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Neural crest patterning and the evolution of the jaw

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ABSTRACT

Here we present ideas connecting the behaviour of the cranial neural crest during development with the venerable, perhaps incorrect, view that gill-supporting cartilages of an ancient agnathan evolved into the skeleton of an early gnathostome's jaw. We discuss the pattern of migration of the cranial neural crest ectomesenchyme in zebrafish, along with the subsequent arrangement of postmigratory crest and head mesoderm in the nascent pharyngeal segments (branchiomeres), in diverse gnathostomes and in lampreys. These characteristics provide for a plausible von Baerian explanation for the problematic inside-outside change in topology of the gills and their supports between these 2 major groups of vertebrates. We consider it likely that the jaw supports did indeed arise from branchiomeric cartilages.

Key words: Jaw evolution; neural crest; branchial arches; pharyngeal arches.

INTRODUCTION: A PROBLEM OF HOMOLOGY IN THE VERTEBRATE BRANCHIAL AND JAW SKELETONS

It is known that a component of the skeleton that early vertebrates may have used to support breathing gills, and perhaps to support teeth for pharyngeal chewing, evolved into the supports of biting jaws. So it has long been supposed that the jaw skeleton is derived from modification of branchial skeletal elements (reviews, Mallatt, 1984, 1996, Janvier, 1996). However, the theory stands in the face of a long-standing problem: namely, the gill supports of lampreys, agnathan (jawless) fishes, and of gnathostomes (jawed vertebrates) are in topologically non-corresponding locations. This problem, recognised during the 19th century, led to the proposal that the skeletal gill supports in agnathans and gnathostomes are not homologous. In consequence, new theories have emerged that try to resolve the conflict (e.g. Forey & Janvier, 1993; Janvier, 1996; Mallatt, 1996, 1997). But these theories have inelegant consequences: Mallatt supposes that an ancient ancestor had 2 sets of branchial cartilages, one medial and one lateral, with the medial set surviving in gnathostomes, and the lateral set surviving in lampreys. Janvier also supposes that lateral cartilages are primitive in vertebrates, and that this was the only set. By this theory, the lateral cartilages are tossed away in gnathostomes, and replaced by a medial set that is invented anew. Here we use developmental evidence to resurrect the discarded homology between the branchiomeric cartilages of agnathans and gnathostomes, and hence the original idea of jaw evolution.

In all vertebrates, a segmental set of pharyngeal (branchial) arches develops along the lateral walls of the foregut. The segments, branchiomeres, notably include a series of aortic arches of mesodermal origin that primitively form the vasculature of the gills (Goodrich, 1930). Supporting the gills is a segmental set of cartilages derived from the neural crest. Muscles, the fibres of which come from mesoderm (Noden, 1983; Schilling & Kimmel, 1994), and the connective tissue of which may derive from neural crest (Köngtes & Lumsden, 1996), are also segmentally arranged (Schilling & Kimmel, 1997). The linings of the gills are epithelial, thought to come either from pharyngeal endodermal outpocketings termed pouches, or from ectodermal inpocketings termed clefts. All of these tissues together comprise the derivatives of a branchiomere in a gill-breathing fish. In gnathostomes the skeletal supports of the gills and jaws alike are jointed.

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In lampreys there are no joints; the cartilage bars supporting the gills are interconnected in a so-called branchial basket. Hence, joints seemingly are derived structures, perhaps invented in the earliest gnathostomes. However, the long-standing problem in considering jaw evolution is not about the structure of the skeletal elements but about their locations. In lampreys the cartilage bars are located laterally to the gills in the pharyngeal wall. In contrast, in all major groups of gnathostome fishes the gill-supporting cartilages are located medially in the wall. One of the essential tests of evolutionary homology is the congruence of the topological arrangements of the elements in a body region, and at first glance the branchiomeres of agnathans and gnathostomes fail this test (for a review, see Mallatt, 1984).

Taking into account newly available evidence, how far does evolutionary homology between branchiomeres in a representative gnathostome and agnathan extend? Since morphological evolution is a consequence of developmental change, it is crucial to understand how development of the branchiomeres compares between these 2 groups. Developmental considerations are particularly important for cases like the one we address here, where there is a problem with the topology. This is because developmental processes like involution and ingression reverse topological relationships between tissues.

To understand how agnathans develop, we are presently limited to a single relict group, the lampreys. Embryos of the other modern agnathan, the hagfish, have been essentially unavailable. Lamprey embryos appear to retain many primitive features, and fortunately we have considerable information about how their branchiomeres develop, including cartilage formation (Horigome et al. 1999; Kuratani et al. 1999; Neideret et al. 2001; Morrison et al. 2000). For gnathostomes we have corresponding information for representatives of a number of different groups, including fish, amphibians, birds and mammals. Among these, we focus especially on the zebrafish because of our new studies with this species (see also Schilling, 1997, for a review). Importantly for the arguments we make below, comparing features of the early branchiomeres in the zebrafish, an actinopterygian (see Metscher & Ahlberg, 1999), with distantly related sarcopterygians, the frog *Xenopus*, the chicken, and the mouse, shows similar patterning among all 4 derived species. Hence, for the features we emphasise here, zebrafish may represent the generalised gnathostome condition. A more complete argument would include chondrichthyans, for which new information is sorely needed.

Here we describe work on the formation and patterning of the pharyngeal segments in the zebrafish embryo, and compare these findings with current understanding from other vertebrates. The observations suggest that the early stages of branchiomere development, including the patterning of head paraxial mesoderm and neural crest migration, are equivalent in both gnathostomes and agnathans. Not long after these early stages, the distribution of postmigratory neural crest in the nascent branchiomeres appears to differ in the 2 groups. We propose that the difference comes about by the addition in gnathostomes of a new morphogenetic movement on to the primitive, generalised pattern.

In our proposal the gnathostome arrangement in which the skeleton is medial is new. Relatively extended morphogenetic movements bring the head mesoderm, the postmigratory neural crest, and the overlying epithelia of both endodermal and ectodermal origin into more intimate contact, the new close cellular associations providing the anatomical basis for new signalling interactions. Included among these is an Endothelin-1 signal that is crucial for dorsoventral patterning of the jointed cartilages of gnathostomes, and for the joints themselves. In this way the extended morphogenesis in gnathostomes underlies the more complex patterning of the gnathostome branchiomeric skeleton.

This 'outside-in' hypothesis, as we term it, allows us to resurrect the theory that the cartilages supporting the gills in all modern vertebrates are evolutionary homologues, and that the jaw skeleton arose from elements within this series, whether or not those elements ever bore gills.

BRANCHIOMERE SEGMENTATION AND PATTERNING IN ZEBRAFISH

Segments are units, modules, present along the principal body axis. Multiple elements make up each module, i.e. the elements all repeat along the axis with the same periodicity (Bateson, 1894). It is clear that branchiomeres meet this definition of segments, by considering their multiple iterated characters along the pharyngeal wall as outlined above. Nevertheless, a problem confronted by anyone wanting to explain an aspect of head patterning by invoking segments is that there is a history of controversy to the idea. Perhaps beginning with Goethe's famous vertebral theory of the skull, and the yet more famous annihilation of this theory by T. H. Huxley in 1858 (for a review, see Jeffs & Keynes, 1990), 'segmentalists' seem to see segmental patterns where others simply cannot. A recent

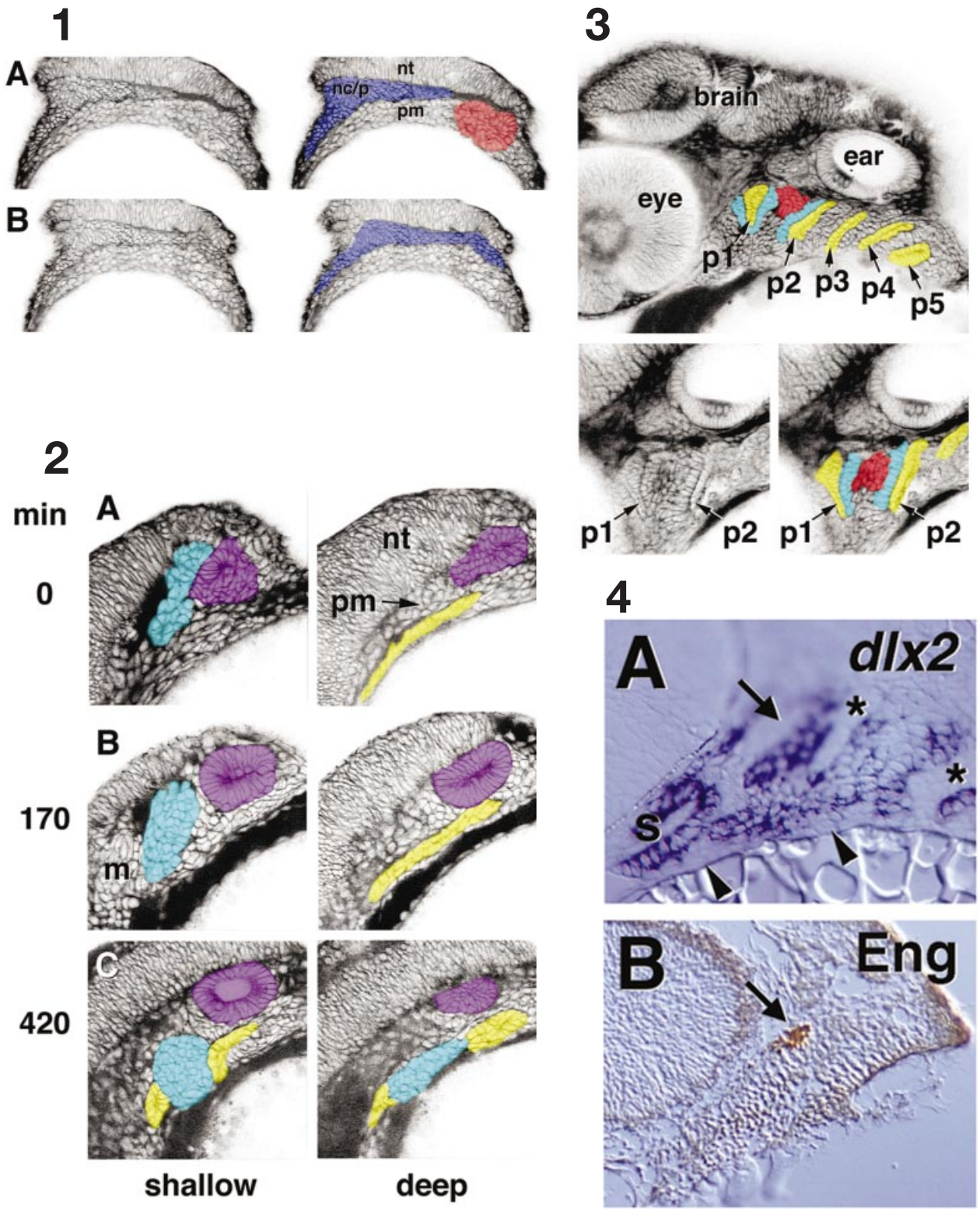


Fig. 1. Head mesoderm and neural crest appear unsegmented before crest migrates in zebrafish. Confocal micrographs (dorsolateral views shown as negative images) of a live embryo labeled with BODIPY ceramide (for method see Henry et al. 2000; Moens et al. 1998). A and B show two planes of focus of the same preparation at the 5-somite stage (12 hours postfertilisation). Dorsal is to the top and anterior is to the left. The images to the right are coloured to show our interpretation of the embryonic tissues: red indicates the first two somites, continuous with the head paraxial mesoderm (pm, uncolored); blue indicates the common primordium of the neural crest and placodes (nc/p), present just ventrolaterally to the neural tube (nt).

Fig. 2. Neural crest migration and the origin of pharyngeal pouches in zebrafish. Confocal micrographs, as in Fig. 1, from a 4D series in which records were taken every 4 min at 12 planes of focus. Two of these planes are shown (left: shallow, right: deep) at three selected intervals, as indicated to the left (*A–C*). The first time point (*A*) is at the 10-somite stage of development. The images are pseudocolored to

example of such confrontation is the case of the ethereal head somitomeres, structures which might be there, but which more probably are not there.

Early mesoderm appears unsegmented

Meier and his associates described early mesodermal segments in the head primordia of several gnathostomes, possibly serially homologous to the somites in the trunk and tail (for a review, Jacobson, 1993). These head somitomeres would be present very early—before the stages when the pharyngeal endoderm makes its series of pouches and before the neural crest migrates away from the developing neural tube. Head somitomeres were described in studies by a single laboratory utilising a single method, scanning electron microscopy (SEM), and proposed to be present widely in gnathostomes. Yet other investigators have doubted their very existence (review, Keynes & Lumsden, 1990) and a recent and thorough SEM study showed no hint of head somitomeres in lamprey embryos (Kuratani et al. 1999). Are they present in gnathostomes?

Somitomeres seem not to be recognisable in all gnathostomes. Using SEM we could not find them in zebrafish (R. Manes & C.B.K., unpublished observations), in spite of head somitomeres having been reported earlier in the medaka, another teleost (Martindale et al. 1987). We turned to what we argue is a better way to see morphological segmentation of a tissue if it is present—confocal microscopy with vitally stained living embryos. The method takes advantage of the optical clarity of the zebrafish embryo, and of new staining and imaging technology (Moens et al. 1998; Henry et al. 2000). Sequential scans over time allow 4-dimensional (4D) reconstruction (x, y, z, time), such that supposed morphogenetic changes can be verified. Because of these features, and because the method eliminates certain problems such as artifacts arising from fixation, the method inherently gives confidence in the results.

At the earliest stages of zebrafish head development, there is no sign of segmentation in any of the tissues. Before neural crest begins to migrate from the dorsolateral sides of the neural rudiment, the head paraxial mesoderm is easily recognised because it is continuous with the somite-forming mesoderm more caudally (Fig. 1). This head mesoderm appears morphologically unsegmented. Anterior to the somites, there are no iterated boundaries within the mesoderm and no consistent swirling arrangements of mesodermal cells such as described previously in the SEM studies of other vertebrates. Confocal scans of the mesoderm during the time when crest migration begins later do not change this conclusion.

Origin of morphological segmentation: neural crest migration and endodermal outpouching

In zebrafish at the stage described above, a well delineated tissue band can be recognised that lies just superficial to the paraxial mesoderm and beside the neural keel, the primordium of the central nervous system (Fig. 1). This dorsolateral band includes premigratory neural crest as well as the primordia of ectodermal placodes. In agreement with the classical study of Landacre (1910) in another teleost, we can find no morphological subdivision of this primordium before neural crest migration begins. However, fate mapping at this stage by single cell labelling reveals

show our interpretation of the embryonic tissues. Purple shows the otic vesicle, beside the neural tube (nt), and adjacent to head paraxial mesoderm (pm). Yellow shows the endodermal lining of the pharynx, outpouching at the last time point. Blue shows migrating neural crest of the hyoid stream (second branchiomere). The mandibular stream (m, uncolored) is evident at the shallow plane in *A* and *B*, present just to the left of the hyoid stream. The postotic stream is just visible posterior to the otic capsule (to the right).

Fig. 3. Tissue arrangements in the nascent pharyngeal segments. Confocal micrographs, as in Fig. 1, at 30 h postfertilisation. The upper panel shows one plane from a z-series, pseudocoloured to show our interpretation of the tissue arrangements. A series of endodermal pouches (p1-5, yellow) form the boundaries of adjacent segments (pouch 1 separates branchiomeres 1 and 2, and so on). The postmigratory neural crest ectomesenchyme (coloured blue in the hyoid segment) lines up along the pouches, forming a distinctive layer that separates the epithelium and the mesoderm-derived mesenchyme (red) that is located deeper within each segment. The lower panels show an image from another preparation at the same stage. The arrangements are identical in the two embryos. The same image is uncoloured on the left and pseudocolored on the right. The optical section is from a 4d series, and during the 4 h recording period some cell movement occurred, but cells did not exchange between the 2 mesenchymal compartments.

Fig. 4. Marker expression shows tissue relationships in the zebrafish pharyngeal segments. Parasagittal sections at 36 h postfertilisation. Dorsal is to the top, anterior is to the left. Both sections include the eye to the left, just anterior to the mandibular segment. (*A*) The neural crest marker *dlx2* shown by in situ RNA hybridisation (unpublished, see Miller et al. 2000). The expressing cells line up next to unlabeled endodermal pouches (*). Unlabelled mesenchyme is evident deep in the mandibular segment. The arrow indicates the dorsal part of this mesodermal domain that appears to correspond to the domain of Engrailed-labelling, as shown in *B*. The arrowheads indicate ventral mesodermal domains in the mandibular and hyoid segments that express *endothelin-1* (see Figs 5, 6). s, stomodeum. (*B*) Engrailed labeling in the mandibular segment (arrow corresponding to the arrow in *A*). The expression is revealed by antibody labelling (as in Hatta et al. 1990), and marks a dorsal mesodermal domain (Edgeworth's 'constrictor dorsalis', see Fig. 8) that will split to form 2 pharyngeal muscles as described in the text.

that individual premigratory neural crest cells contribute all of their cellular progeny to only a single branchiomere (Schilling & Kimmel, 1994). Such clonal restriction in fate indicates that the premigratory cells may already be cryptically specified to their segmental identity.

Morphological segmentation of neural crest, seemingly just somewhat after the stage when migration begins (Fig. 2*A*), forms the well known 3 primary streams (the preotic mandibular and hyoid streams, and a single postotic stream). This morphogenesis occurs about the same time as morphogenetic changes in other local tissues, as if there were a general demarcation of distinctive areas in the region: The placodes, including the otic placode (Fig. 2*A*) become distinctive. The first rhombomere boundaries become visible in the hindbrain primordium (Moens et al. 1998; L. Maves, unpublished studies). Pharyngeal endoderm is recognisable as an epithelial layer deep and ventral to the mesoderm (Fig. 2); at this time in zebrafish there is no discernable outpouching of the endodermal walls, nor yet any segmentation of mesoderm. Our 4D analyses suggest that the bulk of the neural crest migrates superficially to the mesoderm, just beneath the ectodermal epithelium.

As the migrating crest cells reach and accumulate at the ventral level where the pharyngeal endoderm is present, we see the first signs of endodermal pouches (Fig. 2*C*). The neural crest cells now appear to move inward, medially, perhaps along with associated deep mesoderm, to form a thicker accumulation of cells. Simultaneously with this morphogenesis, and occurring both anterior and posterior to the thickened region that includes both types of mesenchyme, the endodermal epithelium outpockets. The anterior (first or hyomandibular) pouch separates branchiomeres 1 and 2 (the mandibular and hyoid), and the posterior (second) pouch separates the hyoid segment from the now largely postmigratory crest of the third (postotic) stream. Segmentation of this posterior mesenchyme occurs secondarily and over a developmental period of about a day, as 4 more pouches appear, probably in an anterior–posterior sequence. The endodermal outpocketings in this postotic region could well be the driving morphogenetic movements that carve out these posterior branchiomeres.

Epithelial–mesenchymal relationships in the nascent branchiomeres

Optical sectioning of the anterior branchiomeres during the period that the posterior ones are still segmenting reveals a new appearance of the tissues comprising each segment. A layer of larger and rounded cells that we take to be neural crest-derived ectomesenchyme now intervenes between putative mesodermal cells (smaller and more spindle-shaped mesenchymal cells) and the endoderm-derived epithelial walls of the pharyngeal pouches (Fig. 3). The arrangement, considering the 3-dimensional structure, is like a cylindrical shell of crest surrounded by superficial epithelium (including both lateral ectoderm and medial endoderm) and the crest itself surrounding a central core of mesoderm. The more posterior segments develop essentially the same arrangement, but the amount of mesenchyme in these segments is smaller.

Expression analysis of developmental regulatory genes supports the same arrangement of embryonic tissues in the branchiomeres, present transiently after neural crest migration and before precartilage condensations form and muscles differentiate. A parasagittal section through the mandibular segment, labeled by RNA in situ hybridisation for the neural crest marker *dlx2* (Fig. 4*A*) shows that the crest does not fill the whole branchiomere along the anterior–posterior axis. Rather the crest has the appearance of oblique dorsal–ventrally oriented walls on either side of an unlabeled core. The posterior *dlx2*-expressing wall in this mandibular segment is directly adjacent to the hyomandibular pouch. That cells in the unlabeled core are mesodermal is supported by labeling, in a similar section, for the homeodomain protein Engrailed (Fig. 4*B*). Engrailed immunoreactivity is present specifically in a dorsal mandibular muscle primordium, which fate mapping has established is mesodermal (Hatta et al. 1990). The primordium is discussed below where we consider muscle patterning in this mesoderm. Here the site of Engrailed labelling in Figure 4*B* appears to correspond to the dorsalmost region of the unlabelled core in Fig. 4*A*.

Other markers extend the argument. The peptide Endothelin-1 (encoded by the *sucker* gene in zebrafish, see below) is expressed by the ventral mesoderm and ventral epithelium (both ectoderm and endoderm), and is presumed to act as a secreted signal on to postmigratory neural crest (Miller et al. 2000). The neural crest responds to the signal by upregulating expression of target genes including, in both the mouse and zebrafish (Clouthier et al. 1998, 2000; Thomas et al. 1998), the gene encoding the bHLH transcription factor dHAND. Examination of whole mounts labelled by RNA in situ hybridisation (Fig. 5) reveals the *dHAND* expressing neural crest cells are present as an essentially complete ring just under the epithelium. Labelling for *endothelin-1* RNA shows the

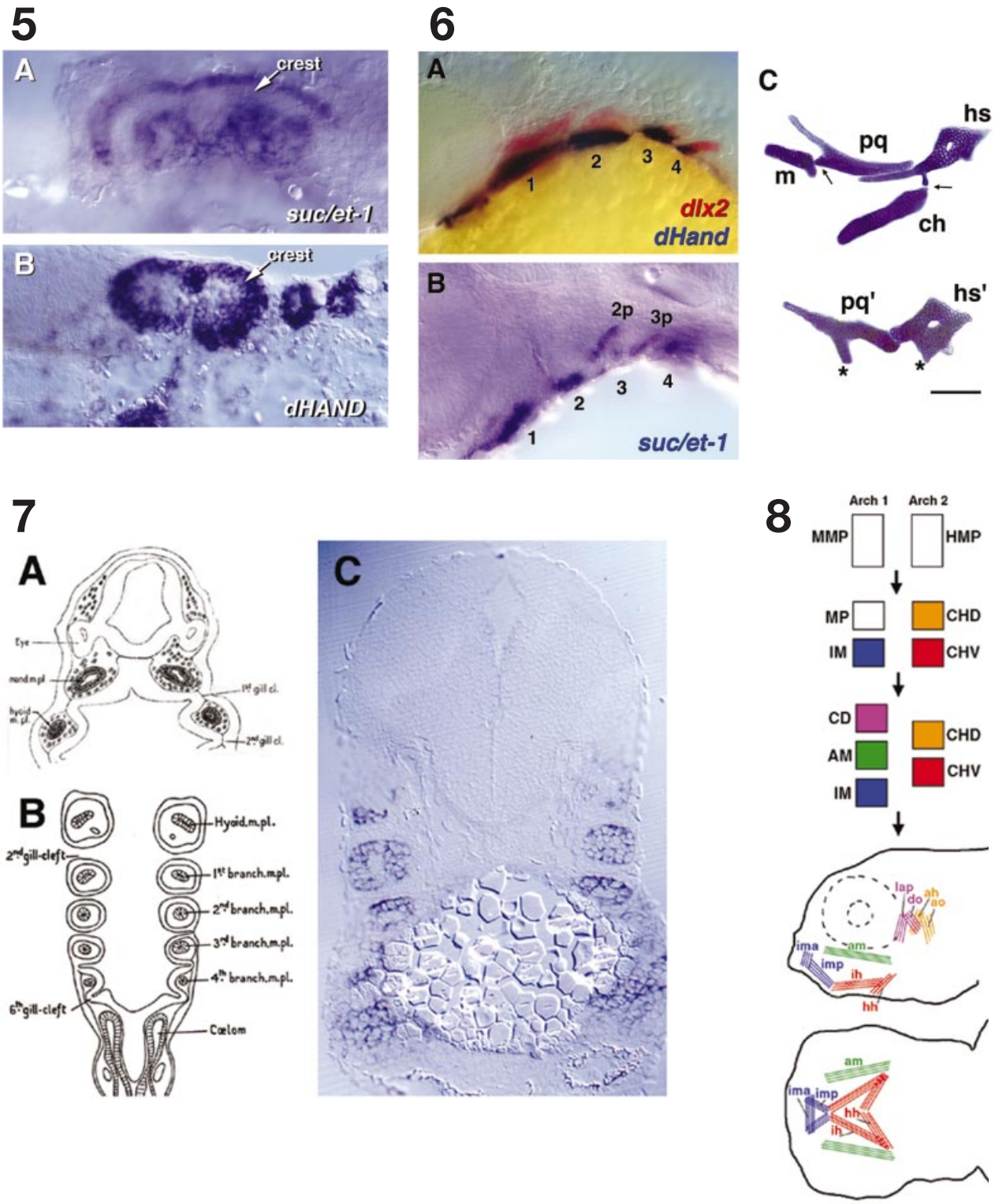


Fig. 5. Concentric tissue arrangements in the ventral branchiomeres, as revealed by marker expression (RNA in situ hybridisation of whole mounted preparations from Miller et al. 2000). Ventral views, anterior to the left. The position of postmigratory neural crest is indicated in the hyoid segment in both panels. (*A*) Expression of *endothelin-1* (encoded by the *suc/et-1* gene in zebrafish) at 28 hours postfertilisation. Expression is present in cores of mesoderm deep to the unlabeled neural crest, and in epithelium superficial to the crest. (*B*) Expression of *dHAND* at 28 h postfertilisation. Branchiomere expression is restricted to the neural crest. Note that in all of the 4 segments shown, *dHAND* is expressed in a ring, much larger in the first 2 (mandibular and hyoid).

Fig. 6. Segmental patterning of gene expression along the pharyngeal wall (RNA in situ hybridisation of whole mounted preparations from Miller et al. 2000). Lateral views with anterior to the left. (*A*) Expression of *dlx2* (red) and *dHAND* (blue) at 28 h postfertilisation. The same dorsal–ventral pattern is present in the anterior 4 branchiomeres shown. (*B*) Expression of *endothelin-1* at 36 h postfertilisation. At this stage there is strong ventral expression in the mesodermal core of the first 4 segments, and in pharyngeal pouches. Two labelled pouches are visible in the figure (2p, 3p).

opposite appearance. The ring is unlabelled: *endothelin-1* expression is limited to the epithelium and the mesodermal core.

Hence the mesenchyme within a branchiomere is concentrically organised along a dorsoventral axis. There is a mesodermal core, mostly or entirely surrounded by a cylindrical wall of postmigratory neural crest ectomesenchyme. We have not yet undertaken a complete 4D analysis to show the morphogenetic movements by which this new arrangement develops. We suppose that following an initial dorsal to ventral migration along the basal face of the (lateral) ectoderm, the neural crest cells subsequently move medialwards along the endodermal pouch epithelium, and so eventually come to surround the medial face of the mesoderm. We propose further (see below) that this medialwards movement of crest occurs in all of the segments, and that at least in all but the mandibular segment, the movement is a new feature in gnathostomes.

Segmental homologies and dorsal–ventral organisation of the branchiomeres

Analysis of the patterning of the pharyngeal cartilages and muscles in zebrafish argues for segmental homology of the branchiomeres and individual elements within them (Schilling & Kimmel, 1997). All of the segments make aortic arches, even if those arches subsequently disappear (as in the gnathostome mandibular segment; Goodrich, 1930). Particularly relevant to the case we develop here is the comparison of the first segment, the jaw-forming mandibular branchiomere, and more posterior ones, especially the second or hyoid branchiomere. These 2 segments both have large early-developing dorsal and ventral cartilages (e.g. the ventral Meckel's cartilage in the first segment corresponding to the ventral ceratohyal cartilage in the second). They both have dorsal and ventral muscles (e.g. the ventral intermandibularis corresponding to the ventral interhyoideus; see below). Our more recent studies of the earlier patterning of the branchiomeres from which these elements develop lend considerable further support to this model of segmental homology. This new evidence is important to mention because of recent argument to the contrary (Smith & Coates, 2000).

By segmental homology, we mean correspondence along a presumed segmental series due to the elements arising from equivalent, iterated primordia. Specialisation of a member of the series ('heterosis'; Bateson, 1894), such as we imagine for the jaw-forming segment, could also be evident, showing up as a unique feature of the specialised primordium for this segment. But shared features, not differences, argue for homology. Our evidence includes shared gene expression domains of the neural crest, mesoderm, and epithelial components of the segments. It also includes phenotypic changes that correspond in adjacent segments in particular mutant strains. The evidence reveals equivalence in features in the control of dorsal–ventral patterning of gnathostome branchiomeres (Miller et al. 2000).

For example, the neural crest-derived ectomesenchyme maintains expression of the homeobox gene *dlx2* in the branchiomeres as a set, as shown for the first 4 in Figure 6*A*. In each branchiomere this domain is subdivided along the dorsal–ventral axis, as is evident in the figure because the ventral cells express *dlx2* and *dHAND*, whereas the dorsal cells express only *dlx2* and not *dHAND*. Sections (not shown) reveal that ventrally in each segment both markers (*dlx2* and *dHAND*) are co-expressed in the hollow cylinder pattern described above. Other markers, including the homeobox gene *msxE*, and the tyrosine kinase receptor gene *EphA3*, also are expressed specifically in the ventral crest mesenchyme in at least the first 6 segments.

Although we have only carried out preliminary fate mapping studies, we suppose from mutational analyses in particular that the ventral cartilages specifically develop from the ventral neural crest domains in the mandibular and hyoid segments. The ventral expression domains and the ventral cartilages are reduced or absent in zebrafish homozygous for loss-of-function mutation of several genes of the so-called

Fig. 7. Conserved arrangement of muscle plates in the branchiomeres of diverse gnathostome fish. (*A*, *B*) Drawings of horizontal sections through embryos, from Edgeworth (1935). (*A*) The mandibular and hyoid segments of a shark *Scyllium* (a chondrichthyan fish). (*B*) Branchiomeres 2–6 in the lungfish *Ceratodus* (a sarcopterygian fish). (*C*) Horizontal section showing *dlx2* expression as a segmental series of rings in branchiomeres of the zebrafish (an actinopterygian fish; see Miller et al. 2000). The muscle-plates form the unlabelled cores of these rings.

Fig. 8. Development of mandibular and hyoid muscles. (*A*) Edgeworth's (1935) interpretation of the series of muscle plate subdivisions in the first 2 pharyngeal segments of an actinopterygian, the bowfin *Amia* (the teleost sister group; see Metscher & Ahlberg, 1999). (*B*) The muscle pattern deriving from the homologous segments in the zebrafish (after Schilling & Kimmel, 1997). AM, adductor mandibulae; CD, constrictor dorsalis; CHD, constrictor hyoideus dorsalis; CHV, constrictor hyoideus ventralis; HMP, hyoid muscle plate; IM, intermandibularis; MP, masticatory plate; MMP, mandibular muscle plate; ah, adductor hyoideus; ao, adductor operculi; do; dilator operculi; hh, hyohyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; ih, interhyoideus; lap, levator arcus palantini.

'anterior arch' phenotypic class, including *sucker*, *schmerle*, and *sturgeon* (Miller et al. 2000, and unpublished data; see Kimmel et al. 2001 for review). The mandibular and hyoid cartilage phenotypes for the most severe of the mutations in this class, *sucker*, is shown in Fig. 6*C*. In *sucker* mutants the dorsal cartilages look relatively normal, but the ventral cartilages are severely reduced and fused onto the dorsal ones in both segments. The phenotype establishes that *sucker*, which we know encodes the zebrafish Endothelin-1 orthologue mentioned above, is required for development of ventral cartilage, and for development of the joint between the dorsal and ventral cartilage in both segments. The mutants also have prominent defects in the segmental homologs of these ventral cartilages of at least the next 3 more posterior segments (Piotrowski et al. 1996; Miller et al. 2000).

This new evidence strongly supports homology of the neural crest domains within the series of branchiomeres. The sizes of the domains change along the series; the first 2 are larger and these give rise to larger, earlier developing cartilages (see Schilling & Kimmel, 1997). Heterosis is supported by marker expression as well; the homeobox genes *goosecoid* and *dlx3* provide examples of genes expressed differently among the segments (Akimenko et al. 1994; Miller et al. 2000).

The expression domains of the *sucker/endothelin-1* gene make a similar argument for segmental homology among branchiomeres, now for the epithelium (including the surface ectoderm and the endodermal pouches) and the mesodermal cores. For example, the same pattern of expression by ventral core mesenchyme is present in at least the first 5 segments (Fig. 6*B* and data not shown). The *sucker/endothelin-1* mutant phenotype not only supports a requirement for Endothelin-1 shared by the anterior segments by ventral ectomesenchymal derivatives mentioned above, but also by muscle. Loss of function of this gene dramatically and specifically reduces the ventral mandibular and hyoid muscles that we presume derive from the *endothelin-1* expressing ventral mesenchyme (Miller et al. 2000).

Expression of other markers and mutant phenotypes reported in other studies support our proposal of serial homology extending to all of the branchiomeres. The homeobox gene *nkx2.5* is expressed specifically in pouch endoderm in the pharynx, and labeling is in all of the pouches, from the first on back (Lee et al. 1996; Piotrowski & Nüsslein-Volhard, 2000). Loss of function mutation of the *lazarus/pbx4* homeobox gene results in loss of all the pharyngeal pouches, fusion of segmental neural crest streams, and fusion together of mandibular and hyoid cartilages that derive from this crest (Pöpperl et al. 2000). We have interpreted the crest and cartilage phenotypes in *lazarus/pbx4* mutants as possibly being homeotic, i.e. a loss of segment-specific features of homologs (see also Kimmel et al. 2001).

EARLY BRANCHIOMERE PATTERNING IS CONSERVED AMONG GNATHOSTOMES

Segmental homologies and dorsal–ventral organisation of the branchiomeres

Branchiomere development in other gnathostomes seems to conform at least in broad outline to the findings outlined above for zebrafish. New support for somitomeres has not been forthcoming, and Lumsden & Krumlauf (1996) infer that head mesoderm is 'patently unsegmented'. A study of the expression of early regulatory genes controlling trunk mesodermal segmentation (Pourquie, 2000) in developing head mesoderm of the chick embryo provides no evidence for a primary segmentation of the head mesoderm into somitomeres (O. Pourquie, personal communication). Our failure to find any segmental patterning in the zebrafish head mesoderm before crest migrates and endodermal pouches form is in accord.

Edgeworth's mesodermal muscle-plates

However, later in development the mesoderm does become patently segmented, sequestered into the interiors of segmentally arranged hollow cylinders of neural crest. We described this above for the zebrafish. That mesoderm comes to form the branchiomere cores in the embryos of the mouse and chick is quite clear from fate mapping (Trainor et al. 1994; Trainor & Tam, 1995; Hacker & Guthrie, 1998). Furthermore, head paraxial mesoderm has been shown to be the source of the majority of avian branchiomeric muscles (Noden, 1983), and of at least some of the branchiomeric muscles of zebrafish (Hatta et al. 1990).

We make special note that the early organisation of mesoderm was known many years ago, and for a large diversity of gnathostomes. This understanding came from the work summarised in F. H. Edgeworth's magnum opus, *The Cranial Muscles of Vertebrates* (1935). By following serial sections of closely staged embryos and larvae, and comparing these with dissected adult heads, Edgeworth and others identified and classified most of the gnathostome head muscles. Further, they traced the development of the muscles

back to the earliest stages when their primordia could be recognised in the embryo. These rudiments, which they termed 'muscle-plates', correspond to the mesodermal branchiomere cores characterised more recently.

In Edgeworth's model, all gnathostome embryos possess a series of muscle-plates, initially present as one undifferentiated core cluster of muscle precursor cells in each segment (Fig. 7). The strong correspondence of these arrangements in chondrichthyan and sarcopterygian fishes with the pattern in zebrafish (an actinopterygian) is evident in this figure. These muscle-plates would then subdivide in a stereotyped sequence, represented for an actinopterygian in Figure 8. At an intermediate stage of development, three dorsoventrally oriented premyogenic condensations are present in the mandibular segment, and 2 in the hyoid. As pointed out earlier by Miyake et al. (1992), the arrangement and location of engrailed-labelled muscle precursors in zebrafish (Hatta et al. 1990) are exactly consistent with Edgeworth's description of the dorsal mandibular primordium, constrictor dorsalis (Fig. 8).

Engrailed labelling also shows that the dorsal mandibular primordium itself splits later in zebrafish to give rise to 2 definitive dorsal branchiomeric muscles, levator arcus palatini and dialator operculi (Fig. 8; Hatta et al. 1990). Remarkably, both muscles insert onto hyoid arch-derived skeletal elements, not mandibular cartilages as might be expected (see Fig. 8). This quite unusual fate (i.e. muscles deriving from one segment connecting onto skeletal elements of another segment) is a derived character in actinopterygians and was well known to Edgeworth. Hence, our confirmation of this feature of his scheme attests to the accuracy of his observations, made at a time when specific labelling of the primordia in question was impossible.

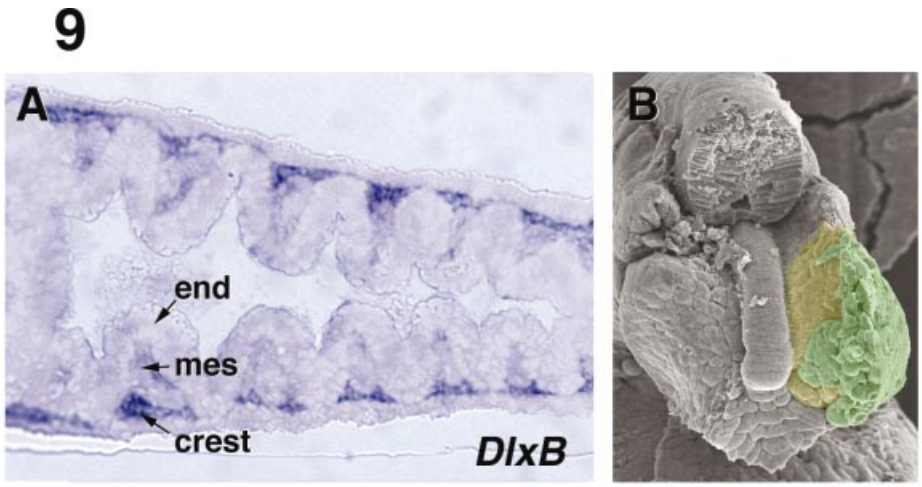
Engrailed labelling has also been mapped onto specific muscles in the mouse (Degenhardt & Sassoon, 2001). In contrast to the zebrafish, in the mouse the muscles attach to the dentary, i.e. they insert on a dermal element deriving from the mandibular segment. As we discuss below, Engrailed-expressing muscles also insert onto the mandibular skeleton in lampreys.

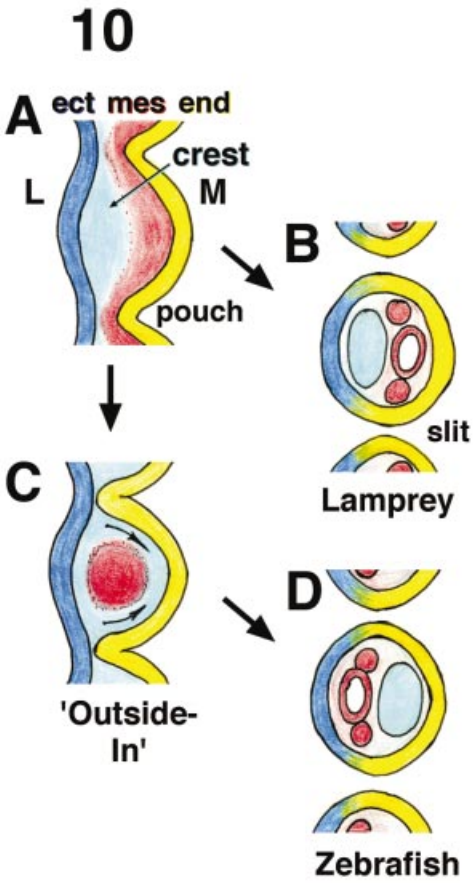
Further support for Edgeworth's scheme comes from our recent discovery of the ventral mesodermal domain of *endothelin-1* expression (Figs 5, 6; Miller et al. 2000). Expression appears to mark Edgeworth's ventralmost primordia deriving from both the mandibular and hyoid muscle plates. At later developmental stages the *endothelin-1*-expressing primordia of the first 2 segments both elongate towards a central median point at the segment-one/two boundary. At this location they form an X-shape (C. T. Miller, unpublished observations). The X-shaped expression domains match the subsequent X-shaped appearance of the definitive primitive ventral muscles in these two segments (the intermandibularis posterior and the interhyals, Fig. 8). Before the muscles differentiate, *endothelin-1* expression is downregulated, and our evidence for the eventual fates of the cells in these ventral domains is incomplete. Nevertheless it is likely that the *endothelin-1* expressing mesenchymal cells are the precursor cells for these ventral muscles, in accordance with Edgeworth's muscle plate scenario.

Neural crest. It also seems likely that the principal features we observe of cranial neural crest patterning in the zebrafish can be generalised to other gnathostomes. Some differences may be important (e.g. the relative timing of crest migration and endodermal outpouching; see Veitch et al. 1999). In chicks, as in zebrafish, the anterior–posterior arrangement of premigratory neural crest cells is preserved after migration (Köntges & Lumsden, 1996). However, clonal restriction of premigratory crest cells to single branchiomeres appears not to be absolute in the chick (Kulesa & Fraser, 2000). In *Xenopus*, the cranial neural crest migrates initially as a continuous sheet that is only later broken up into the 3 separate streams (Sadaghiani & Thiebaud, 1987). Our 4D study showed an initially unbroken sheet in zebrafish (Fig. 1). Three migrating streams, 2 preotic and 1 postotic are well documented in chick, mouse, and *Xenopus* (Sadaghiani & Thiebaud, 1987; Köntges & Lumsden, 1996; see Graham et al. 1996 for review). Fate mapping reveals that the cranial crest migrates as a shell superficial to deep mesoderm in the mouse (Tam & Trainor, 1994; Trainor & Tam, 1995), as our 4D study suggests for zebrafish. Cranial crest may also migrate superficial to mesoderm in the chick (Hacker & Guthrie 1998; A. Graham, personal communication). Important for the case we make below, marker expression shows essentially the same concentric arrangements of postmigratory crest and mesoderm in the nascent branchiomeres of mouse and chick as we described above for zebrafish: neural crest comes to surround a mesodermal core (Bulfone et al. 1993; Maemura et al. 1994).

CONSERVATION AND PERHAPS A DIVERGENCE IN PATTERNING IN LAMPREYS

The telling comparison with respect to jaw evolution is between the gnathostomes and lampreys, for which





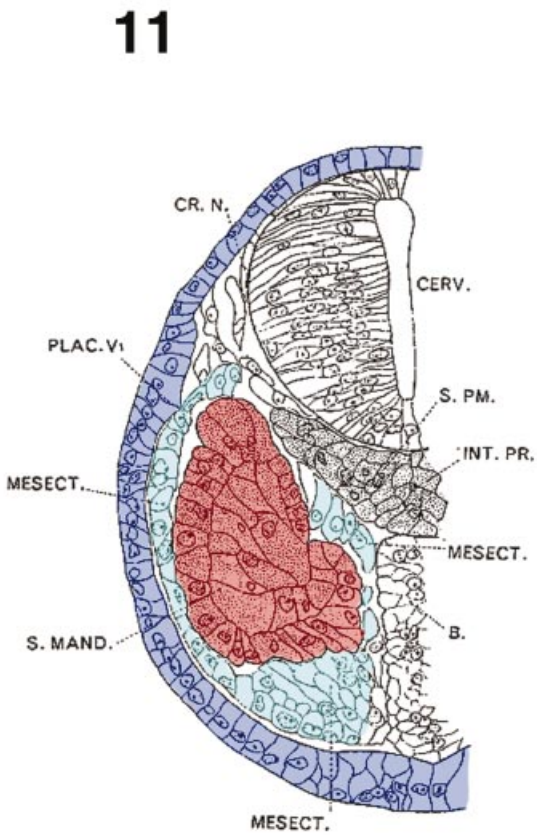


Fig. 9. Neural crest appears to be laterally located in the branchiomeres of lampreys. (*A*) Expression of *DlxB*, a neural crest marker, in pharyngeal segments 2–5 of *Petromyzon marinus*. Horizontal section, with anterior to the left, of a 15-d embryo (from Neidert et al. 2001). Highest expression is located laterally in each segment, in cells just underneath the ectoderm. end, endoderm; mes, mesoderm. (*B*) SEM of a stage 21 *Lampetra japonica embryo* (from Kuratani et al. 1999). Dorsal–anterior view with the brain dissected away. The notochord is the rod-like midline structure, flanked on both sides by the mandibular branchiomere. To the left, the mesenchyme has been removed to show the endodermal hyomandibular pouch. To the right the mesenchyme interpreted by the authors to be head paraxial mesoderm is colored yellow, and the mesenchyme interpreted to be of neural crest origin is coloured green. Most of the neural crest covers the lateral aspect of the mesoderm.

Fig. 10. The outside-in hypothesis of branchiomere development. Development of a typical branchial segment (e.g. branchiomere 3) is sketched out for the left side of the pharynx. (*A*) Neural crest migrates laterally (L) and head mesoderm is medial (M) in the segment. (*B*) Most of the crest remains lateral in the lamprey, and the cartilage bar of the branchial basket develops laterally, external to the gills. (*C*) In a gnathostome branchiomere (and perhaps in the lamprey mandibular segment; see the text) the neural crest relocates medially ('outside-in') and at the intermediate stage shown, it forms a cylindrical wall around a mesodermal core. (*D*) Continued relocation of the crest in gnathostomes brings most of it medial in the segment, where it develops internal gill-supporting cartilages.

much new information is available. Here, as for diverse gnathostomes, we are mainly struck by the marked similarities in pattern that comparative analysis reveals. A possible difference, therefore, may be telling.

The similarities

An SEM analysis of development of head mesoderm in lampreys (Kuratani et al. 1999) shows that the segmentation of preotic mesoderm occurs relatively late, and may be acquired secondarily to segmentation in the other germ layers, notably the endoderm. As we have proposed for the zebrafish, here too Kuratani et al. suggest that endodermal outpockets break the head mesodermal sheet into segments in lampreys. Hence, in the absence of new evidence to the contrary, preotic mesodermal somitomeres would seem not to a feature of head segmentation in any vertebrate.

Lamprey neural crest, studied by DiI fate mapping and by SEM (Horigome et al. 1999), also appears to arise as an initially unsegmented mass of cells (as at least in zebrafish and *Xenopus*). There is anterior–posterior fate mapping of premigratory crest onto the branchiomeres (as in chick and zebrafish). As in gnathostomes, the neural crest sheet in lampreys breaks up into 2 preotic streams and one postotic stream. Unlike the case for mesoderm, this neural crest segmentation may not be imposed by endodermal pouching: the segmentation appears before the neural crest has migrated far ventrally to where it would be in intimate contact with either head mesoderm or the pharyngeal pouches. Hence, neural crest segmentation might be intrinsic to the ectoderm at this stage. This is as we also have supposed for zebrafish. However, we need to qualify this interpretation for there are important caveats, as may apply to all vertebrates. Piotrowski & Nusslein-Volhard (2000) suppose an early patterning role of the endoderm in zebrafish, as do Veitch et al. (1999) for the chick. A recent study in the mouse points to an importance of head mesoderm in signalling segmental features of neurectoderm, including signalling to the neural crest (Trainor & Krumlauf, 2000; Trainor et al. 2000). Hence, even if overt segmentation is intrinsic to the neural crest at the stage when it is migrating, this patterning may have been imposed onto it earlier, by inductive signalling.

In lampreys, neural crest migrates superficially to the head mesoderm (Horigome et al. 1999), as we have argued above for gnathostomes, and in lampreys postmigratory crest forms a superficial shell, laterally overlying the head mesoderm (Fig. 9; Kuratani et al. 1999; Neidert et al. 2001). This too is as we suppose for the early postmigratory crest in zebrafish and mouse.

A possible difference

However, in lampreys a subsequent reorganisation of the branchiomeres into the cylindrical tissue arrangement has not been described and may not occur, at least in the segments posterior to the mandibular branchiomere (see below). That is, perhaps the embryonic tissue arrangements in a lamprey gill-bearing segment remain almost as they appear in Fig. 9, with the crest located mostly lateral to the mesoderm. This arrangement would perfectly fit the definitive structure, with crest-derived cartilages located laterally, external to the gills.

A hypothesis

As we have proposed above for gnathostomes, a specific neural crest medial relocation after a lateral migration underlies the formation of the neural crest cylinder walls. However, cylinders cannot be a terminal stage in morphogenesis, for gnathostome branchial cartilages are not hollow structures with mesoderm inside. In our 'outside-in' hypothesis, the cylinder is a transient structure in a developing gnathostome branchiomere (Fig. 10). After the cylinder stage, movement of the crest continues, and brings much of the crest medial to the mesoderm, where it then forms the precartilage condensations. By this hypothesis, the internal branchial skeleton of gnathostomes arises only after an extended period of complex morphogenesis. Such complex morphogenesis creates successive new tissue arrangements, that in turn provide new opportunities for cells to interact. The cylinder stage provides for intimate contacts between the postmigratory neural crest and both mesoderm and epithelium, and our evidence, as reviewed above, shows that signals present during the cylinder stage pattern the complex, dorsal–ventral arrangement of jointed pharyngeal cartilages, including the jaw cartilages, of the gnathostomes.

Fig. 11. Neural crest may form a cylinder in the lamprey mandibular branchiomere. Transverse section through a 32–34 somite embryo (from Damas, 1944, with colours added). Dark blue, ectodermal epithelium; light blue, neural crest-derived ectomesenchyme; red, head mesoderm.

If outside-in morphogenesis does allow for the signalling and segregation of the dorsal and ventral precartilage domains characteristic of gnathostomes specifically, then such separate domains should not be present in lamprey embryonic branchiomeres. In accord, Neidert et al. (2001) show that several *Dlx* genes present in lampreys are co-expressed apparently throughout a single domain of cartilage-forming postmigratory neural crest. In contrast, in gnathostomes only a subset of *Dlx* genes are so ubiquitously expressed (e.g. *dlx2* in zebrafish, and *Dlx1* and *Dlx2* in the mouse). Expression of other *Dlx* genes is ventrally restricted (e.g. *dlx3* in zebrafish, and *Dlx3*, *Dlx5* and *Dlx6* in the mouse; Robinson & Mahon 1994; Depew et al 1999; Miller et al. 2000). Neidert et al. propose that such dorsal-ventral patterning of the branchiomeres is new in gnathostomes. We concur; invention of the cylinder stage provides the anatomical basis for this new patterning.

Hence we suppose that outside-in morphogenesis, a lateral-to-medial relocation of neural crest, is either absent or much abbreviated in the developing gill-bearing segments of lampreys. Accordingly, the change in neural crest morphogenesis between lamprey and gnathostomes accounts for the difference in the topology of the gill supports and gills, the very difference that has so concerned the evolutionary theorists as discussed in the Introduction. By our argument, the gill supports are evolutionary homologues in the 2 groups. One only need suppose that adding an extra morphogenetic movement to a developmental sequence does not necessesarily mean that creation of a neomorphic (i.e. nonhomologous) structure will result. We would argue further that the lamprey pattern of morphogenesis is the primitive one. The same lateral migration route being present in gnathostomes follows von Baer's rule: There is retention of a developmentally early and evolutionarily primitive aspect of the morphogenesis. Subsequent cylinder formation is a derived feature. Just where and when was it derived? One possibility is that cylinders first developed specifically in the mandibular segment of an ancient agnathan.

JAWS EVOLVED SOMEWHERE, SOMEHOW; THE MANDIBULAR SEGMENT IN LAMPREYS

The invention of jaws appears to be a crucial innovation in vertebrate evolution. A perhaps tongue-in-cheek argument that 'jaws have always been jaws' (Janvier, 1996; p. 258) is not compelling. Jaws seem to be missing in the early vertebrates as recognised from fossil remains (Janvier, 1996), including newly discovered soft-bodied fossil early vertebrate remains (Chen et al. 1999; Shu et al. 1999). The modern vertebrate outgroups (amphioxus, urochordates) do not have them, nor do they have anything like them during early development. Rather jaws seem to have come from somewhere during evolution, modified from some structure or structures present in a jawless ancestor, or added onto a primitive pattern by invention anew. Jaws, as we recognise them, are highly derived, specialised and complex structures. They need and have a strong supporting jointed skeleton, and opener and closer muscles that act on this skeleton. It is a wonderful problem, long debated in the literature, to consider how they evolved.

We have reviewed above new evidence that adds to an already persuasive case that the gnathostome mandibular segment is a part of the same series as the other branchiomeres. If one accepts this argument, it is then not much of a theoretical leap to propose that lamprey and gnathostome mandibular segments are evolutionarily homologous. The comparative anatomical evidence supporting homology has been reviewed in detail by Mallatt (1996). Developmental evidence builds upon this foundation: Mandibular muscle precursors in lampreys, as in gnathostomes express Engrailed (Holland et al. 1993). As in gnathostomes, lamprey mandibular mesenchyme, presumably ectomesenchyme, expresses an *Otx* homeobox gene (Ang et al. 1994, Ueki et al. 1998; Tomsa & Langeland, 1999). Lamprey mandibular mesenchyme, and an endodermal pouch-like region just caudal to this mesenchyme (perhaps homologous to the hyomandibular pouch in gnathostomes) expresses a *Pax* gene. So does the mouse mandibular ectomesenchyme and hyomandibular pouch epithelium (Peters et al. 1998; Ogasawara et al. 2000). Just as the numerous shared developmental features strongly support segmental homology of the gnathostome mandibular segment and the other branchiomeres, parsimony strongly supports evolutionary homology of the mandibular segment in lampreys and gnathostomes. An independent (convergent) acquisition of the growing list of shared developmental regulators seems just too fantastic to consider seriously.

If lampreys have a mandibular segment, and if this segment forms jaws in gnathostomes, then it certainly follows that the jaw-forming domain was present before jaws evolved. One does not have to propose evolutionary novelty in the sense that a new domain has to be invented.

Can we take the argument any farther? The lamprey mandibular segment is highly specialised, and by

inference it is highly derived. Included is a velum, a specialised structure posterior to the mouth used in feeding and ventilation (Mallatt, 1996). A velar cartilage, the internal velar bar, lies medial to muscles and blood vessels in the segment. The medial position of this cartilage supports an argument based on topology for evolutionary homology between the internal velar cartilage and the gnathostome jaw cartilages (Janvier, 1996; Ogasawara et al. 2000). Muscles deriving from Engrailed-expressing primordia connect to the velar cartilages (Holland et al. 1993) providing connectional evidence for the same thing. However, there are considerable uncertainties. The velar supports are made of so-called mucocartilage, with a special histology that matches neither the cartilages of the branchial basket nor any of the cartilages in gnathostomes. Fate mapping by crest extirpation yielded no evidence for a neural crest origin of the velar cartilages (Langille & Hall, 1988). The fact that Engrailed expressing muscles insert on the velar cartilages is no nail-in-the-coffin argument, considering the extent to which presumed evolutionary homologues of these muscles have promiscuously attached to one skeletal element or another during gnathostome evolution.

There is also a laterally located mandibular cartilage in lampreys, the external velar bar (Mallatt, 1996). It seems to us (see also Mallatt, 1996) that this lateral element is just as suitable a candidate for a jaw cartilage homologue as is the lamprey medial cartilage. To get around the topological difference, of course we could use exactly the same argument that we made above for the gill-bearing segments. Namely, there is a new 'outside-in' phase of morphogenesis.

New developmental studies can and will provide far more understanding than we have now for the developmental anatomy of the lamprey mandibular arch. The adult muscle pattern has been well described (for a review, see Mallatt, 1996). Given that Engrailed expression is present, it seems reasonable to look also for an *endothelin-1* homologue expressed in mandibular mesoderm, and hence to possibly learn whether conserved features of an Edgeworthian-style muscle-plate can be found. Further, is the neural crest confined laterally? An intriguing old drawing (Fig. 11; Damas, 1944) suggests that in the developing lamprey mandibular segment the neural crest-derived mesenchyme forms the walls of a hollow cylinder surrounding a central mesodermal core. If Damas is correct in his interpretation of this structure, it may mean that we are already seeing the 'gnathostome style' of branchiomere development in the mandibular segment in lampreys. If so, might not the lamprey-style of involving the mandibular segment in feeding be a preadaptation (or exaptation; Gould & Vrba, 1982) to jaw evolution? Janvier (1996) proposes a similar scenario.

We complete our outside-in hypothesis with an admittedly far-out scenario. We assume that the velar cartilages will turn out to be neural crest-derived, and disregard the concern about mucocartilage (in agreement with Mallatt, 1996). Further, we propose that the outside-in movement of neural crest begins in the mandibular arch of an ancient, derived agnathan. Crest forms a cylinder, as drawn by Damas. However, in contrast to the gnathostomes, in the ancient ancestor and in modern lampreys a pronounced medial movement does not continue. In lampreys, crest remaining laterally forms the external cartilage bar, and medial crest forms the internal bar. By this proposition, both cartilages are segmental homologs of the external branchial cartilages in lampreys. Both are evolutionary homologues of the internal jaw cartilages of gnathostomes.

'AN INITIALLY UNIFORM SERIES' (GOODRICH, 1930)

A question arising repeatedly in the literature on jaw evolution is whether the mandibular branchiomere was ever used in gill breathing. Either it was (e.g. Mallatt, 1997) or it was not (e.g. Janvier, 1996; Smith & Coates, 2000). Much evidence and learned discussion has been brought to bear on both sides of the issue. We have no new evidence to bring to this debate, but we do wish to conclude with 2 observations that seem to have been largely overlooked.

If, as we and others have supposed, the pharyngeal walls are made of a series of segments, and if the mandibular segment is one of these branchiomeres, then it immediately follows that an ancient mandibular segment may have functioned in just the same way as the others. That the series was initially uniform is basic in the concept of a modular-style segmental organisation, and lies at the foundation of segmentation theory (Goodrich, 1930). Accordingly, if one accepts this view, differences between segments must be derived.

Since the generalised function of the branchiomeres in vertebrates is gill breathing, it would seem at first glance that a necessary consequence of this segmentalist line of argument is that the mandibular segment once was a gill-breathing structure. However, generalised does not always mean primitive. According to the wonderful theory of Gans & Northcutt (1983), early vertebrates invented neural crest, the cartilaginous supports of the pharyngeal walls, and

the muscles to pump water through these perforated walls. By this theory these inventions provided for breathing gills. But even if this is correct, it is entirely plausible that the inventions were first used to increase efficiency of filter feeding, the primitive function of the perforated pharynx in chordates. We deduce this function from analysis of vertebrate outgroups; tunicates and particularly the vertebrate sister group amphioxus use their pharyngeal slits to filter feed (Barrington, 1965). Hence gill breathing is derived, and a straightforward consequence is that the vertebrate mandibular branchiomere may never have been primarily used in gill breathing.

There is another possibility as well. It is just as plausible, and against the 'strong' version of the segmentalist argument outlined above, that the ancestral mandibular segment was regionally distinct from its neighbours at inception. Segmentation could have been superimposed in an ancestor (an early chordate, or yet earlier in evolution) on body tissues that were already regionally variant. For example, segmentation patterning genes may have been co-opted into territory already regionalised along the A–P axis by *Hox* genes. In this light it is interesting that one of the main arguments of Bateson's *Materials for the Study of Variation* (1894), founded on the detailed study of variations in segmental identity in a wide range of species, was that ancestral segments were not necessarily identical. In debating the early and primitive functions of the branchiomeres we agree with Mallatt that one needs to delve much more deeply into our evolutionary past than has been possible just with investigations of modern vertebrates and fossil fish.

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