck et al., 2002).

Once cells initiate migration, there are several regions from which migrating neural crest cells are conspicuously absent: one in the mesencephalon and two in the rhombencephalon adjacent to r3 and r5 (Fig. 2). Rhombomere 3 separates the mandibular from the hyoid and r5 the hyoid from the common branchial neural crest stream. The mesencephalic neural crest-free space is likely to separate the maxillo-mandibular arch neural crest from the more anterior, frontonasal neural crest (Figs. 1G,H). Despite its presence in illustrations of whole mounts of frog, chick and mouse (Alfandari et al., 2001; Chai et al., 2000; Kuratani and Eichele, 1993; Linker et al., 2000; Mayor et al., 1995; Osumi-Yamashita et al., 1994; Robinson et al., 1997; Sadaghiani and Thiébaud, 1987; Serbedzija et al., 1992), the mesencephalic neural crest-free space has not been described previously. Several mechanisms may account for the formation of neural crest-free spaces and subsequent patterning of streams. These include apoptotic elimination (Graham et al., 1993), selective proliferation, tissue interactions such as paraxial exclusion zones (Farlie et al., 1999) and/or specific signaling molecules such as Eph/ephrin interactions (Holder and Klein, 1999; Robinson et al., 1997; Smith et al., 1997), FGF2 (Kubota and Ito, 2000) or *collapsin 1* (Eickholt et al., 1999).

In axolotl, the random distribution of apoptotic cells (Fig. 3) is inconsistent with a role for cell death in shaping neural crest streams. In the chick, neural crest cells at r3 and r5 have been proposed to undergo cell death (Hirata and Hall, 2000; Lumsden et al., 1991) mediated by *BMP-4* and *Msx-2* (Graham et al., 1993; Smith and Graham, 2001). However, the influence of apoptosis on early neural crest patterning is controversial, not only in chick (Farlie et al., 1999) but also in zebrafish (Cole and Ross, 2001; Ellies et al., 1997), *Xenopus* (Hensey and Gautier, 1998) and mice (Trainor et al., 2002).

A regionally different rate of neural crest proliferation is another possible cell-intrinsic mechanism that might pattern neural crest streams. However, we found no differences in the rate of proliferation between even and odd rhombomeres using an anti-Phosphohistone-3 antibody (Hendzel et al., 1997; Saka and Smith, 2001). In frog, chick and mouse

embryos, there is some evidence that r5 generates more neural crest cells than r3 (Anderson and Meier, 1981; Robinson et al., 1997; Sechrist et al., 1993; Trainor et al., 2002). However, this is unlikely to influence the patterning of neural crest cell migration.

Intrinsic properties contribute to segmental migration of cranial neural crest

We explored the possibility that cranial neural crest cells themselves may contribute to the observed segmental migration in the hindbrain by examining their behavior in isolation. To this end, cranial neural crest cells from stage 20–22 embryos were grown on fibronectin-coated substrates in vitro. Surprisingly, we found that streams of neural crest cells from r2, r4 and r6 were clearly visible even in the absence of neighboring tissue. The streams remained separate for at least 30 h, becoming fused thereafter (Fig. 4). Thus, early steps of cranial neural crest cell migration and subsequent patterning into separated streams appear to be autonomous. These data suggest that there are indeed intrinsic differences between adjacent neural crest populations that may lead to sorting behavior and thus contribute to the genesis of neural crest streams.

When r3 and r5 cells were labeled focally with DiI, it was clear that the gaps in migration corresponded to the odd numbered rhombomeres. However, DiI-labeled neural crest cells emerged from these rhombomeres, but joined the streams of neural crest cells from even numbered rhombomeres (Fig. 4D). This is consistent with in vivo labeling experiments in chick and mouse, suggesting that r3 and r5 generate neural crest cells that are redirected anteriorly or posteriorly joining the lateral streams from r2, r4 and r6 (Birgbauer et al., 1995; Kulesa and Fraser, 2000; Trainor et al., 2002).

What molecular properties might account for the separation of adjacent neural crest populations even in the absence of other tissue types? In *Xenopus*, the complementary expression of Eph receptors and ligands in adjacent populations of migrating neural crest cells appears to segregate streams and ensure neural crest migration into the correct arch (Holder and Klein, 1999; Smith et al., 1997). At later stages, other molecules may contribute to neural crest migration; for example, FGF8 is known to affect the morphogenetic movements of the pharynx and subsequent segregation of neural crest streams (Trumpp et al., 1999).

Tissue interactions help maintain segmental migration of cranial neural crest

We explored the possibility that interactions between neural crest and adjacent tissues might influence neural crest migration and patterning. In other systems, paraxial mesoderm is favored for influencing neural crest migration (Farlie et al., 1999; Noden et al., 1999; Trainor and Krumlauf, 2000a; Trainor et al., 2002). However, in axolotl, cranial neural crest cells migrate and form streams well before they reach the paraxial mesoderm (Figs. 1C,D, 2 and 5A–C). This makes it clear that the mesoderm has no influence on initial steps of neural crest cell migration. Its migration, however, might have been influenced by interactions between the neural tube surface and epidermis and/or may be an autonomous process as suggested from observations in culture (Fig. 4).

Interestingly, we observed apposed thickenings of the neural tube and epidermis adjacent and ventral to r3 and at r5 that form well-defined exclusion zones for migrating neural crest cells (Figs. 5 and 6). Careful analyses revealed tight appositions between zones of adjacent tissues in register with r3 and r5 (Fig. 5). This results in the formation of a "channel" adjacent to r4 through which hyoid neural crest cells migrate ventrally. Similar channels form at the mandibular and branchial levels as well. Such exclusion zones have been previously proposed though not understood mechanistically (e.g., Farlie et al., 1999). A similar barrier mechanism had been documented for the otic placode at r5 before in fish (Sadaghiani and Vielkind, 1989), newt (Jacobson and Meier, 1984), turtle (Meier and Packard, 1984), chick (Sechrist et al., 1993) and mouse (Trainor et al., 2002). Our data suggest that such zones exist for r3 as well as r5 in axolotl. In both odd numbered rhombomere regions, migrating neural crest cells are mechanically constrained. In species in which the otic placode initially develops closer to r4, a conspicuous neural crest stream migrates from r5 (Bradley et al., 1992; Del Pino and Medina, 1998; Horigome et al., 1999; Sadaghiani and Thiébaud, 1987). This supports the idea that extrinsic mechanisms are important for regulating neural crest migration. Moreover, grafting r5 to other locations results in lateral migration of r5-derived neural crest cells (Saldivar et al., 1996; Trainor et al., 2002) and extirpation of epidermis at r3 leads to the lateral migration of neural crest cells from r3 (Golding et al., 2002). When small grafts from the paraxial area consisting of cranial mesoderm or surface ectoderm were transplanted next to r4, they were unable to inhibit neural crest cell migration (Trainor et al., 2002). Therefore, these tissues do not appear to secrete inhibitory signals. We conclude that at the beginning of cranial neural crest migration, mechanical interactions between the neuroepithelial and the epidermal layer create well-defined neural crest-free zones that maintain streams of neural crest cells in channels between neighboring tissues.

Our results show that there are regions through which the cranial neural crest cells can migrate with facility, that arise between regions of close apposition that appear to block neural crest migration. These channels are likely to result partially from mechanical influences of the environment but may also be created by the migrating neural crest cells themselves burrowing their way through permissive tissue.

Tissue channels help to guide neural crest cells to the pharynx

Our results demonstrate that cranial neural crest cells migrate laterally in compact streams in which they are funnelled through adjacent tissues en route to the pharynx (Fig. 5). As nicely illustrated in the chick (Veitch et al., 1999), pharyngeal arch patterning is not coupled to neural crest migration and cranial tissue is regionalized even in the absence of the neural crest. Similarly in zebrafish, the endoderm has been suggested to form segment boundaries between arches to constrain neural crest migration (Schilling and Kimmel, 1994). Moreover, in the zebrafish *vgo* mutant, pharyngeal endoderm does not form pouches and consequently neural crest streams fuse at a ventral (pharyngeal) but not dorsal level where neural crest cells migrate in normal streams (Piotrowski and Nüsslein-Volhard, 2000).

We find that ablating the cranial epidermis in axolotl causes fusion of neural crest streams and finally cessation of neural crest migration (Fig. 7). These results suggest that interactions between both ectoderm and endoderm may be critical for neural crest migration in streams. Without directed navigation of neural crest streams through the ecto-/endodermal interface, cranial neural crest cells cannot reach their final destinations and ectomesenchymal derivatives fail to develop properly (Fig. 7D). We assume that this mechanical interaction is a main part of common evolutionary conserved mechanisms which keep cranial neural crest streams separate and guide neural crest cells along correct pathways during craniofacial development of vertebrates.

Outside-in movement

The embryonic pharyngeal arch of vertebrates consists of a central mesodermal core ensheathed by neural crest (e.g., Hacker and Guthrie, 1998; Meulemans and Bronner-Fraser, 2002; Noden et al., 1999; Trainor and Tam, 1995). However, the precise spatiotemporal order in which both tissues interact during arch morphogenesis is not known.

We have shown that cranial neural crest streams in axolotl migrate exclusively along the lateral (subepidermal) pathway and neither mix with mesoderm nor use a medial route (Fig. 8). This seems to be a unique feature of axolotl embryos, since in other vertebrates in addition to the predominant lateral route, neural crest cells invade the cranial mesoderm (e.g., Noden, 1988; Noden et al., 1999; Sadaghiani and Thiébaud, 1987; Serbedzija et al., 1992; Tan and Morris-Kay, 1986; Trainor and Tam, 1995). However, the exact way that the mesoderm becomes eventually ensheathed by neural crest cells is not known. Kimmel et al. (2001) have put forth an elegant model in zebrafish suggesting that neural crest cells migrate laterally along the arch mesoderm and encircle it by moving from "outside-in".

Here, we document for the first time in gnathostomes the dynamic development of an arch ensheathment via an outside-in movement of neural crest cells (Fig. 9) that supports the model proposed by Kimmel et al. (2001). In the axolotl, neural crest streams initially move subepidermally. At a pharyngeal level, however, neural crest streams turn medially circumnavigating the mesodermal core of each arch and progress toward the pharyngeal endoderm, first from a posterior, then from an anterior location, and in a slight ventral to dorsal fashion. In this manner, the entire arch mesoderm becomes encircled by neural crest cells. The two cell types do not intermix nor do neural crest cells use a medial pathway as in the trunk. The ventral to dorsal fashion in which axolotl cranial neural crest cells envelop the arch mesoderm reminiscent of the manner in which cranial and trunk neural crest cells colonize their derivatives in chick and mouse (Lumsden et al., 1991; Serbedzija et al., 1989, 1992). Early migrating neural crest cells first populate the ventral branchial arches, later ones the mid-pharynx, whereas the latest contribute to sensory ganglia situated dorsally.

In contrast to previous views (e.g., Kimmel et al., 2001), cranial neural crest cells were observed to completely surround the mesodermal core of branchial arches in lamprey, a basal agnathan vertebrate (McCauley and Bronner-Fraser, 2003; Meulemans and Bronner-Fraser, 2002). Thus, this resolves the long-standing controversy of whether neural crest cells are being distributed around the mesoderm. However, many differences between gnathostomes and agnathans are still apparent: lamprey neural crest cells fail to fill their derivatives in a ventral to dorsal order and a medial migratory route of cranial neural crest cells is clearly observed (McCauley and Bronner-Fraser, 2003). Thus, the neural crest sheaths around the arches appear to be an ancient and conserved vertebrate trait, whereas the ventral-to-dorsal filling of derivatives appears to have arisen after the agnathan/gnathostome transition. Still more precise neural crest cell tracing experiments are needed in both vertebrate groups to understand how developmental pathways were changed during the agnathan/gnathostome transition. One might speculate that the failure of cranial neural crest cells to migrate along the medial migratory pathway in gnathostomes was the first step in the modification of the gnathostome body plan away from their agnathan forerunners.

In the axolotl, the neural crest sheath around the mesoderm starts to lose its homogeneity after completion of the outside-in movement (stage 34–35). Some individual neural crest cells are found within the mesoderm from about stage 37 onward where they gradually differentiate into connective tissue components of cranial muscles as in other vertebrates (Noden, 1988; Olsson et al., 2001). By this stage, axolotl neural crest cells on the medial side of the arches condense into cartilage, for which an interaction with pharyngeal endoderm is required (Epperlein and Lehmann, 1975). The outside–inward movement therefore might function to bring neural crest cells close to signals from the endoderm and therefore is a prerequisite for the development of the visceroskeleton. Thus, the ring of neural crest around the arch mesoderm may be regarded as a developmentally and evolutionarily conserved feature and the

outside-in movement as an alternative developmental mechanism with which the colocalisation of neural crest and mesoderm can be achieved.

Conclusions

In this study, we have documented an autonomous migratory pattern of axolotl cranial neural crest cells in vitro and interactions of neural crest streams with neighboring tissue in vivo. The ease of visualization coupled with the availability of neural crest markers in this species has allowed us to uncover several novel mechanisms contributing to neural crest patterning. First, we note that neural crest cells migrating from cranial neural tubes migrate in distinct streams in vitro on a permissive substrate without adjacent tissue. Then we observed that close local appositions between the cranial epidermis and adjacent tissues (neural tube, mesoderm and endoderm) lead to the formation of channels that constrain laterally migrating neural crest cells and maintain distinct streams. Third, we experimentally show that removal of the epidermis leads to the fusion of neural crest streams, indicating a requirement for interactions between neural crest cells and at least one adjacent tissue. Fourth, we demonstrate that cranial neural crest cells—migrating initially only laterally—surround the mesodermal core in the branchial arches via an outside-in movement that has been proposed but never proven in other gnathostomes. Taken together, autonomy of initial cranial neural crest stream formation with stabilizing interactions between more advanced neural crest cell streams and neighboring tissues appear to play the predominant role in establishing the migratory pattern of cranial neural crest streams. The exact migratory pathways of neural crest cells seem to differ significantly across vertebrates, irrespective of conservative patterns of cranial neural crest streams. We suggest that these subtle differences in neural crest migration are mirrored later and provide morphological variations of vertebrate types.

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PAPER 2:

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RESEARCH ARTICLE

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

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

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INTRODUCTION

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

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

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

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

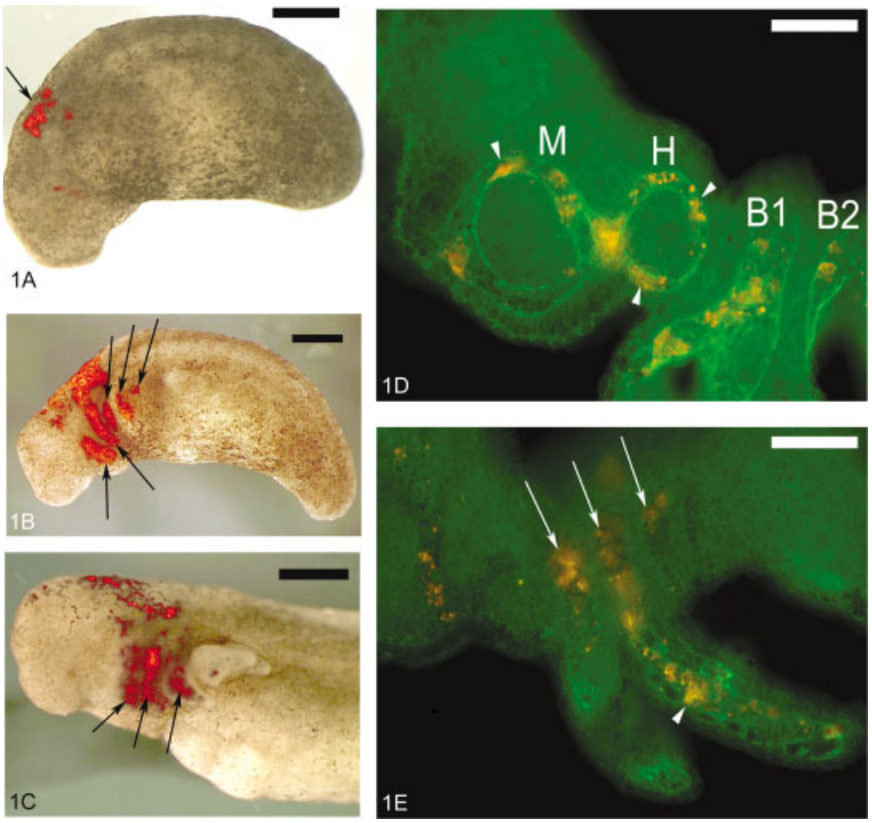


Fig. 1.

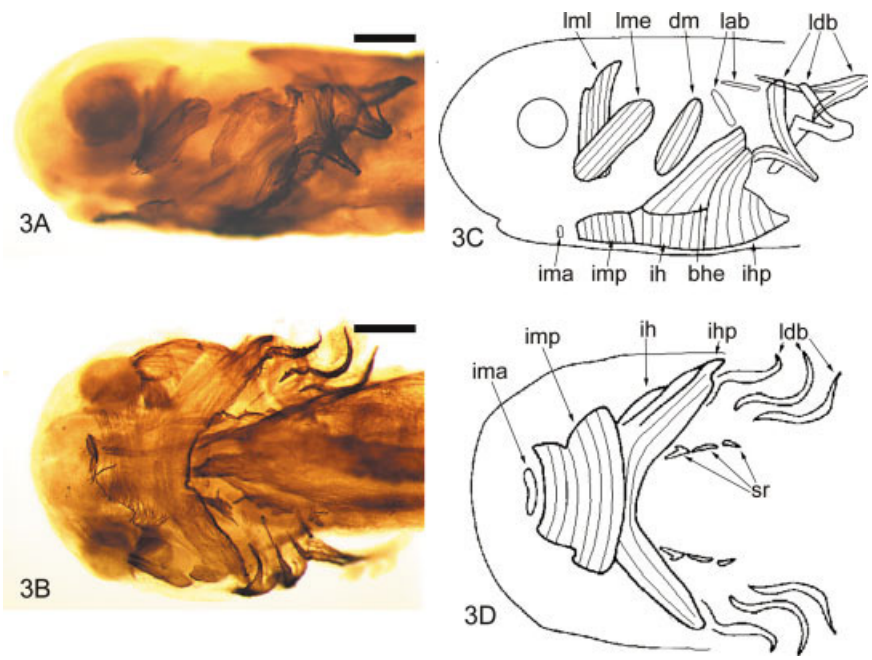


Fig. 3. **A:** Lateral view of stage 44 nonextirpated axolotl embryo. Muscles are stained with the 12/101 antibody and visualized with a horseradish peroxidase–conjugated secondary and diaminobenzidine. **B:** Ventral view of A. **C:** Schematic view of the visceral arch muscles of A. **D:** Schematic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

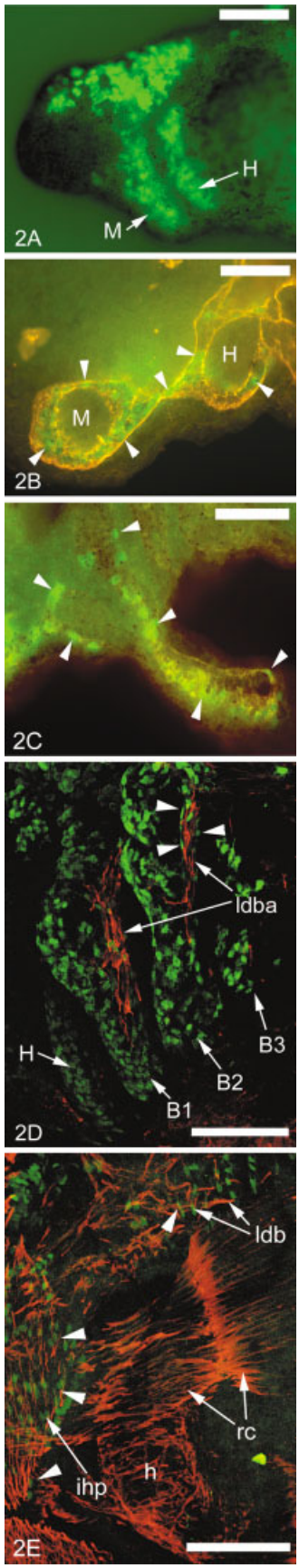


Fig. 2.

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

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

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

RESULTS

Fate Mapping

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

Extirpations

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

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

Mandibular stream.

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

Hyoid stream.

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

Branchial stream.

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

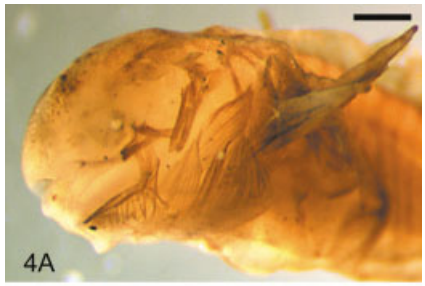
Controls.

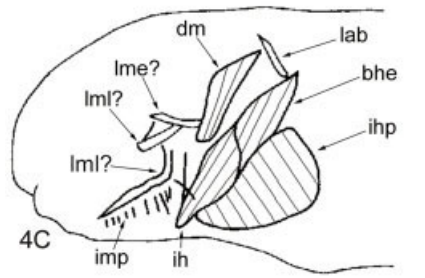
Several controls were made to test the accuracy of the extirpations. To find out if we managed to extirpate the majority of migrating neural crest, we extirpated the hyoid stream of DiI-injected embryos. No DiI-containing cells were observed migrating in the hyoid stream area upon extirpation (data not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

DISCUSSION

Fate of Cranial Neural Crest Cells

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





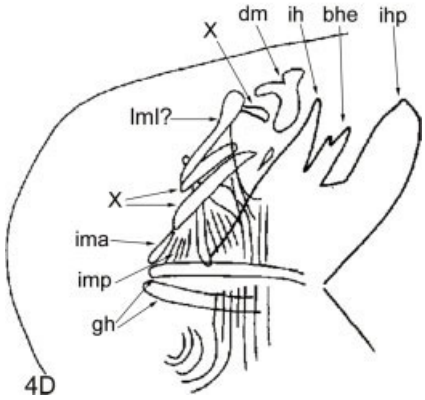
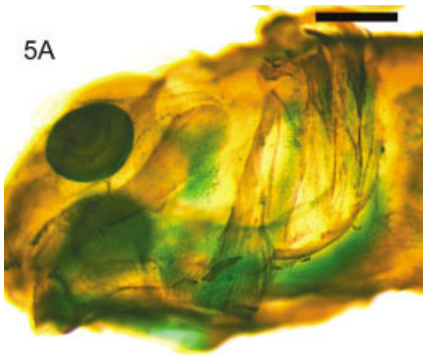
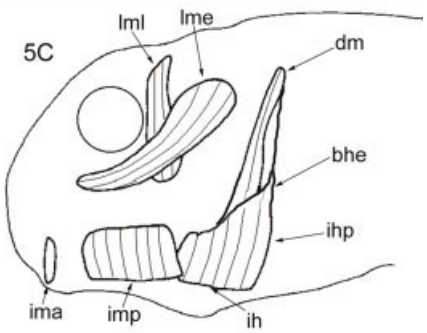


Fig. 4.





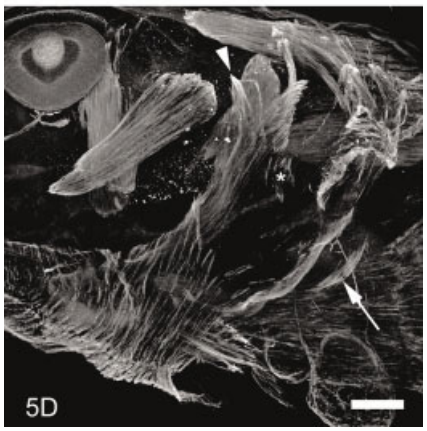
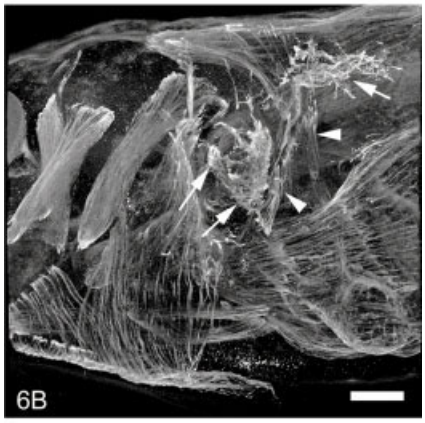


Fig. 5.



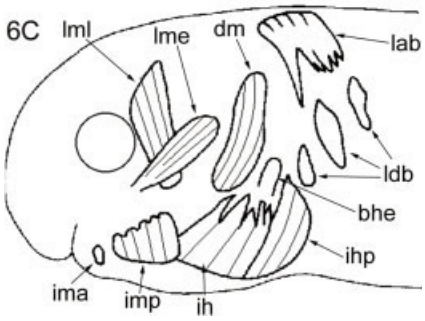
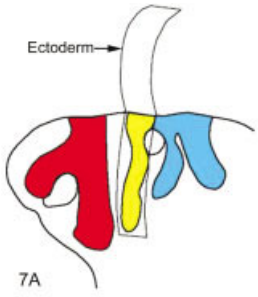


Fig. 6.



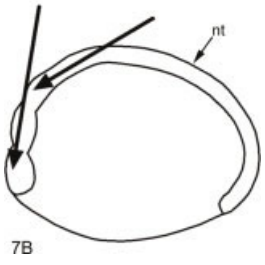


Fig. 7.

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

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

Cranial Neural Crest and Positioning and Morphogenesis of Cranial Muscles

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

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

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

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

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

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

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

EXPERIMENTAL PROCEDURES

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

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

Immunohistochemistry

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

DiI Injections

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

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

GFP mRNA Injections

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

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Developmental origins and evolution of jaws: new interpretation of "maxillary" and "mandibular"

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Abstract

Cartilage of the vertebrate jaw is derived from cranial neural crest cells that migrate to the first pharyngeal arch and form a dorsal "maxillary" and a ventral "mandibular" condensation. It has been assumed that the former gives rise to palatoquadrate and the latter to Meckel's (mandibular) cartilage. In anamniotes, these condensations were thought to form the framework for the bones of the adult jaw and, in amniotes, appear to prefigure the maxillary and mandibular facial prominences. Here, we directly test the contributions of these neural crest condensations in axolotl and chick embryos, as representatives of anamniote and amniote vertebrate groups, using molecular and morphological markers in combination with vital dye labeling of late-migrating cranial neural crest cells. Surprisingly, we find that both palatoquadrate and Meckel's cartilage derive solely from the ventral "mandibular" condensation. In contrast, the dorsal "maxillary" condensation contributes to trabecular cartilage of the neurocranium and forms part of the frontonasal process but does not contribute to jaw joints as previously assumed. These studies reveal the morphogenetic processes by which cranial neural crest cells within the first arch build the primordia for jaw cartilages and anterior cranium.

Keywords: Jaw cartilages; Head; Arches; Maxillary; Mandibular; Axolotl; Chick; Neural crest; Evolution

Introduction

Jawed vertebrates, or gnathostomes, represent the majority of extant vertebrate species. The evolution of jaws allowed gnathostomes to become effective predators and probably accounted for much of their subsequent success (Mallatt, 1996). The classical view is that jaws evolved via modifications of ancient gill arch cartilages (viscerocranial elements), but little is known about the underlying mechanisms (for review, see Hall, 1999a; Janvier, 1996; Mallatt, 1996). In the embryo, the jaw cartilage supports the mouth and is derived from the first pharyngeal (mandibular) arch, which consists of the palatoquadrate cartilage (contributing to the upper jaw) and Meckel's cartilage (forming the presumptive lower jaw) (de Beer, 1937; Depew et al., 2002; Hall, 1999a; Köntges and Lumsden, 1996; Liem et al., 2001). These endoskeletal jaw cartilages form a developmental and evolutionary framework for adult vertebrate jaws (Kardong, 1995; Liem et al., 2001; Smith and Schneider, 1998). Because Hox genes have marked inhibitory effects on jaw formation (Grammatopoulos et al., 2000; Pasqualetti et al., 2000), it has been proposed that the origin of jaws was facilitated by a loss of Hox (homebox transcription factor) expression from the first pharyngeal arch (Cohn, 2002; but see Takio et al., 2004). The jaw and visceral skeleton owe their embryonic origins entirely to the neural crest (Hall, 1999b; Le Douarin and

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Kalcheim, 1999; Santagati and Rijli, 2003), a migratory and multipotent cell population unique to vertebrates (Le Douarin and Dupin, 2003).

Cranial neural crest cells emerge from the dorsal neural tube and migrate extensively to differentiate into the craniofacial skeleton and the neurons and glial cells of the peripheral nervous system, among other derivatives (Le Douarin and Kalcheim, 1999). In all vertebrates, cranial neural crest cells migrate in a conserved and characteristic pattern of three distinct streams termed trigeminal (or mandibular), hyoid (or preotic), and common branchial (or postotic) (Epperlein et al., 2000; Lumsden et al., 1991; Meulemans and Bronner-Fraser, 2002; Trainor et al., 2002). The most rostral (trigeminal) neural crest stream populates the entire anterior head. These cells emerge from the neural tube and eventually contribute to the trigeminal ganglion and to all mesenchymal derivatives of the first pharyngeal arch (mandibular arch sensu lato). Within the first arch, neural crest cells condense to form a dorsal primordium (termed maxillary) below the eye, and a ventral primordium (termed mandibular) below the oral cavity (Chai et al., 2000; Francis-West et al., 1998; Meulemans and Bronner-Fraser, 2002). Interestingly, this pattern represents a common trait of those vertebrate embryos examined to date. In most tetrapods, the maxillary and mandibular condensations appear to progressively proliferate to fill facial prominences, swellings of mesenchyme encased in epithelium termed maxillary and mandibulary prominences (Richman and Lee, 2003). It has been assumed that the embryonic skeleton of the lower jaw (Meckel's cartilage) is formed from the ventral, mandibular component of the first pharyngeal arch whereas the palatoquadrate cartilage is formed from the upper, maxillary component of the arch (Depew et al., 2002; Francis-West et al., 1998; Larsen, 1993; Mina, 2001). Palatoquadrate is therefore often referred to as the "maxillary cartilage" and Meckel's element as the "mandibular cartilage" (Depew et al., 2002; Larsen, 1993). However, there has been no direct evidence that supports or refutes this prevailing assumption.

In the present study, we utilize direct lineage labeling and careful morphological observations in an anamniote (axolotl) and amniote (chick) to observe and compare the patterns of late neural crest migration as they form these distinct cartilages. Contrary to the generally held notion, our data suggest that both palatoquadrate and Meckel's cartilage, as fundamental cartilaginous structures of jawed vertebrates, derive from a common ventral condensation within the first pharyngeal arch that is here referred to as maxillomandibular. The dorsal condensation (here termed "trabecular") of the first pharyngeal arch was found to give rise to anterior neurocranial structures, predominantly to the trabecula cranii. The data show that the so-called first arch of vertebrates is a much more complex structure than previously assumed, emphasizing the necessity for precise fate mapping of this region of the vertebrate embryo.

Materials and methods

Embryos

Embryos of the Mexican axolotl (*Ambystoma mexicanum*) were obtained, reared, and staged as previously described (Epperlein et al., 2000). Before operating on the embryos, they were decapsulated manually. Fertilized chicken eggs were obtained from commercial sources, incubated at 38°C as indicated elsewhere (Sechrist et al., 1993), and staged according to Hamburger and Hamilton (1951).

Injections of DiI

A fixable form of DiI, Cell Tracer CM-DiI (Molecular Probes), was dissolved in absolute ethanol and diluted to a working concentration of about 0.1 mg/ml in 0.3 M sucrose. To visualize continuous migration of neural crest streams, DiI was injected into anterior cranial neural folds of axolotl embryos between stages 19 and 22 using an Inject + Matic microinjector.

For injections at a pharyngula stage, a small amount of DiI was focally injected into either dorsal or ventral aspects of the mandibular arch of axolotl (dorsal or ventral neural crest cell condensations, respectively; Figs. 3A and D) using an IM 300 microinjector and fine glass micropipettes attached to a needle holder (Oxford instruments). Those embryos in which the DiI stained neural crest cells stayed precisely at the same place and did not spread out within 5 min ($n = 10$ for dorsally, and $n = 27$ for ventrally injected ones) were developed to stage 40, sectioned and analyzed. In chick, small focal injections of DiI were made into either the dorsal "maxillary" ($n = 8$) or ventral "mandibular" ($n = 9$) facial processes (Figs. 3G and J) in stage 13–14 chick embryos and analyzed at E7.

Injections of FITC-dextran and GFP followed by cranial tube transplantations

For expression of FITC-dextran or green fluorescent protein (GFP), about 1–5 ng GFP mRNA (in 20-nl solution) was injected using an IM 300 microinjector into one blastomere of a two- to four-cell axolotl embryo. For injection, the embryos were placed in an agar dish containing 5% Ficoll in 1/10 Steinberg solution and antibiotics (Gibco). After 1 day, the embryos were transferred into normal strength saline. Embryos were then allowed to develop to the neurula (stages 15–17) and used for grafting. Using tungsten needles, dextran- or GFP-stained head neural folds were grafted orthotopically into uninjected host sibling embryos in which the corresponding part of neural fold had been excised before.

Sectioning and immunostaining

Axolotl embryos were anaesthetized in a solution of tricaine methane-sulfonate (MS-222, Sandoz) and fixed in

4% paraformaldehyde in 0.1 M phosphate-buffered saline (PFA/PBS) at least overnight. After washing in PBS, specimens were dehydrated through a graded series of ethanol and embedded in JB4 (Polysciences, Inc.). Sections (5 μm) were cut with a Reichert-Jung microtome (Biocut 2035), stained with Azure B-Eosin (SERVA), and mounted in DePeX (SERVA).

Embryos processed through in situ hybridization were stored in 100% methanol and cut (100 μm) using a Vibratome Series 1000 sectioning system (Ted Pella, Inc.). Axolotl sections were counterstained with a primary polyclonal anti-fibronectin antibody (1:100, Dako) followed by a goat anti-rabbit Cy3 secondary antibody (1:100, Dako) in order to visualize tissue borders. Afterwards, these sections were stained with DAPI (0.1–1 μg/ml PBS) to mark cell nuclei. Some sections were stained with the skeletal muscle marker 12/101 (Developmental Studies Hybridoma Bank).

Chick embryos fixed in PFA/PBS were sectioned (25 μm) using a Leica cryostat. Rabbit anti-collagen II (IgG, BABCO) antibody was used diluted at 1:200 to detect chondrocytes. As a secondary antibody, Alexa-Fluor 488 anti-rabbit IgG1 (Molecular Probes) was used at 1:200 dilution. Immunostained sections were counterstained with DAPI, rinsed in PBS, and mounted on slides using Perma Fluor (Immunon). Separate brightfield and fluorescence images were captured with a Zeiss Axiocam or SPOT RT camera, merged, and optimized using Spot, MetaView, and Adobe Photoshop software.

In situ hybridization

In situ hybridization was performed on axolotl albino embryos as described by Henrique et al. (1995) with the addition of an extra wash in MAB-T (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween 20) overnight at 4°C (Meulemans and Bronner-Fraser, 2002). Hybridization was performed at 65°C. Preparation of riboprobes was done as previously described (Cerny et al., 2004).

Scanning electron microscopy and cartilage staining

Scanning electron microscopy (SEM) and staining of cartilage using Alcian Blue followed our protocols as described in detail elsewhere (Cerny et al., 2004).

Results

We examined the developmental origins of jaw cartilages from neural crest condensations in axolotl and chick embryos as representatives of early and late tetrapods. From an embryological standpoint, axolotl embryos represent an advantageous model system for studying tissue morphogenesis (Beetschen, 1996). Their relatively large size and slow development (Cerny et al., 2004; Epperlein et al., 2000) allow accurate tracing of neural crest cells during condensation into cartilage and early jaw development. In contrast, chick embryos have smaller cells and large cell numbers but represent the best-studied and best-understood model for amniote neural crest development (Le Douarin and Kalcheim, 1999), and are used here for comparison to axolotl.

The postoptic portion of the trigeminal neural crest stream covers the surface of the first (mandibular) arch

As in other vertebrates, the trigeminal neural crest stream in axolotl originates from the posterior prosencephalon, mesencephalon, and anterior rhombencephalon (Cerny et al., 2004; Epperlein et al., 2000). These anterior neural crest cells are further distinguished from posterior cranial neural crest cells by their lack of Hox gene expression. These cells migrate as a broad sheet that first uniformly populates the anterior head but soon becomes mechanically subdivided by the optic vesicle into neural crest cells situated preoptically and mandibular arch neural crest cells situated postoptically, which cover the first pharyngeal (mandibular) arch mesoderm (Cerny et al., 2004) (Figs. 1A–C).

Dorsal "maxillary" and ventral "mandibular" neural crest condensations of the first arch appear to be a conserved feature of vertebrates

Mandibular arch neural crest cell migration ceases around the pharyngula stage (axolotl stage 34; Figs. 1B and C) with neural crest cells distributed homogeneously over the surface of the central mandibular arch mesoderm (Cerny et al., 2004). Subsequently, neural crest cells become depleted from the middle of the first arch by an as yet unknown mechanism and accumulate into two condensations (Fig. 1D), one dorsally, closer to the optic vesicle and nose, and another at the ventralmost aspect of the mandibular arch (Fig. 1D). Although a thin layer of neural crest cells remains over the surface of the arch mesoderm at this time, the majority of neural crest cells appear in two aggregated condensations (not shown). The dorsal (often referred to as "maxillary") and ventral (termed "mandibular") condensations of the first arch neural crest cells are morphologically visible in all vertebrate embryos examined, including jawless lampreys (Meulemans and Bronner-Fraser, 2002).

At the end of the pharyngula stage, the basic vertebrate body plan represents a vertebrate phylotype, a conserved developmental stage shared by both gnathostomes and agnathans (Kuratani et al., 2001, 2002). From this point in development onwards, body plans among vertebrates diverge. In agnathans, neural crest cell condensations develop into cartilages of the larval oral apparatus. In lower gnathostomes, the dorsal condensation has been thought to develop into the upper jaw cartilage and the ventral one into lower jaw (Kardong, 1995; Larsen, 1993; Liem et al., 2001).

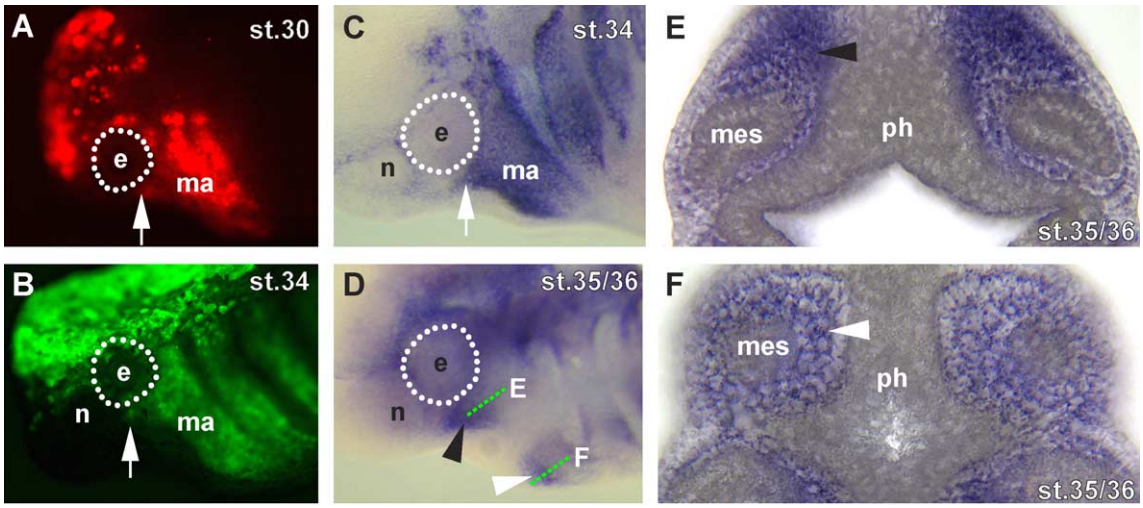

Fig. 1. Axolotl cranial neural crest streams and early development of facial condensations. Neural crest cells are visualized by DiI (A) or GFP (B) labeling, or after in situ hybridization using the *snail* riboprobe (C–F). (A) The first neural crest stream covers the entire anterior head; the arrow points to the "maxillary" part situated close to the eye (e, dotted circle). (B) GFP labeling of all neural crest cells shows cranial streams. The arrow points again to the "maxillary" part. (C) At the pharyngula stage, neural crest cells populate the first arch homogeneously. (D) Shortly thereafter, neural crest cells appeared reduced in the middle of the arch. Black and white arrowheads point to dorsal and ventral accumulations of neural crest cells, respectively. Green dotted lines show the position of horizontal sections through the dorsal (E) and ventral (F) neural crest condensations within the same mandibular arch. The dorsal center develops anteromedially whereas the ventral one develops close to the central mesodermal core (mes) of the first branchial arch. ma, mandibular arch; n, nose; ph, pharynx.

In amniotes, these two neural crest condensations contribute to the corresponding facial prominences that are also referred to as maxillary and mandibular. It has been predicted that these prominences give rise to the upper and lower jaw structures, respectively. However, direct evidence for such contributions, particularly into skeletal derivatives of the jaw and face, has been lacking (Richman and Lee, 2003).

Both palatoquadrate and Meckel's cartilages originate solely from the ventral condensation

In axolotl, obvious morphological differences between the dorsal versus ventral condensation become apparent around stage 35, as documented in longitudinal sections (Figs. 1D–F). Cranial neural crest cells in the "maxillary" area appear as a dorsally enlarged accumulation of cells, predominantly along the anterior portion of the arch (Fig. 1E), as visualized by *snail* expression. In contrast, the ventrally localized "mandibular" condensation (Fig. 1F) forms a homogeneous tube that extends slightly toward the medial portion of the arch.

In order to better visualize the changes that occur within condensations of neural crest mesenchyme as a function of time, we transplanted neural folds labeled with either FITC-dextran or GFP into unlabeled host embryos. This allowed distinction of neural crest cells from the tissues through which they migrate and differentiate. As an alternative labeling approach that involved no microsurgery, we labeled neural folds in some embryos with focal injections of the lipophilic dye DiI. We then carefully analyzed the morphogenetic changes of labeled neural crest cells at consecutive stages in order to understand how mesenchymal condensations differentiate into jaw cartilages and build facial primordia. Both labeling methods yielded identical results.

Our analysis of sectioned embryos revealed that neural crest cells from the ventral, commonly designated mandibular, condensation contribute to both Meckel's (Fig. 2D) and palatoquadrate cartilage (Fig. 2H). Neural crest cells at the ventralmost aspect of the mandibular condensation (Fig. 2A) subsequently develop into Meckel's cartilage (Figs. 2B–D). Careful analysis of sections reveals that the palatoquadrate cartilage (Fig. 2H) derives from a more dorsal part of this same ventral, "mandibular" condensation (Fig. 2E). The palatoquadrate arises from a compact cell mass (Figs. 2E and F) that is initially localized in the medial part of the mandibular condensation. With time, this rearranges to a more posterior position within the arch (compare Figs. 2E, F, G and H). The identity of the cartilage was confirmed by the presence of a group of mandibular levator muscles (Fig. 2H, m.l.m.) that are always associated with the palatoquadrate (Ericsson and Olsson, 2004; Piatt, 1938). Progressive development of this muscle from an initially undifferentiated central mesodermal mass within the mandibular arch can be visualized by comparing Fig. 2F (undifferentiated central mesodermal mass indicated by white asterisk) with Fig. 2G (condensation now splits into a lateral and medial mass, indicated by two white asterisks) and finally with Fig. 2H (group of three levator mandibulae muscles, m.l.m., recognized by staining with the skeletal muscle marker 12/101 in red).

These results suggest that the cranial neural crest mass of the ventral ("mandibular") condensation becomes sub-

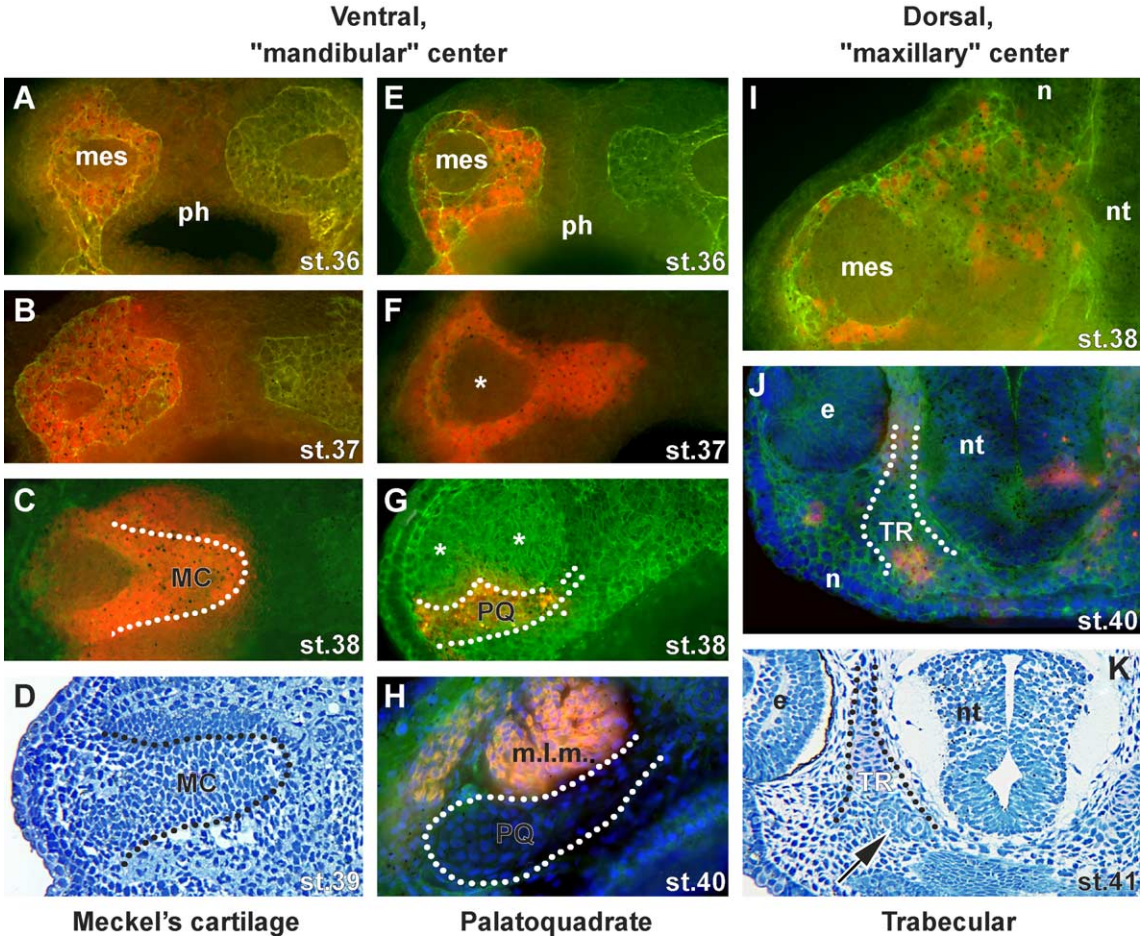

Fig. 2. Both Meckel's (MC) and palatoquadrate (PQ) cartilages develop from a single ventral ("mandibular") center, whereas the dorsal ("maxillary") condensation gives rise to trabecular cartilage (TR). Development of the ventral ("mandibular") center (first two columns; A–H) and dorsal ("maxillary") center (right column; I–K) of the first arch is shown in horizontal (A–I) or transverse (J and K) sections, primarily of the left side of each section. Neural crest cells are visualized by DiI (G and J), fluorescent dextran (A–C, E, and F), or GFP (I) labeling. Neural crest cells stained by DiI, GFP, or fluorescent dextran are red; anti-fibronectin staining reveals tissue borders (green) and DAPI shows cell nuclei (blue) (H and J). For detailed explanation, see the text. mes, arch mesoderm; n, nose; ph, pharynx; nt, neural tube.

divided, with a portion ultimately contributing to the dorsal palatoquadrate cartilage and the remainder to the ventral Meckel's cartilage in the mandibular process. This finding was unexpected as it contradicts the previously held assumption that the upper jaw (palatoquadrate) cartilage arises from the "maxillary" condensation of the first arch in all vertebrates (Depew et al., 2002; Hall, 1999a; Kardong, 1995; Larsen, 1993). However, this is not entirely surprising since, even at early stages, two prechondrogenic cell masses within the single ventral (mandibular) condensation resemble by their shape and position the mature palatoquadrate and Meckel's cartilage of axolotl, similar to the situation observed during formation of early hyoid cartilage in zebrafish (Kimmel et al., 1998). Therefore, based on careful morphological analysis, these results suggest that both jaw cartilages that were previously thought to be derived from "maxillary" and "mandibular" condensation arise from a single ventral neural crest condensation.

The dorsal neural crest cell condensation gives rise to trabecula cranii, a neurocranial element

The above findings raise the intriguing question of what forms from the dorsal "maxillary" neural crest mass. To address this, we examined the morphogenetic movements and differentiation of the dorsal condensation by following labeled neural crest (Figs. 2I–K). Some cells derived from the dorsal condensation contributed to dermis and head mesenchyme (not shown). However, most of labeled cells contributed to a cranial element that was clearly identified as the trabecula cranii (Figs. 2J and K), with no contribution to the palatoquadrate as had been previously suggested. The trabeculae of the neurocranium were discovered first by Rathke in the grass-snake (de Beer, 1931) and later recognized as the essential element of the cartilaginous anterior cranial base (skull) in all vertebrates (Bertmar, 1959; de Beer, 1937; Kuratani et al., 1997). Our results suggest that the dorsal condensation gives rise to trabecular cartilage and

some other neural crest derivatives, but does not contribute to either the palatoquadrate or Meckel's cartilage.

Interestingly, the differentiation of the dorsal neural crest cell mass is about 1–2 days delayed compared to the ventral one. The first signs of chondrocyte differentiation as visualized by collagen type II staining in both palatoquadrate and Meckel's cartilage are detectable from stage 38/39 onwards (not shown), whereas those neural crest cells in the dorsal center are still present as an undifferentiated condensation at the same time.

Direct lineage labeling in axolotl demonstrates that both palatoquadrate and Meckel's cartilage arise from the ventral condensation

The above results strongly suggest that the dorsal condensation gives rise to trabecula of the neurocranium and the ventral condensation gives rise to cartilaginous lower (Meckel's) and upper (palatoquadrate) jaw elements. Because neural crest cells were labeled prior to migration, however, we cannot rule out the possibility that some mixing between these populations may have occurred but was missed in our analysis of sectioned material.

We next turned to direct lineage labeling of these cranial neural crest cell masses using focal injections with the vital dye DiI. Direct labeling allowed us to experimentally confirm the nature of the structures that arose specifically from the "dorsal" and "ventral" condensations within the first arch in axolotl. Embryos received a single injection of DiI into either the dorsal or ventral condensation. Only those embryos with relatively small and focal injections were selected for subsequent analysis in stages following chondrogenesis. When DiI injections were made into the dorsal condensation at stages 34–36 (n = 10, Fig. 3A), labeled neural crest cells were clearly observed several days after injections in the trabecular cartilage between the eye and brain (as best viewed in transverse sections, Figs. 3B and C). However, no staining was seen in the palatoquadrate (Fig. 3C) or Meckel's cartilage. In contrast, focal DiI injections to the "ventral" cranial neural crest cell mass (n = 27, Fig. 3D) revealed a contribution from this portion of the arch to both the palatoquadrate and Meckel's cartilage, but not to the trabecular cartilage (Figs. 3E and F). These results validate our morphological analysis by using direct neural crest lineage labeling and confirm that dorsal condensation contributes to trabecular cartilage of the neurocranium and ventral condensation contributes to both the palatoquadrate and Meckel's cartilage. Although these injections undoubtedly labeled some adjacent cranial mesoderm as well, this tissue makes no contribution to cartilage.

Direct lineage labeling of dorsal and ventral neural crest cell condensations in the chick

To further test whether the contribution of the ventral condensation to both Meckel's cartilage and the palatoquadrate is a general feature of tetrapods, we turned to the chick that is currently the best-studied amniote model for neural crest development (Le Douarin and Kalcheim, 1999). Parallel to the DiI injections performed in axolotl embryos, focal injections of DiI were made into either the dorsal "maxillary" (n = 8) or ventral "mandibular" (n = 9) facial processes in stage 13–14 chick embryos and analyzed at E7. Analogous to our findings in axolotl, DiI-labeled neural crest cells from dorsal injections (Fig. 3G) contributed to trabecular cartilage (also called interorbital) with generally no contribution to palatoquadrate or Meckel's cartilage (Figs. 3H and I). In contrast, ventral injections (Fig. 3J) contributed labeled neural crest cells to both the palatoquadrate and Meckel's cartilage (Figs. 3K and L). Thus, similar findings were observed for representatives of two groups of gnathostomes, anamniote (axolotl), and amniote (chick). Surprisingly, in chick, we found that ventral injections contributed a few neural crest cells to the trabecular cartilage as well; suggesting that there must be at least some very late neural crest cell movements or some intermixing within the first arch.

FGF8 expression prefigures the maxillomandibular region and in axolotl is restricted to the ventral portion of the arch

What might account for the specification of the maxillary and mandibular region within the first neural crest stream? In many vertebrate embryos, the early distribution of FGF8 in the ectoderm correlates with the presence of the nascent maxillomandibular region (Crossley and Martin, 1995; Ferguson et al., 2000; Trumpp et al., 1999) and has been proposed to play a role in its determination (Shigetani et al., 2000; Tucker et al., 1999). Interestingly, FGF8 expression precedes the influx of cranial neural crest cells into the first arch and their condensation into dorsal and ventral centers (Richman and Lee, 2003; Shigetani et al., 2002). Therefore, these embryonic craniofacial regions may be specified early in development and be independent of patterning by cranial neural crest streams. In the axolotl, surprisingly, we observe that FGF8 expression in the ectoderm is present only adjacent to the ventral but not the dorsal neural crest condensation (Fig. 4, sections not shown), consistent with a possible role in influencing the neural crest contribution to both palatoquadrate and Meckel's cartilage.

Discussion

By combining lineage analysis with careful morphological observations, our results have established the descendants of the dorsal, previously called "maxillary", and ventral, "mandibular", neural crest condensations that derive from migrating cranial neural crest cells at the trigeminal level. Based on these observations, we propose the following model of first arch neural crest cell regionalization and subsequent jaw cartilage morphogenesis (Fig. 5). Initially,

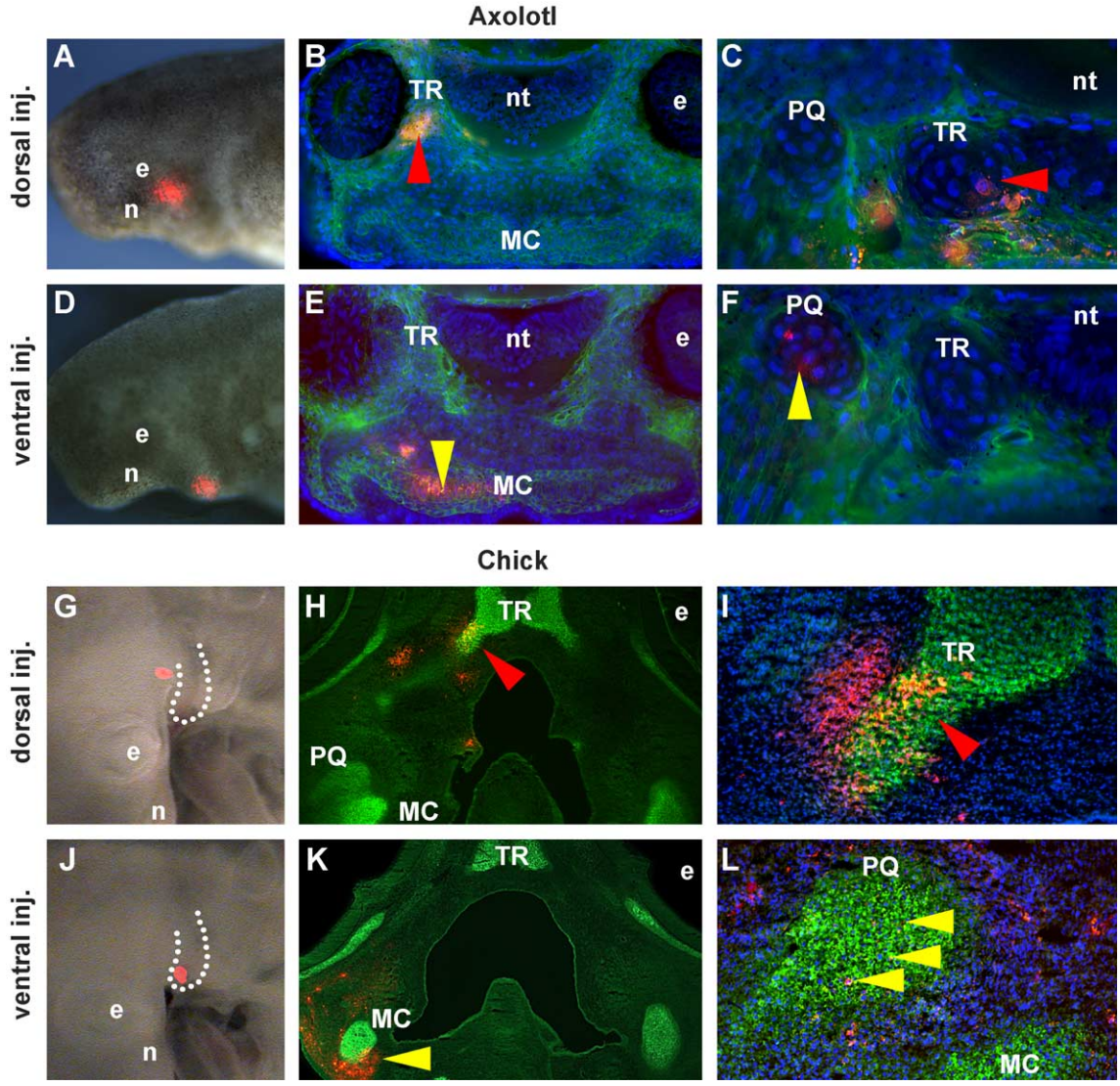

Fig. 3. Focal DiI injections into either dorsal (A and G) or ventral (D and J) neural crest condensation in both axolotl (A–F) and chick (G–L) confirm that both Meckel's (MC) and palatoquadrate (PQ) cartilages arise from a single ventral condensation, whereas the dorsal condensation develops into trabecular cartilage (TR). Transverse sections show the position of stained cells several days after the injection; anti-fibronectin counterstaining (green in B, C, E, and F) reveals tissue borders in axolotl and anti-collagen type II (green in H, I, K, and L) stains cartilage in the chick. DAPI stains cell nuclei (blue). Red arrowheads point to stained cells after the dorsal injection, yellow arrowheads point to stained cells after the ventral injection. e, eye; n, nose, nt, neural tube.

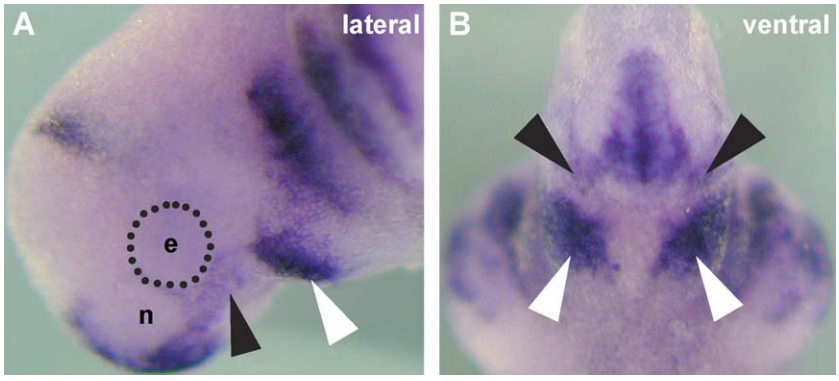

Fig. 4. FGF8 expression in the first arch of axolotl (stage 30). Staining is visible in the ventral portion of the first arch only (white arrowhead), which develops into both the palatoquadrate ("maxillary") and Meckel's ("mandibular") cartilage. Black arrowheads point to the upper part, incorrectly named as maxillary. e, eye; n, nose.

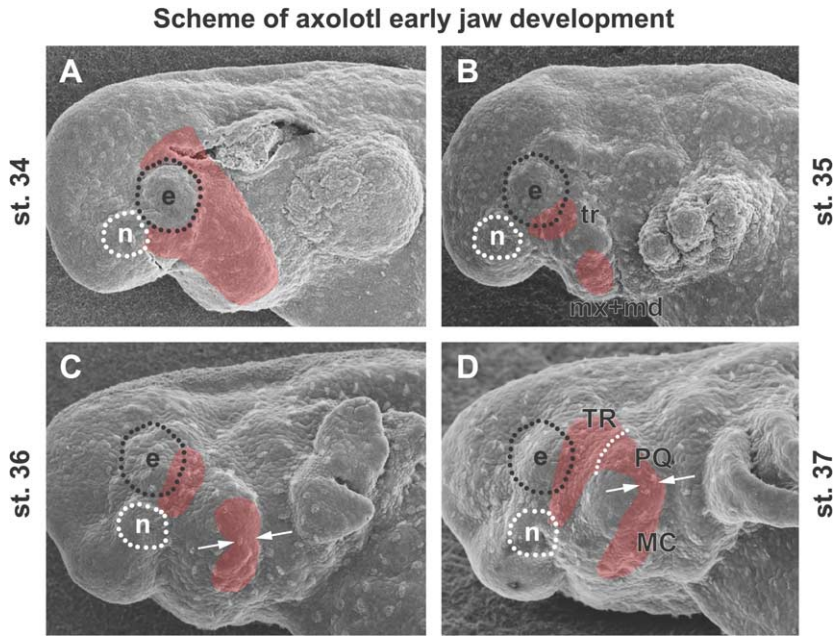

Fig. 5. Early steps in jaw morphogenesis in axolotl. SEM images at stages 34–37, head to the left. Development of neural crest cells (red) in relation to the first pharyngeal arch, nose (n), and eye (e) is schematically outlined. (A) After extensive migration, neural crest cells homogeneously cover the entire surface of the first arch mesoderm. Soon after (B), they condense into a dorsal trabecular (tr) center and a ventral, maxillomandibular (mx + md) center. Subsequently (C and D), the ventral center extends more dorsally and differentiates into palatoquadrate (PQ) and Meckel's (MC) cartilage. The dorsal center gives rise into trabecular cartilage (TR) instead. White arrows point to the position of the jaw-joint (in C and D), white dotted line in D indicates schematically the border between trabecula and palatoquadrate cartilage.

cranial neural crest cells populate the entire surface of the mandibular arch mesoderm (Fig. 5A). They establish a sheath of neural crest around the central mesodermal rod. Later, neural crest cells split into two distinct condensations: (1) a dorsal trabecular condensation in the vicinity of the eye and nose that probably prefigures the maxillary prominence and (2) a ventral "maxillomandibular" condensation within the mandibular arch (Fig. 5B). The dorsal center contributes to trabecular cartilage of the neurocranium and probably to portions of the frontonasal process as well, comprising fundamental elements of the anterior cartilaginous skull of all vertebrates. The ventral center becomes subdivided such that its ventralmost portion elongates medially to develop into Meckeĺs cartilage, the endoskeletal framework for the adult lower jaw. The more dorsal portion extends further dorsally and develops into the palatoquadrate cartilage (the proximal part of which later ossifies to become the quadrate). This then connects Meckeĺs cartilage of the lower jaw with the trabecular cartilage and neurocranium, and forms the primary jaw-joint in all vertebrates (Figs. 5C,D and 6A,B).

Early morphogenesis of jaw cartilages in the evolutionary context of anterior pharyngeal arches

During the course of evolution, the vertebrate cranium and jaw have served the common functions of protecting the brain/sensory organs and capturing or biting food, respectively. However, the way in which individual cranial components participate in these functions has been extensively modified between species. Thus, function has remained constant while form has changed. A well-known example of such structural remodeling during evolution is the change of jaw structure in mammals that culminated in a new jaw joint and two additional auditory ossicles in the middle ear (Janvier, 1996; Liem et al., 2001; Smith and Schneider, 1998).

Cranial elements like trabeculae and anteriormost pharyngeal arch cartilages are another notable example of structural remodeling during evolution. The trabeculae and their direct developmental derivatives (Miyake et al., 1992) have been shown to be of neural crest origin in a variety of vertebrates (Couly et al., 1993; Langille and Hall, 1988a,b; Matsuo et al., 1995; Olsson and Hanken, 1996; Schneider and Helms, 2003). This suggests that these elements are originally viscerocranial (pharyngeal arch) components enlarged during the course of evolution in order to protect anterior brain and sensory organs finally forming a new, prechordal neurocranium (for a review, see de Beer, 1937; Kuratani et al., 1997).

Classical studies based on careful histological observations have suggested that the trabeculae originate within the "maxillary process of the mandibular arch" in a variety of animals (e.g., Allis, 1923, 1924; de Beer, 1931; Haller, 1923; but see others, e.g., Bertmar, 1959; Goodrich, 1930, for a different opinion). Here, we confirm the first view experimentally using a combination of vital dye labeling of late-migrating cranial neural crest cells and morphological

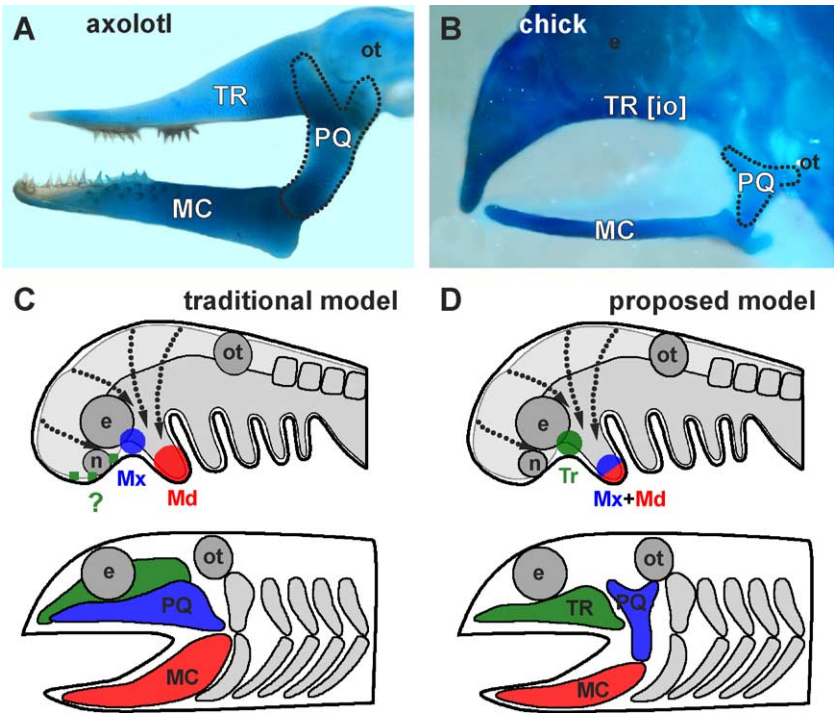

Fig. 6. Alcian Blue staining of axolotl (A) and chick (B) cartilaginous skull. In both, Meckel's cartilage (MC) is a framework for the lower jaw whereas trabeculae (TR) contribute to the upper jaw. Palatoquadrate (PQ, black dotted lines) connects these two elements. In larval axolotl, even teeth develop on trabecular cartilage. (C) A traditional model of developmental origin of jaw cartilages in gnathostomes. The dorsal (maxillary, Mx) neural crest condensation has been proposed to give rise to palatoquadrate cartilage (PQ), which is often described to form the entire upper jaw, whereas the ventral (mandibulary, Md) condensation develops into Meckel's cartilage (MC) that is the lower jaw element. Neurocranium (green) is expected to originate from cranial neural crest cells (green dots and question mark). (D) Our data suggest an alternative interpretation for modern tetrapods, in which the dorsally situated condensation of neural crest cells (here referred to as trabecular, Tr) contributes to trabecular cartilage of the neurocranium (TR). Trabeculae or their developmental derivatives like inter-orbital [io] cartilage and the nasal septum are connected to the anterior palatoquadrate, which forms the hinge of the upper jaw in modern tetrapods. The ventral condensation (probably fused maxillary and mandibular, Mx + Md) gives rise to both Meckel's and palatoquadrate cartilages. Therefore, we conclude that the embryonic mandibular arch of recent animals consists of several fused elements (compare C and D, upper parts). e, eye; n, nose; ot, otic vesicle. Dashed curves represent pathways of neural crest migration.

analysis (Figs. 2 and 3). Moreover, we also show that both jaw cartilages can be traced back to a single, ventral neural crest cell condensation for which we have coined the term "maxillomandibular" (Figs. 2, 3, and 5).

Trabeculae are primarily considered to be bars of a "preoral" or "premandibular" arch based on embryological and topographical homology as well as the supposed metameric organization of cranial nerves (Allis, 1923; de Beer, 1931; Kuratani et al., 1997; Platt, 1897). Following this kind of view, the structure called the mandibular arch of recent vertebrates, in fact, comprises precursors of mandibular arch elements (jaw cartilages) on its ventral aspect and precursors of another cranial arch on its dorsal side (Fig. 5).

Developmental changes in positions of the precursors to these structures are reflected by evolutionary changes of adult jaw structures and probably provide the mechanism through which changes in evolution of the jaws may have arisen. We propose the following model as the ancestral state of the cartilaginous head of basal jawed vertebrate (Fig. 6C, bottom): the anterior braincase is likely to have consisted of a trabecular element (which had enlarged and moved into a horizontal position to protect the brain) to which a large, anteriorly extending palatoquadrate cartilage had been articulated. The palatoquadrate as an upper jaw structure was connected to Meckel's (mandibular) cartilage representing the lower jaw (Fig. 6C, bottom). The embryological position of the condensation of Meckel's cartilage would then be expected in the ventral aspect of the mandibular arch (mandibular prominence), palatoquadrate condensation in the dorsal (maxillary) portion of the mandibular arch (maxillary prominence), and trabecular condensations somewhere in a "premandibular area", closer to the anterior tip of the head (upper part of Fig. 6C).

In modern tetrapods, however, the precursors to both palatoquadrate and Meckel's cartilage apparently have merged into a single "maxillomandibular" condensation in the ventral portion of the first pharyngeal arch (Fig. 6D, upper). The embryonic precursor to the premandibular element, the trabecular cartilage, appears to occupy a dorsal portion of the first arch (Fig. 6D, upper) as conclusively demonstrated by our morphogenetic studies and direct tracing experiments (Figs. 2 and 3).

Meckel's and palatoquadrate cartilages are clearly homologous across vertebrate taxa. However, these two elements are reduced in size and importance in multiple vertebrate lineages such that they no longer define the

functional adult jaws. This is particularly clear in the case of the palatoquadrate, which is relegated to a more posterior place (Liem et al., 2001; Smith and Schneider, 1998). This reduction culminates in modern tetrapods, where the palatoquadrate often forms only the jaw-joint (quadrate bone) such that the upper jaw is entirely comprised of dermal bones (predominantly by maxilla and premaxilla), the endoskeletal framework of which represents the trabecula (in axolotl, Fig. 6A) or its developmental derivatives like the interorbital septum (in chick, Fig. 6B). Dermal bones like maxillary and palatine undergo a direct ossification without any contribution from the endoskeletal cartilage components and belong to another likely independent skeletal system—the dermatocranium.

Possible homology between agnathans and gnathostomes

Recent experiments in which cranial neural crest migration in a basal vertebrate, the sea lamprey, was followed by DiI labeling (McCauley and Bronner-Fraser, 2003) suggest that migration patterns in the first pharyngeal arch may be highly conserved between agnathans and gnathostomes (Kuratani et al., 1999, 2001). In lamprey, cranial neural crest cells migrating into the first arch bifurcate ventrally to surround the mouth (McCauley and Bronner-Fraser, 2003; Meulemans and Bronner-Fraser, 2002) in a pattern similar to that observed in gnathostomes. This particular migratory pattern of lamprey resembles the behavior of the ventral condensation in axolotl, suggesting that they may be homologous. Although agnathans possess trabecular cartilage, its developmental origin is not yet known nor is it clear whether this trabecular cartilage is homologous to that of gnathostomes (Damas, 1944; Kuratani et al., 2001).

Our results suggest strong similarities between the general patterns and the processes of neural crest migration that form the jaws of anamniote (axolotl) and amniote (chick) vertebrates. However, some interesting species differences were noted as well. Following injections made into the ventral mandibular condensation, we observed a contribution of a few neural crest cells to the trabecular cartilage in the chick but never in axolotl. This is an interesting difference between species and may result from the fact that there are many more (and smaller) neural crest cells in birds than in axolotl, allowing more regulative development or simply more cell migration and intermixing. Such mixing between dorsal and ventral populations in chick has been observed in later fate mapping studies of facial primordia, suggesting that these regions are not strictly segregated lineage compartments (McGonnell et al., 1998). Clearly, facial regions of chick and axolotl are not identical because the anamniote mandibular arch, in principle, does not display clear maxillary and mandibular prominences. This again points to the fact that there is a strong need for precise fate-mapping experiments to accurately map this region.

Of course, one would expect that DiI injections made directly into the dorsal and ventral condensations would also label some cranial mesoderm in addition to neural crest. Because the mesoderm does not contribute to cartilage, it remains unambiguous that this contribution came from the neural crest.

Patterning of cranial neural crest cells

Patterning of cranial neural crest cells that contribute to the jaws appears to involve a combination of extrinsic and intrinsic factors. Some neural crest populations exhibit plasticity and loss of Hoxa2 gene expression when transplanted from hyoid to mandibular arch (Trainor and Krumlauf, 2001), suggesting an environmental influence on their gene expression. However, there is also some evidence for prepatterning within the neural crest itself; for example, transplantation of quail and duck neural crest into the homologous position in chick embryos results in beak patterning characteristic of the donor tissue (Schneider and Helms, 2003). In terms of molecular mechanisms underlying arch patterning, there is an interesting nested distribution of members of the distalless (Dlx) gene family within the first pharyngeal arch, such that Dlx1/2 are expressed most dorsally, Dlx5/6 genes expressed in the middle of the arch, and Dlx3/7 in the most ventral portion (Depew et al., 2002). Dlx genes appear to be critical for dorsoventral specification of the first arch: such that null mutations in Dlx5/6 result in mirror image duplications of the upper jaw (Beverdam et al., 2002; Depew et al., 2002). The differential expressions of signaling molecules like fibroblast growth factors (FGFs), Sonic hedgehog (SHH), retinoic acid (RA), and bone morphogenetic proteins (BMPs) are also important for patterning of the facial primordial and appear to modulate expression of Hox genes therein (Barlow and Francis-West, 1997; Barlow et al., 1999; Francis-West et al., 1998; Hu and Helms, 1999; Schneider et al., 2001).

The steps between initial molecular specification of the first arch neural crest cells and subsequent jaw formation have yet to be elucidated. Our data provide the first detailed view of the morphogenetic processes by which mesenchymal condensations of facial primordia give rise to the jaws of gnathostomes. There are several comprehensive papers describing the contribution of migrating cranial neural crest cells from different axial levels into facial prominences (e.g., Imai et al., 1996; Köntges and Lumsden, 1996; Osumi-Yamashita et al., 1994; Richman and Lee, 2003), as well as the mesenchymal cell expansion within each primordium (McGonnell et al., 1998). The present study is the first to provide direct cell lineage analysis in vivo relating neural crest cell condensations of the first arch and of developing facial prominences to the cartilagenous structures that prefigure the bones of the jaw and face. In a recent review of face and jaw development in vertebrates, Richman and Lee (2003) proposed possible scenarios

regarding the origin of the maxillary prominence. One possibility, commonly referred to in textbooks, is that the first pharyngeal arch gives rise to both maxillary and mandibular prominences. An alternative possibility is that the mandibular arch contributes only to the posterior portion of the maxillary process (palatoquadrate), but more dorsal postoptic tissues may also make a significant maxillary contribution. By direct lineage analysis of this region, the present study distinguishes between these alternatives for the first time to demonstrate that the latter is the case.

Conclusions

In summary, our findings show that ventral ("mandibular") condensation of neural crest cells within the first pharyngeal arch makes a dual contribution to the palatoquadrate and to Meckel's cartilage. The more dorsally localized ("maxillary") neural crest condensation that appears to presage the maxillary prominence contributes to trabecular cartilage. This suggests that the common terms of "maxillary" and "mandibular" condensations are misleading since the palatoquadrate is a component of the upper jaw and yet derived from ventral, mandibular neural crest. Our data provide the first detailed view on the morphogenetic aspects of the processes in vertebrates by which mesenchymal condensations of facial primordia give rise to jaws.

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