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Sequence and developmental expression of *AmphiDll*, an amphioxus *Distal-less* gene transcribed in the ectoderm, epidermis and nervous system: insights into evolution of craniate forebrain and neural crest

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SUMMARY

The dynamic expression patterns of the single amphioxus *Distal-less* homolog (*AmphiDll*) during development are consistent with successive roles of this gene in global regionalization of the ectoderm, establishment of the dorsoventral axis, specification of migratory epidermal cells early in neurulation and the specification of forebrain. Such a multiplicity of *Distal-less* functions probably represents an ancestral chordate condition and, during craniate evolution, when this gene diversified into a family of six or so members, the original functions evidently tended to be parcelled out among the descendant genes. In the amphioxus gastrula, *AmphiDll* is expressed throughout the animal hemisphere (presumptive ectoderm), but is soon downregulated dorsally (in the presumptive neural plate). During early neurulation, *AmphiDll*-expressing epidermal cells flanking the neural plate extend lamellipodia, appear to migrate over it and meet mid-dorsally. Midway in neurulation, cells near the anterior end of the neural plate begin expressing *AmphiDll* and, as neurulation terminates, these cells are incorporated into the dorsal part of the neural tube, which forms by a curling of the neural plate. This group of *AmphiDll*-expressing neural cells and a second group expressing the gene a little later and even more anteriorly in the neural tube demarcate a region that comprises the anterior three/fourths of the cerebral vesicle; this region of the amphioxus neural tube, as judged by neural expression domains of craniate *Distal-less*-related genes, is evidently homologous to the craniate forebrain. Our results suggest that craniates evolved from an amphioxus-like creature that had the beginnings of a forebrain and possibly a precursor of neural crest – namely, the cell population leading the epidermal overgrowth of the neural plate during early neurulation.

Key words: amphioxus, *Distal-less*, forebrain, neural crest, evolution

INTRODUCTION

Because the molecular machinery directing animal development is remarkably conserved among phyla, developmental genetic data can indicate homologies between body regions of distantly related animals. Of such comparisons, one of the most interesting is between craniates and amphioxus, which is the best living proxy for the ancestor of the craniates (Wada and Satoh, 1994; Chen et al., 1995). Early work was focused on the central nervous system and studies of an amphioxus *Hox* gene (P. W. H. Holland et al., 1992, 1994a,b) indicated that a relatively extensive hindbrain was present at the beginning of craniate evolution. In contrast, the evolutionary origins of the more anterior regions of the craniate brain remain contentious. For instance, Gans and Northcutt (1983) suggested that the forebrain may be a derived feature of craniates, but others (like Lacalli et al., 1994) have proposed that at least the beginnings of a forebrain were already present in the proximate ancestor of the craniates.

The question of whether the ancestor of the craniates had a forebrain can be addressed by studying amphioxus genes homologous to craniate genes known to be forebrain markers, such as: *Emx-1*, *Emx-2* (Boncinelli et al., 1993) and most of the *Distal-less*-related genes (Porteus et al., 1991, 1994; Price et al., 1991, 1992; Robinson et al., 1991; Salinas and Nusse, 1992; Bulfone et al., 1993a,b; Dirksen et al., 1993; Papalopulu and Kintner, 1993; Price, 1993; Akimenko et al., 1994; Boncinelli, 1994; Robinson and Mahon, 1994; Simeone et al., 1994; Zhao et al., 1994; Tole and Patterson, 1995). The primary goal of the work described here is to use the developmental expression of *Distal-less*-related genes to analyze possible forebrain homologies between amphioxus and the craniates. However, our results also provide insights into establishment of embryonic body plan and evolution of neural crest.

Amphioxus establishes its embryonic body plan with diagrammatic clarity, because morphogenesis is influenced neither by the relatively enormous yolk content of non-mammalian craniate embryos nor by the extensive extraem-

bryonic tissues of mammalian embryos. The early development of amphioxus is echinoderm-like: cleavage results in a hollow ball of cells, the blastula, and from this the gastrula is produced by a symmetrical invagination from the vegetal pole (Conklin, 1932; Hirakow and Kajita, 1990, 1991, 1994). Yet, in spite of this early simplicity, the amphioxus embryo soon develops such craniate-like features as a notochord, a dorsal hollow nerve cord, segmentally arranged muscular somites and a perforated pharynx. A crucial event separating the early, echinoderm-like development from the later, craniate-like development is the establishment of a dorsoventral axis at approximately right angles to the animal-vegetal axis. The *Distal-less*-related gene that we found in amphioxus is the earliest known marker of this dorsoventral polarity.

The neural crest accounts for much of the structural complexity that sets craniates apart from invertebrates (Gans and Northcutt, 1983; Northcutt and Gans, 1983; Hall and Hörstadius, 1988) and there have been frequent speculations about its evolutionary origin. The consensus has been that neural crest arose in the craniate line and has no precedents in amphioxus or any other invertebrate. However, our data suggest that amphioxus neurulae include cells having some resemblance to craniate neural crest cells in their topography, *Distal-less* expression and migratory behavior.

MATERIALS AND METHODS

Obtaining genomic DNA from adults and cDNA from larval amphioxus

Adults of the Florida amphioxus, *Branchiostoma floridae*, were collected in Tampa Bay, Florida. Gametes from electrically stimulated adults were fertilized, and the embryos and larvae were raised in laboratory culture (N. D. Holland and Holland, 1993). A cDNA library was constructed from mRNA pooled from 2- to 4-day larvae and genomic DNA was extracted from a sample of 20 adults (methods in L. Z. Holland et al., 1996).

DNA probe isolation, library screening and sequencing clones

Nested PCR was used to amplify a 147 bp (base pair) portion of the homeobox from DNA purified from the larval cDNA library in λZAP II (Stratagene, La Jolla, CA). Forward and reverse primers included, respectively, *Eco*RI and *Bam*HI sites at their 5′ ends. The primers corresponded to the following sequences: MRKPRT and KIWFQN for the first PCR reaction, and KPRTIY and KIWFQN for the second PCR reaction. The PCR products were cloned into PCR II vector (Invitrogen, San Diego, CA). The 147 bp insert was used to screen about 250,000 clones from the larval cDNA library under moderate stringency. Hybridization was in 6× SSC, 10× Denhardt's, 1 mM EDTA, 0.2% SDS and 0.1 mg/ml tRNA at 60°C. Washes were in 1× SSC, 0.1% SDS at 50°C. 7 full-length cDNA clones (approximately 2,400 bp) were identified, all with identical base sequences. Double-stranded sequencing was with the Fmol sequencing kit (Promega, Madison, WI).

Southern blot analyses

Southern blots of genomic amphioxus DNA were prepared according to L. Z. Holland et al. (1996). To determine the number of amphioxus genes related to *Distal-less* and gene copy number, low stringency hybridization with a 741 bp *Kpn*I-*Pst*I fragment (Fig. 1), comprising the homeobox and flanking coding regions, was in 6× SSC, 0.2% SDS, 10× Denhardt's, 0.1 mg/ml tRNA overnight at 55°C. Washes were at 50°C in 2× SSC, 0.1% SDS.

In situ hybridization and histology

Gene expression was studied by in situ hybridizations on developmental stages of *Branchiostoma floridae* fixed at frequent intervals from the blastula through the 5-day larva (Table 1). Fertilization envelopes of prehatching stages were removed with insect pins to insure penetration of reagents. Methods of fixation and in situ hybridization were according to L. Z. Holland et al. (1996). To maximize the signal intensity, two antisense riboprobes were combined. The first corresponded to about 900 bp of the 3′ untranslated region, and the second corresponded to about 720 bp of a *Kpn*I-*Eco*RI fragment linearized with *Hin*dIII (Fig. 1), which included the coding region 3′ from the homeobox plus a downstream portion of the 3′ untranslated region. After the in situ preparations had been photographed as whole mounts, they were counterstained pink in Ponceau-S, embedded in Spurr's resin and prepared as 3.5 μm sections. For scanning electron microscopy (SEM), developmental stages of amphioxus were fixed in 2% glutaraldehyde in sea water, dehydrated in ethanol, dried by the CO_2 critical point method, mounted on stubs with double-stick tape, sputter coated with a palladium/gold mixture and observed by SEM.

RESULTS

Sequence analysis

Fig. 1 shows the nucleotide and deduced amino acid sequences of full-length cDNA. Of the 2,426 bp, 117 constitute the 5′ untranslated region and there is a polyadenylation signal 22 bases upstream from the poly(A) tail. The longest open reading frame contains 321 amino acids, if one assumes that translation starts at the ATG underlined in Fig. 1. This ATG is not downstream from any in-frame stop codons, but its neighboring bases agree well with the initiation context of Kozak (1987). About midway in the translated region, there is a 61-amino acid homeodomain of the sort encoded by *Distal-less* genes in other animals (Bürglin, 1994). We have thus named our gene *AmphiDll*.

Fig. 2 compares the homeodomains encoded by *AmphiDll* and *Drosophila Dll*, which have 92% amino acid identities, and compares homeodomains encoded by *AmphiDll* and craniate *Distal-less*-related genes, which have amino acid identities ranging from 85% (for *Dlx-1*) to 82%. When other regions of

Table 1. Developmental stages of amphioxus (*Branchiostoma floridae*)

Stage*	Age†	Size‡
Blastula	4 hour	190 μm
Early gastrula (G1)	4.5 hour	200 μm
Cap-shaped gastrula (G3)	5.5 hour	220 μm
Cup-shaped gastrula (G4)	6 hour	195 μm
Late gastrula (G5)	7.5 hour	200 μm
Very late gastrula (G6)	8.5 hour	220 μm
Early neurula begins (N1)	9.5 hour	240 μm
Early neurula ends (N1)	9.8 hour	245 μm
Hatching neurula (N2)	11 hour	260 μm
Late neurula (N3)	17 hour	430 μm
2-gill slit larva (L3)	2 day	1.1 mm
3-gill slit larva	5 day	1.2 mm

*Stages in parentheses from Hirakow and Kajita (1991, 1994).
†Time after fertilization; 23°C culture.
‡*Diameters* from blastula through late gastrula; *lengths* from very late gastrula through larval stages. Data for living specimens; after in situ hybridization or SEM, specimens shrink to 70-85% of size when alive.

these proteins are compared – between amphioxus and *Drosophila* on one hand, or between amphioxus and craniates on the other – there are some amino acid identities in short stretches immediately flanking the homeodomain, but relatively little conservation elsewhere.

Genomic Southern blots

In Southern blots probed at low stringency with a stretch of *AmphiDll* including the homeobox, a single hybridization band resulted from digestion by six out of nine restriction enzymes (Fig. 3). This pattern leaves little doubt that *AmphiDll* is the sole member of the *Distal-less* family present in the genome of *Branchiostoma floridae*. The other three enzymes yielded two hybridization bands, presumably as a result of polymorphism and/or cutting within introns included in the probed region. The preponderance of single hybridization bands also indicates that *AmphiDll* is a single copy gene.

Developmental expression of *AmphiDll* and correlated morphogenesis

Transcripts of *AmphiDll*, undetectable in the blastula (Fig. 4A), are first seen throughout the animal hemisphere in the earliest gastrula (defined by the flattened vegetal pole) (Fig. 4B). When the cap-shaped gastrula forms, expression persists throughout the ectoderm (Fig. 4C) but is not detectable in the invaginated mesendoderm (Fig. 4D). The cap-shaped gastrula soon develops into a cup-shaped gastrula, and the ectodermal expression of *AmphiDll* becomes down-regulated on what subsequent development reveals to be the dorsal side (Fig. 4E,F). By late gastrula, it is convenient to substitute the orientation terms anterior and posterior, respectively, for animal and vegetal. On the dorsal side of the late gastrula, ectoderm lacking detectable transcripts of *AmphiDll* begins to flatten into the definitive neural plate (Fig. 4G,H). Elsewhere, expression persists throughout the rest of the ectoderm (now the epidermis), most conspicuously at the ventrolateral borders of the blastopore (Fig. 4G) and at the margins of the neural plate, both anteriorly (Fig. 4I) and laterally (Fig. 4J). Similar expression patterns (Fig. 4K,L) are detected until the gastrula stage ends with blastopore closure.

At the early neurula stage (Fig. 4M-O), rapid anteroposterior elongation begins and mesodermal somites evaginate from the archenteron. *AmphiDll* is still expressed throughout the epidermis, but less intensely than before (Fig. 4M,O), except anteriorly (Fig. 4M), where transcripts are abundant chiefly in epidermal cells (Fig. 4N, tandem arrow) bordering the neural plate and in a few lateral cells located within the neural plate itself (Fig. 4N, single arrow).

At the boundary between the epidermis and neural plate, intercellular contacts are evidently disrupted and the epidermis from either side appears to migrate mediad across the dorsal surface of the neural plate (Fig. 4N,O). These epidermal cells are still expressing *AmphiDll*, although less strongly than before. During the overgrowth of the neural plate, which takes only about 20 minutes, the epidermis remains a coherent cell sheet. SEM (Fig. 5A,B) reveals that many of the epidermal cells at the leading edge of this migration have lamellipodia splayed out on the surface of the neural plate. The morphology of the lamellipodial cells suggests that they may be acting collectively to help pull the epidermis mediad from either side of the neural plate. Additional morphogenetic forces (reviewed by Schoenwolf and Smith, 1990) cannot be ruled out, although epidermal expansion due to cell division seems unlikely, because migration requires only a few minutes and many of the epidermal cells have cilia (Fig. 5B), structures that are

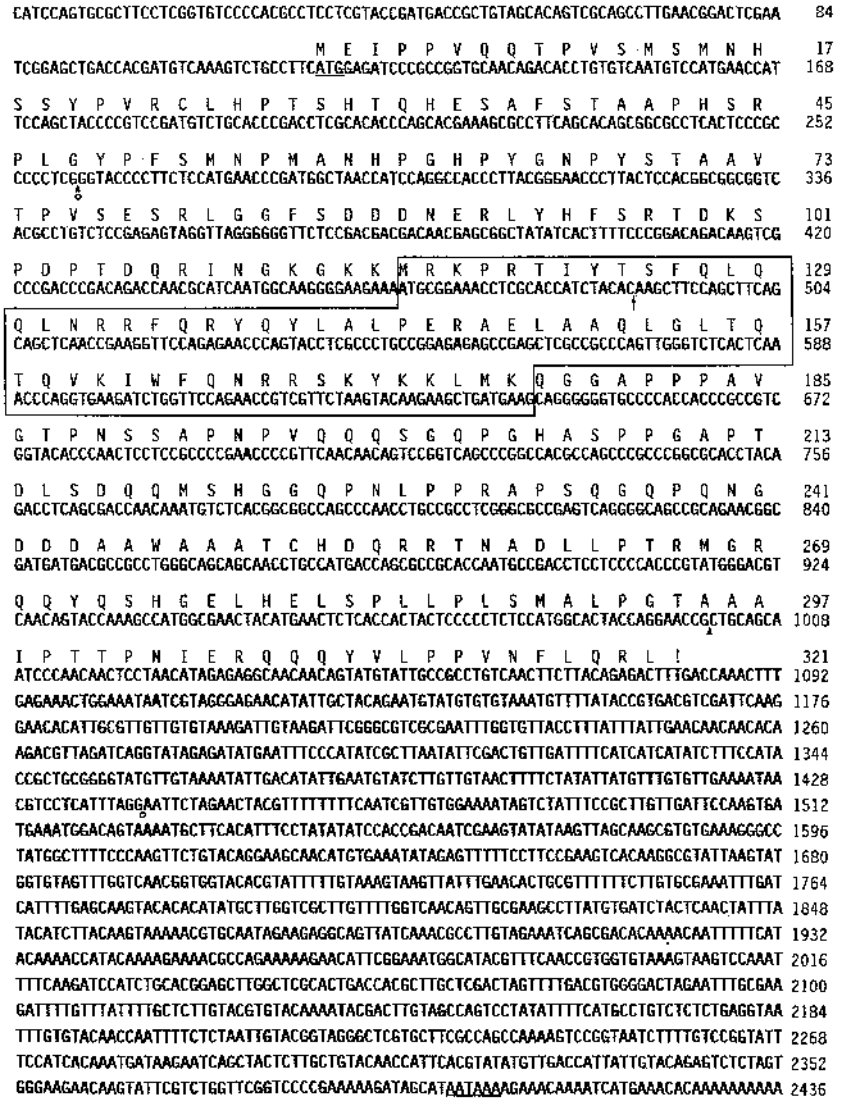


Fig. 1. *AmphiDll* isolated from cDNA of 2- to 4-day larvae of an amphioxus, *Branchiostoma floridae*: nucleotide sequence and deduced amino acid sequence (GenBank accession number U47058). The box encloses the homeobox and homeodomain. The presumed translational start site is underlined, as is the polyadenylation signal. The segment delimited by the two arrowheads was used in the Southern blot analysis. A riboprobe was made to a *Kpn*I-*Eco*RI fragment (delimited by the open circles) and linearized with *Hin*dIII (indicated by the arrow).

always resorbed around the time of mitosis in embryonic ectoderm cells (Stephens, 1995).

At the next stage (Fig. 6A,C), an uninterrupted epidermal sheet overlies the neural plate – except anteriorly (Fig. 6A,B). Expression of *AmphiDll* is still general throughout the epidermis and is especially intense anteriorly in epidermal cells bordering the hole where the epidermis has not yet closed over the neural plate. In this anterior region, strong expression also occurs in some lateral cells of the neural plate itself (Fig. 6B).

By the time the neurula hatches, the epidermis has closed over the neural plate (except at the small anterior neuropore) and is still expressing *AmphiDll* throughout, but most strongly at the anterior and posterior ends of the embryo (Fig. 6D). In the neural plate itself, there is strong expression of *AmphiDll* in two clusters of cells (Fig. 6D,E) located bilaterally a short distance posterior to the level of the neuropore; in addition, there is weak expression in some lateral cells of the neural plate that are scattered in disjunct groups (Fig. 6D, arrowheads) along the anteroposterior axis of the embryo. At this stage, the notochord is just beginning to form as a mid-dorsal evagination from the archenteron (Fig. 6F, arrowhead).

In the late neurula (Fig. 6G-J), the epidermis is still expressing *AmphiDll*, most strongly at the anterior and posterior ends of the embryo and in a few dorsal midline cells. By this stage, a definite notochord is present and the lateral edges of the neural plate have rolled up dorsally to form the neural tube, which encloses a neural canal. The neural canal is inconspicuous except where it is slightly dilated within the anteriormost few hundred micrometers of the neural tube; this anterior extremity of the amphioxus neural tube is termed the cerebral vesicle. Due to the rolling up of the neural tube, the two lateral clusters of *AmphiDll*-expressing neural plate cells (Fig. 6E) are brought together as a single group of cells occupying the dorsal half of the neural tube (Fig. 6I) a little posterior to the midpoint of the cerebral vesicle. More posteriorly in the neural tube, the primary pigment spot (which does not express *AmphiDll*) has formed (Fig. 6H, arrow). In the vicinity of the pigment spot, relatively weak expression is detected in a few roof plate cells and in a few cells located ventrally or ventrolaterally in the neural tube (Fig. 6H,J).

In the 2-gill slit larva (Fig. 6K,L), the dorsal wall of the cerebral vesicle still comprises the cells strongly expressing *AmphiDll* and described above for the neurula. By this stage, the neuropore has shifted from an anterodorsal to a dorsal position, such that there is now a short preneuroporal region at the anterior extremity of the cerebral vesicle. A second cluster of cells strongly expressing *AmphiDll* comprises the dorsal part of this preneuroporal region of the neural tube (Fig. 6L, inset). More posteriorly, in the vicinity of the primary pigment spot (Fig. 6K, inset), some *AmphiDll* expression is still detectable in a few scattered cells of the neural tube. Epidermal expression resembles that of the late neurula, but is somewhat weaker. By the stage of the 3-gill slit larva (fifth day of development), little or no further expression of *AmphiDll* can be detected anywhere in the whole-mount in situ preparations.

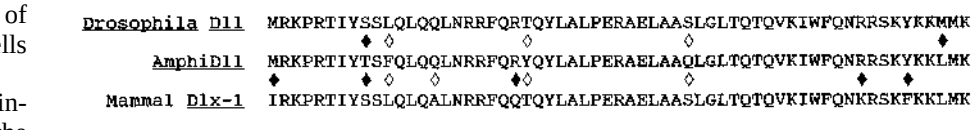


Fig. 2. Comparison between homeodomain amino acids of amphioxus AmphiDll with homeodomain amino acids of mammalian Dlx-1 (identical in mouse and human; data from Boncinelli, 1994) and *Drosophila* Dll (data from Vachon et al., 1992). Conservative amino acid substitutions indicated by filled diamonds and non-conservative substitutions by open diamonds.

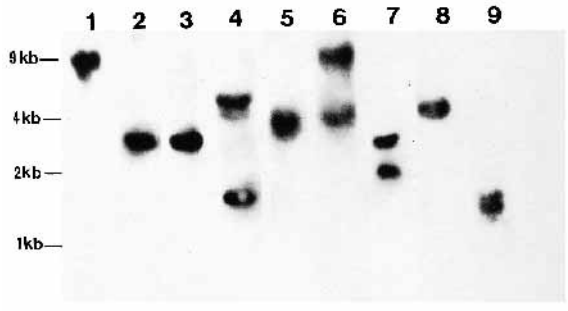


Fig. 3. Genomic Southern blot analysis of DNA pooled from 20 amphioxus adults. Numbers at top of lanes refer to digestion in following restriction enzymes: 1, *Bst*EII; 2, *Bst*XI; 3, *Eco*RI; 4, *Hin*dIII; 5, *Nco*I; 6, *Pst*I; 7, *Sma*I; 8, *Spe*I; 9, *Xba*I. Blot probed at low stringency with a 740 bp stretch of *AmphiDll*, including the homeobox. Size markers are at left.

DISCUSSION

AmphiDll compared to *Distal-less*-related genes of arthropods and craniates

Distal-less-related genes have so far been studied only in arthropods, craniates and amphioxus. Arthropods, like amphioxus, have only one *Distal-less* gene in the genome and, like *AmphiDll*, arthropod *Dll* is expressed both in the epidermis (Cohen, 1990; Cohen et al., 1991; Panganiban et al., 1994, 1995) and in the central nervous system (Cohen in Bulfone et al., 1993a; Kaphingst and Kunes, 1994; Panganiban and Skeath, personal observations). In comparison to arthropod *Dll*, *AmphiDll* is apparently involved in a wider range of epidermal functions (and perhaps earlier in developmental gene cascades), because the epidermal expression in arthropods is limited to the developing appendages.

Whereas amphioxus has only one *Distal-less* gene in the genome, craniates have five or six (Boncinelli, 1994), which probably evolved from a single ancestral gene (Price, 1993) that had much in common with *AmphiDll*. The presence of a single *Distal-less*-related gene in amphioxus and multiple members of this gene family in craniates is yet another indication that the size of developmental gene families increased abruptly early in craniate evolution. This increase may well have permitted the evolution of the complex features distinguishing the craniates from their invertebrate ancestors (P. W. H. Holland et al., 1995b; P. W. H. Holland and Graham, 1995).

Amphioxus *AmphiDll*, in comparison to any given *Distal-less*-related gene of craniates, appears to serve a wider range of functions and probably represents an ancestral chordate

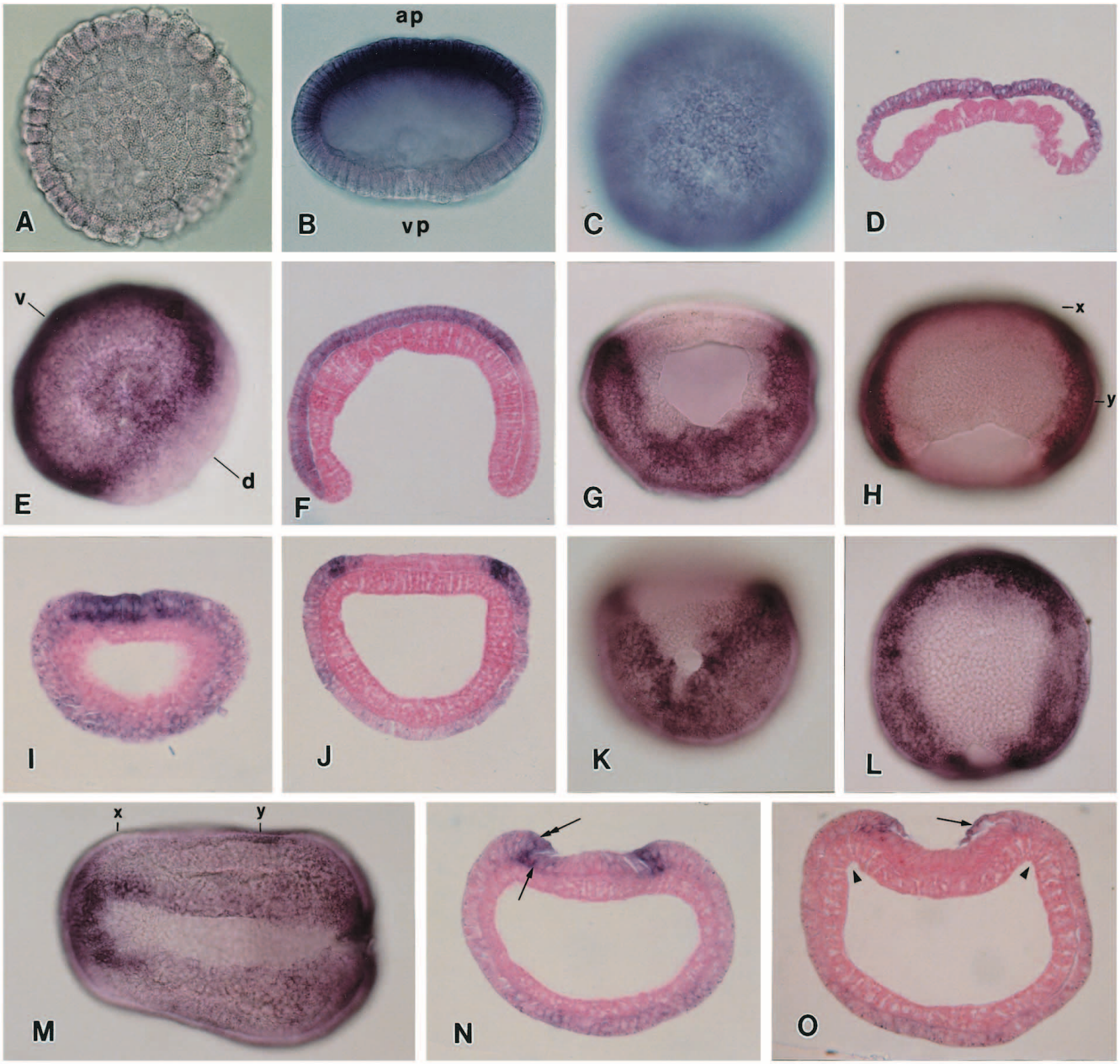


Fig. 4. Expression of *AmphiDll* in embryonic stages (sizes in Table 1) in whole mounts and in sections (counterstained pink). (A) Side view of blastula without detectable expression. (B) Side view of early gastrula with animal pole (ap) at top and vegetal pole (vp) at bottom; expression throughout animal hemisphere (top), but undetectable in vegetal hemisphere (bottom). (C) Animal pole view of cap-shaped gastrula with expression throughout ectoderm. (D) Cross section of preceding gastrula with blastopore toward bottom; detectable gene expression in ectoderm, but not in the invaginating mesendoderm. (E) Animal pole view of cup-shaped gastrula with expression in ectoderm except where down-regulated on presumptive dorsal side of embryo (d-v indicates dorsoventral axis). (F) Cross section of preceding gastrula in plane d-v with detectable expression limited to ectoderm, except dorsally (at right). (G) Vegetal pole (=posterior) view of late gastrula showing conspicuous blastopore; dorsal is at top and ventral at bottom; expression is in epidermal ectoderm, except dorsally (neural plate). (H) Dorsal view of preceding gastrula with blastopore toward bottom; expression is detectable in epidermis but not neural plate. (I) Cross section through x in Fig. 4H; dorsal is at top and ventral at bottom; epidermal expression is especially strong in cells at anterior border of neural plate. (J) Cross section through y in Fig. 4H; expression is especially strong in epidermal cells bordering neural plate. (K) Approximately posterior view of very late gastrula with nearly closed blastopore; dorsal is at top and ventral is at bottom; expression detectable throughout epidermis but not in neural plate. (L) Dorsal view of preceding gastrula with blastopore toward bottom; expression is detectable in epidermis, but not neural plate. (M) Dorsal view of early neurula with anterior toward left; expression not detectable in neural plate, but moderate to strong in epidermis. (N) Cross section through x in Fig. 4M showing expression in epidermis (tandem arrow), especially where it borders neural plate anteriorly; in addition, a few of the anterior neural plate cells (single arrow) now show strong expression. (O) Cross section through y in Fig. 4M showing *AmphiDll*-expressing epidermis (arrow) beginning to overgrow neural plate; arrowheads indicate where mesodermal somites are evaginating from archenteron.

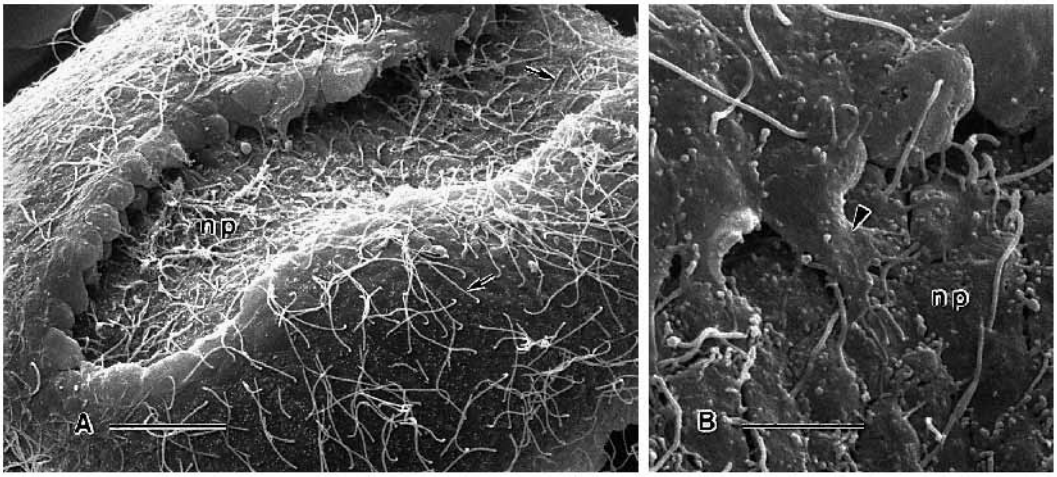


Fig. 5. SEM views of amphioxus embryo at beginning of early neurula stage. (A) Dorsal side of neurula with anterior toward the bottom left; the epidermis is beginning to overgrow the neural plate (np); most of the epidermal and neural plate cells have a single, apical cilium (arrows). Scale bar, 20 μm. (B) Enlargement of cells at edge of advancing epidermal sheet (top left); lamellipodia (one indicated by arrowhead) from epidermal cells extend on surface of neural plate (np). Scale bar, 5 μm.

condition. Presumably, during early craniate evolution, the functions of the original *Distal-less*-related gene were parcelled out among half a dozen related genes (P. W. H. Holland, 1992; Price, 1993), although with some overlap and possible redundancy.

Early in development, *AmphiDll* first appears as a general ectodermal marker, although its expression is soon repressed in the forming neural plate. Similarly, in early embryos of lower craniates, *Xenopus Xdll2* (Dirksen et al., 1994), and possibly its zebrafish homolog, *dlx3* (Akimenko et al., 1994), are expressed generally in ectoderm and then repressed in the neural plate. In higher craniates, *Dlx-3* genes are also expressed in the developing epidermis, but only in localized regions such as: limb buds, branchial arch, tail tip, nose, genital tubercle and tooth germs (Porteus et al., 1991, 1994; Dollé et al., 1992; Bulfone et al., 1993a; Robinson and Mahon, 1994; Morasso et al., 1995). By contrast, the other *Distal-less*-related genes of craniates have little or no epidermal expression and are instead conspicuous markers of neural tissue.

Finally, several *Distal-less*-related genes of developing craniates are expressed in some of the ectoderm-derived neural crest cells (considered in the last section of this discussion) as well as in some mesodermal structures sensu stricto. This is especially true of *Dlx-5* and *Dlx-6* (Simeone et al., 1994; Zhao et al., 1994; Ferrari et al., 1995). Because no *AmphiDll* expression is ever detected in mesodermal tissues of amphioxus, the mesodermal expression of *Distal-less*-related genes in craniates evidently represents the co-option of ancestral genes involved in epidermal and neuroepidermal development for new functions.

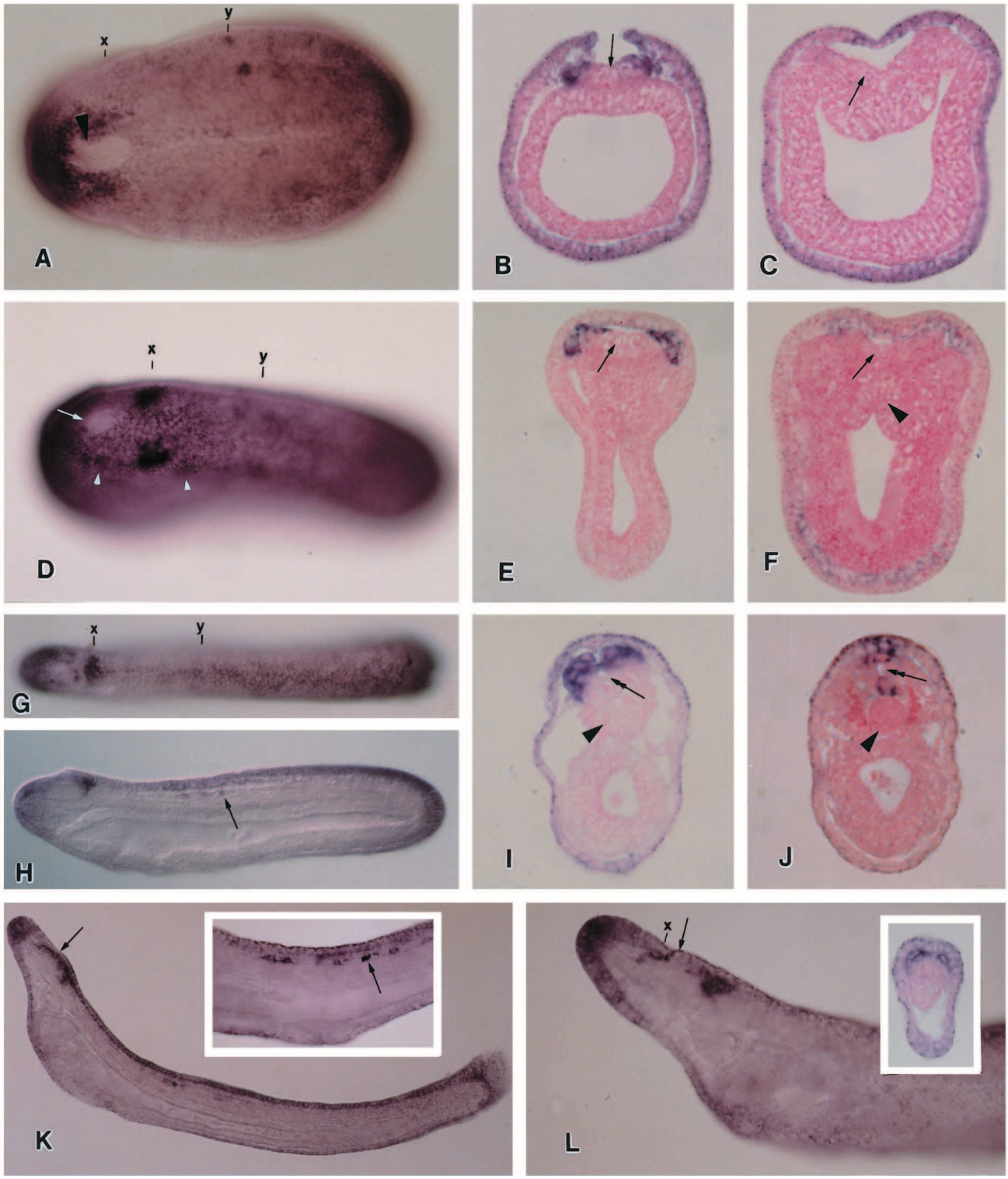
AmphiDll expression in the central nervous system

In craniate embryos, neural expression of *Distal-less*-related genes is exclusively in the forebrain – specifically, in most of the alar region of the ventral thalamus (flexed brain terminology) and in ventrolateral zones (unflexed brain terminology) of the alar regions of more anterior parts of the forebrain (Bulfone et al., 1993a,b; Puelles, 1995). Because the major neural expression domain of amphioxus *AmphiDll* is in the anterior three-fourths of the cerebral vesicle, we suggest that this region of the neural tube is homologous to parts of the craniate forebrain. This conclusion is strongly supported by

Fig. 6. Expression of *AmphiDll* in later embryonic and larval stages (sizes in Table 1). (A) Dorsal view of a whole mount at end of early neurula stage with anterior toward the left; moderate to strong expression in epidermis, which covers most of neural plate except at its anterior end (arrowhead). (B) Cross section through x in A showing that some of the strong expression is in lateral cells of neural plate; at this anterior level, epidermis has not yet closed over the underlying neural plate (arrow). (C) Cross section through y in A; neural plate (arrow) roofed over by epidermis. (D) Dorsal view of hatching neurula embryo with the anterior toward the left; epidermis roofs over neural plate except at the small, persistent anterior neuropore (arrow); expression at either side of neural plate – in two strongly expressing groups of neural plate cells just posterior to the neuropore and also in weakly expressing groups (two of which are indicated by arrowheads) at several levels along the anteroposterior axis. (E) Cross section through x in D, showing neural plate (arrow) with strongly expressing cells at either side. (F) Cross section through y in D showing neural plate (arrow) roofed over by epidermis; notochord (arrowhead) evaginating from archenteron. (G) Dorsal view of late neurula showing a single median cluster of neural tube cells strongly expressing *AmphiDll*. (H) Lateral view of preceding embryo showing strongly expressing neural tube cells anteriorly; weak expression in a few neural tube cells near primary pigment spot (arrow). (I) Cross section through x in G; definitive notochord (arrowhead) and neural canal (tandem arrow) now present; strong expression in dorsal half of neural tube. (J) Cross section through y in G; notochord (arrowhead) and neural canal (tandem arrow) have formed; a dorsal epidermal cell and a dorsal and a ventral cell in the neural tube are expressing *AmphiDll*. (K) Lateral view of a 2-gill slit larva with neuropore indicated by arrow; strong *Amphidll* expression dorsally in anterior neural tube in cells anterior and posterior to neuropore; the *inset* shows an enlarged lateral view of region near primary pigment spot (arrow) with weaker expression in some scattered cells of the neural tube. (L) Lateral view of anterior end of the preceding larva with neuropore indicated by arrow; *AmphiDll* expressed strongly in first group of cells posterior to neuropore and in second group of cells immediately anterior to neuropore; the *inset* is a cross section through x, showing strong expression in cells of second group.

three-dimensional, computer-assisted reconstructions of the neural tube of amphioxus based on serial transmission electron microscopy (Lacalli et al., 1994). At the neuroanatomical level, a number of detailed homologies are indicated between the anterior three-fourths of the amphioxus cerebral vesicle and the diencephalic region of the craniate forebrain. If one assumes that the amphioxus condition fairly represents the nervous system of the proximate ancestor of the craniates, one can suggest that they evolved from a creature that had the beginnings of a forebrain.

In the craniate nervous system, *Distal-less*-related genes are involved in regional specification, control of transition between cell proliferation and terminal differentiation and maintenance of terminal differentiation (Porteus et al., 1994; Beauchemin and Savard, 1992; Robinson and Mahon, 1994). In the amphioxus nervous system, *AmphiDll* may not only specify



brain regions, but could also have later functions concerned with neural differentiation, because a few cells scattered along the length of the neural tube express this gene near the future photoreceptive organs of Hesse (Nakao, 1964). Similarly, in craniates (Price et al., 1991; Dirksen et al., 1993) and arthropods (Kaphingst and Kunes, 1994), *Distal-less*-related genes may also be involved in the differentiation of neural areas receiving or transmitting optic signals.

Amphioxus and insights into the origin of craniate neurulation and neural crest

Development of deuterostomes above the level of echinoderms is characterized by neurulation, which is the overall process leading to the formation of the dorsal, hollow nerve cord. Neurulation is driven by a combination of morphogenetic events within the neural plate ectoderm and nearby areas of epidermal ectoderm (Schoenwolf and Smith, 1990; Moury and Schoenwolf, 1995). Neurulation in ascidian tunicates (Nicol and Meinertzhagen, 1988) and most craniates, termed primary neurulation, is essentially an invagination of the neural plate. In addition, some craniates (i. e. lampreys and teleosts) have an evidently derived form of neurulation, termed secondary, in which the neural tube forms by cavitation of a solid neural keel. In comparison, amphioxus neurulation is highly distinctive. It occurs in two relatively disjunct phases – the first being a rapid epidermal overgrowth of the neural plate without invagination, and the second being a slow rolling up of the neural plate into the neural tube. It is not clear whether this kind of neurulation is unique to amphioxus among deuterostomes, or also occurs in hemichordates (Morgan, 1891). It is possible that neurulation in amphioxus represents the primitive type of neurulation in deuterostomes and the problem needs more study in hemichordates, the most appropriate outgroup for establishing polarity of chordate characters.

In the early neurula of amphioxus, the epidermal cells bordering the neural plate exhibit three features suggestive of craniate neural crest cells. (1) Topographically, these amphioxus cells are at the neural plate border, which approximates the location of neural crest cells when first specified in craniates (Moury and Jacobson, 1990; Mayor et al., 1995; Selleck and Bronner-Fraser, 1995). (2) *Distal-less*-related genes are expressed in premigratory and migratory epidermal cells in amphioxus and also in migratory and differentiating neural crest cells of craniates (Dollé et al., 1992; Bulfone et al., 1993a; Dirksen et al., 1993, 1994; Akimenko et al., 1994; Robinson and Mahon, 1994; Qiu et al,. 1995). However, in amphioxus, the cells in question contain abundant transcripts of *AmphiDll* before cell migration begins, whereas, in craniates, cells derived from the neural crest do not contain detectable transcripts of *Distal-less*-related genes until after migration has commenced. (3) The migratory behavior of amphioxus epidermal cells overgrowing the neural plate has some resemblance to craniate neural crest cell migration. However, there are obvious differences. Unlike neural crest cells, most of which migrate individually within the interior of the embryo, the amphioxus cells migrate, not individually, but at the edge of a cell sheet, and they follow a path over the exterior of the embryo. Finally, amphioxus cells do not differentiate into the wide variety of cell types produced by the craniate neural crest cells. In amphioxus, morphological data (which should be confirmed with cell tracer methods) indicate that the epidermal cells with lamellipodia, after migrating mediad from either side of the neural plate, meet mid-dorsally to re-establish a coherent cell sheet and their fate thus appears to remain epidermal.

It would be premature to construct a detailed scenario for the evolutionary origin of neural crest based on the expression of only one of the genes known to be useful markers for neural crest cells in craniates; for example, amphioxus homologs of craniate *Wnt-1*, *Wnt-3a*, *Slug* and *Snail* (Essex et al., 1993; Nieto et al., 1994; Dickinson et al., 1995; Mayor et al., 1995; Sechrist et al., 1995). Even so, as a starting point for further discussion, we would tentatively propose that the proximate ancestor of the craniates formed the dorsal nerve cord by neurulation of the amphioxus type: the epidermal cells bordering the neural plate became motile and roofed it over. Then, in the earliest craniates, amphioxus-like neurulation gave rise to primary neurulation by invagination. The motile epidermal cells no longer played an important part in roofing over of the neural plate, but instead remained contiguous with it as invagination carried them into the dorsal part of the nerve cord. Some of these former epidermal cells (and possibly their immediate neighbors on either side of the neural plate-epidermis boundary) could have given rise to sensory neurons and others could have penetrated the basal lamina around the nerve cord (much as suggested by Fritzsch and Northcutt, 1993) to enter the remnant of the blastocoel, thus becoming definitive neural crest. From this point, the scenario of Gans and Northcutt (1983) can begin to complete the craniate body plan. In retrospect, the invention of neurulation by higher deuterostomes can be regarded not just as a key innovation in itself, but also as setting the stage and providing raw material for the subsequent evolution of the craniate neural crest.

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