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Anteroposterior Patterning in Hemichordates and the Origins of the Chordate Nervous System

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Summary

The chordate central nervous system has been hypothesized to originate from either a dorsal centralized, or a ventral centralized, or a noncentralized nervous system of a deuterostome ancestor. In an effort to resolve these issues, we examined the hemichordate *Saccoglossus kowalevskii* and studied the expression of orthologs of genes that are involved in patterning the chordate central nervous system. All 22 orthologs studied are expressed in the ectoderm in an anteroposterior arrangement nearly identical to that found in chordates. Domain topography is conserved between hemichordates and chordates despite the fact that hemichordates have a diffuse nerve net, whereas chordates have a centralized system. We propose that the deuterostome ancestor may have had a diffuse nervous system, which was later centralized during the evolution of the chordate lineage.

Introduction

Despite considerable paleontological work and molecular analysis, mystery still surrounds the origin of our own phylum, the Chordata (Gee, 1996). The two closest invertebrate groups, the echinoderms and hemichordates, along with chordates, constitute the deuterostomes, which shared a common ancestor in Precambrian metazoan evolution (Adoutte et al., 2000; Cameron et al., 2000; Peterson and Eernisse, 2001). Early deuterostomes were clearly established by the Lower Cambrian, as documented in recent excavations (Shu et al., 2003). Chordates possess distinctive morphological features such as a dorsal hollow nerve cord, notochord, gill slits, and a post-anal tail (Gee 1996). Were these traits already present in the deuterostome ancestor, or did they originate entirely in the chordate line? Identifying morphological homologies among these phyla has been fraught with difficulties, as their adult body plans appear so divergent (Gee, 1996). Yet, underlying the development of morphology are distinct gene networks that are expressed at specific locations within the bodies of all members of the chordate phylum. A comparison of such domains among the three extant deuterostome phyla could reveal similarities in axial organization obscured by divergent morphology and facilitate the reconstruction of the basic body plan of the hypothetical deuterostome ancestor.

Hemichordates may hold more promise for these comparisons than do echinoderms since they have several proposed morphological affinities with chordates. Indeed, Bateson (1886b) originally classified hemichordates as chordates based on gill slits; a stomochord with uncertain homology to the chordate notochord (Balser and Ruppert, 1990); dorsal and ventral nerve cords, each of which has been proposed as the homolog of the chordate dorsal hollow cord (Morgan, 1894; Nübler-Jung and Arendt, 1999); and a ventral post-anal extension, proposed as the homolog of the chordate dorsal tail (Burdon-Jones, 1952). However, the homologies are easily controverted and hemichordates were reclassified into their own phylum by the 1940s.

The nervous system is the key element in most hypotheses on chordate origins. Three hypotheses currently account for the origin of the chordate nervous system, all consistent with recent molecular phylogenies, yet all mutually incompatible. In the auricularian hypothesis of Garstang ([1894, 1928] see also Lacalli [1994] and Nielsen [1999]), the chordate nerve cord was thought to have originated from the nervous system of a motile auricularia larva of an ancestral sessile deuterostome adult. Bilateral rows of cilia and the associated nerves were said to have converged to the dorsal midline to form the nervous system. In the hemichordate hypothesis, Bateson (1886b) proposed that chordates evolved directly from a hemichordate-like adult ancestor, not a larva. According to him, the ancestor, like the extant adult hemichordate, had a chordate-like dorsal hollow nerve cord (most prominent in the collar region, Figure 1A). In the inversion hypothesis (Geoffroy-St. Hilaire, 1822; revised by Arendt and Nübler-Jung, 1996; DeRobertis and Sasai, 1996), a worm-like ancestor of annelids, arthropods, and chordates already had a well-formed central nervous system, ventrally placed. An adult ancestor of deuterostomes inverted its body dorsoventrally, placing the nerve cord dorsal in chordates. Recent support for this hypothesis comes from the inverse disposition of neurogenic and nonneurogenic ectoderm in the embryos of chordates and *Drosophila*, correlated with the inverse disposition of TGF-β signals and their antagonists (De Robertis and Sasai, 1996).

A more classical perspective of nervous system evolution that has not enjoyed much support from recent molecular analyses is the proposal that the bilaterian ancestor had a diffuse nervous system that was centralized independently in different bilaterian lineages (Brusca and Brusca, 1990). One reason for resistance

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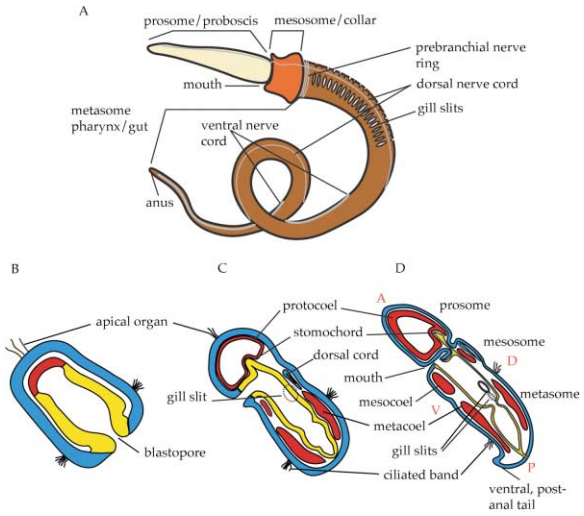

Figure 1. Summary of *S. kowalevskii* Adult Anatomy and Embryology

(A) Schematic view of the *S. kowalevskii* adult. Note the tripartite body of prosome, mesosome, and metasome, each containing a coelom or paired coeloms. Length: 10–20 cm. The dorsal and ventral nerve cords as well as the prebranchial nerve ring are drawn in; these are not visible from the surface but are located at the basal face of the epidermis.

(B) A late gastrula (36 hr postfertilization) shown in longitudinal section. Anterior is to the top left. Ectoderm shown in blue, mesoderm in red, and endoderm in yellow.

(C) A late neurula embryo (3 days postfertilization) shown in sagittal section. This is the orientation of many stained embryos of Figures 2–6. Anterior is to the top left. Dorsal is to the top right. The location of the first gill slit pair is indicated as a dashed line as they initially bud from the endoderm above and below the plane of section. Length: 1 mm.

(D) The two gill slit embryo (14 days postfertilization) in sagittal section. Note the extended stomochord protruding into the prosome, two paired gill slits, and ventral post-anal tail.

to this idea might be that the bilaterian ancestor would necessarily have had a diffuse nervous system with a complex anteroposterior pattern including, for example, a Hox pattern common to arthropods and vertebrates. Since there has been no molecular evidence for an extant group of animals with such a well-patterned but diffuse nervous system, it was not clear such an organism could exist.

To address these hypotheses of nervous system origins and, indeed, to address the origin of chordates, we have investigated a direct developing hemichordate, *Saccoglossus kowalevskii*, known by the common name of acorn worm. We have isolated orthologs for 22 neural patterning genes whose domains have been carefully mapped in the chordate neural plate and nervous system. At least 14 of these chordate domains are similarly expressed in protostomes, whereas at least four of them appear to be chordate specific in their expression. Surprisingly, the expression maps in chordates and hemichordates are very similar for all the genes despite major organizational and morphological disparity of the two nervous systems. Moreover, the expression of complex regulatory gene networks during hemichordate nervous system development suggests that despite its diffuse and noncentralized organization, it is nonetheless highly patterned. Thus, we confirm the presence of a well-patterned and diffuse nervous system in the deuterostomes and propose that the common ancestor of hemichordates, chordates, and perhaps even protosomes resembled the acorn worm in this way.

Results

Anatomy of Select Embryonic Stages

The anatomy of adult and embryonic stages of *S. kowalevskii* is shown in Figure 1. The adult has tripartite, tricoelomic organization (Figure 1A). At the anterior is the muscular proboscis or prosome, used for burrowing and collecting food particles. It contains the heart, kidney, a section of the dorsal nerve cord, and the protocoel. The middle region, which is the collar or mesosome, contains the mouth, a section of dorsal nerve cord formed by neurulation (Morgan, 1894), the paired mesocoels, and the base of the stomochord, which projects forward into the prosome. The posterior region or metasome contains the gill slits, the remainder of the dorsal nerve cord, the entire ventral nerve cord, paired metacoels, gonads, a long through-gut, and terminal anus. At juvenile stages, a ventral post-anal extension (called a tail or sucker) is present.

Gastrulation entails uniform and simultaneous inpocketing of the vegetal half of the hollow blastula. As the blastopore closes, a gumdrop-shaped gastrula is formed (Figure 1B). As the embryo lengthens, two circumferential grooves indent and divide the length into prosome, mesosome, and metasome regions (Figures 1C and 1D). Mesodermal coeloms outpouch from the gut anteriorly and laterally (Figures 1C and 1D). The first gill slit pair appears externally by day 5 (Figure 1C), and the animal bends from the dorsal side. The hatched juvenile elongates and adds further pairs of gill slits successively (Figure 1D). The animal is nearly bilaterally symmetric, except that the prosome excretory pore (the proboscis pore) from the kidney is reliably on the left, defining a left-right asymmetry.

Pervasive Neurogenesis in the Ectoderm

Postdating the hypotheses described in our Introduction, Bullock (1946, 1965) and Knight-Jones (1952) found that the hemichordate adult nervous system is not centralized but is a diffuse intraepidermal, basiepithelial nerve net. Nerve cells are interspersed with epidermal cells and account for 50% or more of the cells in the proboscis and collar ectoderm and a lower percentage in the metasome. Axons form a meshwork at the basal side of the epidermis. The two nerve cords are through-conduction tracts of bundled axons (Cameron and Mackie, 1996) and are not enriched for neurogenesis. This general organizational feature of the nervous system has been largely underemphasized in recent literature that focuses on possible homologies between chordate and hemichordate nerve cords (Nübler-Jung and Arendt, 1999).

Although neurons are dispersed throughout the epidermis in the adult, it has not been demonstrated that neurogenesis in the embryo is uniform. To determine the site of neurogenesis, we localized the domains of expression of three orthologs of pan-neural genes of

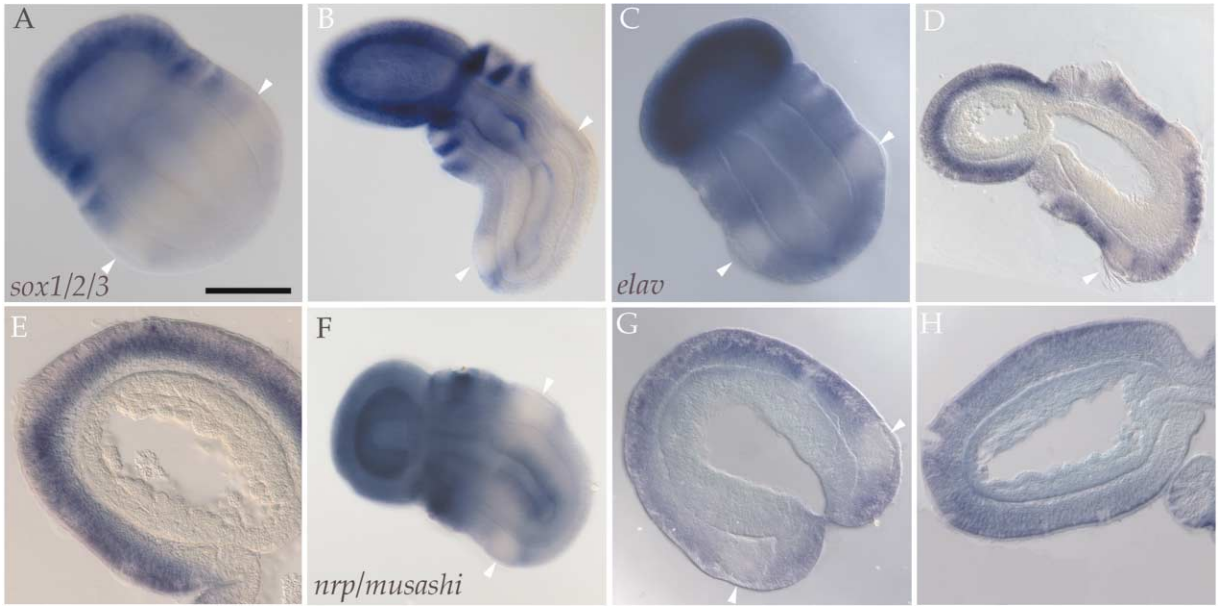

Figure 2. Pervasive Neurogenesis in Ectoderm of *S. kowalevskii*

Whole-mount in situ hybridization and sections. In *S. kowalevskii* embryos, orthologs of pan-neural genes expressed in the vertebrate neural plate are widely expressed throughout much of the ectoderm. White arrowheads indicate ciliary band cells of ectoderm. Scale bar = 100 μm. Unless otherwise indicated, panels are optical sections, with anterior to the left. (A and B) Expression of *sox1/2/3* (A) in late gastrula, (B) in one gill slit stage embryo, side view. Note basiepithelial staining. (C–E) Expression of *elav/hu*. (C) In early neurula, side view. (D) Sagittal cryosection of late neurula. (E) High magnification of sagittal cryosection of late neurula proboscis. Note basiepithelial staining. (F–H) Expression of *nrp/musashi*. (F) Neurula in side view, (G) sagittal cryosection of late gastrula. (H) Sagittal cryosection of proboscis of one gill slit embryo.

chordates and *Drosophila*, namely, *nrp/ musashi, sox1/ 2/3/ soxneuro*, and *hu/elav*. The first two are markers of proliferating neuron precursors, whereas the third is a marker of differentiating neurons (Kim and Baker, 1993; Kaneko et al., 2000; Sasai, 2001). All are expressed in the neural plate of various chordates, but not in the epidermis. As shown in Figure 2, *nrp/musashi* and *sox1/ 2/3 /soxneuro* are expressed in the entire ectoderm of the early *S. kowalevskii* embryo (except for the ciliated band, which all probes except *emx* fail to stain) (Figures 2A, 2B, and 2F–2H). In later stages, the expression remains strong in the prosome and declines in the metasome, correlating with Bullock's observation of decreasing neuron density posteriorly (Figure 2B). In sections, weak expression of *nrp/musashi* can be detected in the posterior endoderm, possibly correlated with a sparse endodermal nerve net (Figure 2F, and data not shown) (Bullock, 1965). *Hu/elav* exhibits similar diffuse staining throughout the ectoderm in early stages (Figure 2C). Additionally, *Hu/elav* staining remains strong along the posterior dorsal midline at later stages, in a punctate pattern perhaps reflecting a concentration of early-differentiating nerves at this site. In sagittal sections of embryos, *hu/elav* expression appears localized toward the basal side of the ectoderm (basiepithelial) (Figures 2D and 2E); it is absent from the mesoderm. Thus, *S. kowalevskii* shows pervasive neurogenesis with no large, contiguous nonneurogenic subregion, as occurs in chordates.

Ectodermal Expression of Putative Neural Patterning Genes

We isolated 22 full-length coding sequences of orthologs associated with neural patterning in chordates. Orthology was first tested by blastx followed by gene tree analysis (see Supplemental Figures online at http://www.cell.com/cgi/content/full/113/7/853/DC1). These genes are probably present as single copies in *S. kowalevskii* because orthologs of most of them are present as single copies in lower chordates and echinoderms, and many of the genes were recovered multiple times in the EST analysis without our finding any closely related sequences.

Using full-length probes for in situ hybridization, we found that all 22 genes are expressed strongly in the ectoderm as single or multiple bands around the animal, in most cases without dorsal or ventral differences (*rx, hox4, nkx2-1, en, barH, lim1/5*, and *otx* are exceptions). Circumferential expression is consistent with diffuse neurogenesis in the ectoderm. The domains resemble the circumferential expression of orthologs in *Drosophila* embryos. In chordates, by contrast, most of these neural patterning genes are expressed in stripes or patches only within the dorsal neurectoderm and not in the epidermal ectoderm. Also, in chordates, the domains are often broader medially or laterally within the neurectoderm, and there are usually additional expression domains in the mesoderm and endoderm. In most of the 22 cases in *S. kowalevskii*, the ectodermal domain is the only expression domain (*six3, otx, gbx, otp, nkx2-1, dbx, hox11/13*, and *irx* are exceptions).

Although each of the 22 genes has a distinct expression domain along the anteroposterior dimension of the chordate body, we have attempted to divide them into three broad groups to facilitate the comparison with hemichordates: anterior, midlevel, and posterior genes. Anterior genes are those which in chordates are expressed either throughout or within a subdomain of the

forebrain. Midlevel genes are those expressed at least in the chordate midbrain, having anterior boundaries of expression in the forebrain or midbrain, and posterior boundaries in the midbrain or anterior hindbrain. Posterior genes are those expressed entirely within the hindbrain and spinal cord of chordates. Many of the chordate genes have additional domains of expression elsewhere in the nervous system and in other germ layers, but we restrict our comparisons to domains involved in specifying the neuraxis in the anteroposterior dimension. Taking these groups of genes one at a time, we ask where the orthologous genes are expressed in *S. kowalevskii*. In all comparisons, no morphological homology is implied between the subregions of the chordate and hemichordate nervous systems.

Anterior Neural Domains

Six genes were examined, namely *sine oculis-like* or *optix-like* (*six3*), *retinal homeobox* (*rx*), *distal-less* (*dlx*), *ventral anterior homeobox* (*vax*), *nkx2-1*, and *brain factor 1* (*bf-1*). As diagrammed in Figures 3i–3iii, these six chordate neural patterning genes are expressed within the forebrain, each with its own contour and location (Shimamura et al., 1995, Rubenstein and Shimamura, 1997; see Supplemental Table S1 for a comprehensive references list).

In *S. kowalevskii*, the orthologs of these six genes are expressed strongly throughout the ectoderm of the prosome. Within the prosome ectoderm, the domain of each gene differs in its exact placement and contours. *vax* is expressed just at the anterior tip of the prosome near the apical organ (Figures 3A and 3B). *six3* and *rx* are expressed throughout most of the prosome (Figures 3C–3F). *rx* expression is exclusively ectodermal, as shown in section in Figure 3M. The section also reveals the absence of *rx* expression in the apical region of ectoderm where *vax* is expressed. *Six3* is expressed ectodermally and at low levels mesodermally in the developing prosome (Figure 3N), and the domain extends slightly into the mesosome ectoderm (Figures 3E and 3F). Expression of *six3* is strongest in the most anterior ectoderm and attenuates posteriorly (Figure 3N). *dlx* and *bf-1* are both expressed strongly in a punctate pattern of numerous individual cells or cell clusters throughout most of the prosome ectoderm and also in a diffuse pattern at a lower level throughout the prosome ectoderm (Figures 3I–3L). The *bf-1* domain is interrupted by a band of nonexpression in the midprosome (Figure 3J). Figures 3O and 3P show *dlx* expression in sections through the proboscis and highlight the apical position of individual cells strongly positive for *dlx* and also the basal position of ectodermal cells giving the widespread low-level expression. *dlx* is also expressed more posteriorly in a dorsal midline stripe (Figure 3K) and will be discussed elsewhere. *nkx2-1* is specifically expressed in a ventral sector of the prosome ectoderm (Figures 3G and 3H). In chordates, *nkx2-1* is expressed in the ventral (subpallial) portion of the forebrain (Sussel et al., 1999). It is also expressed less strongly in a ring in the hemichordate pharyngeal endoderm, a domain of interest in relation to this gene's involvement in the chordate endostyle and thyroid (as found by Takacs et al., 2002, in the hemichordate *Ptychodera flava*).

In conclusion, these six orthologs, whose chordate cognates are expressed entirely within the forebrain, all have prominent expression domains in the prosome ectoderm of *S. kowalevskii*, the hemichordate's most anterior body part.

Midlevel Neural Domains

Ten genes were examined, namely *tailless* (*tll*), *paired box homeobox 6* (*pax6*), *emptyspiracles-like* (*emx*), *barH, orthopedia* (*otp*), *developing brain homeobox* (*dbx*), *lim domain homeobox 1/5* (*lim1/5*), *iroquois* (*irx*), *orthodenticle-like* (*otx*), and *engrailed* (*en*). As indicated in Figures 4i–4v, these genes are all expressed in chordates at least in the midbrain of the central nervous system, and thus, as a group, their domains are more posteriorly located than the anterior set (Rubenstein et al., 1998; Tallafuss and Bally-Cuif, 2002; see Supplemental Table S1 for a comprehensive references list). Some have the anterior border of the domain in the forebrain (*tll, pax6, emx, lim1/5*, and *otx*), and some have it in the midbrain (*otp, barH, dbx, irx*, and *en*). Most have posterior borders in the midbrain, but two (*en* and *irx*) have posterior borders in the anterior hindbrain (Glavic et al., 2002). Thus, while all are expressed in the midbrain, each differs in its anterior and posterior extent. Several of the chordate genes (*pax6, dbx, en, and irx*) have separate posterior expression domains running the length of the chordate hindbrain and spinal cord at different dorsoventral levels of the neural tube. We will not discuss these additional domains because, as the data will show, there are no comparable domains of expression in *S. kowalevskii*.

In *S. kowalevskii*, these ten orthologs are expressed in circumferential bands in the ectoderm at least of the mesosome (collar) or anterior metasome, that is, more posteriorly than the anterior group. Each gene differs in the exact anteroposterior extent of its domain; some are expressed in part or all of the prosome. The most broadly expressed orthologs of this group are *pax6, otp, lim1/5, irx*, and *otx* (Figures 4A and 4B, 4I and 4J, and 4M–4R). All are expressed in the prosome (relatively weakly for *otx*), mesosome (weakly in the case of *otp* and *lim1/5*), and anterior metasome, all ceasing by the level of the first gill slit. *pax6* is strongest at the base of the proboscis (Figure 4B), and *lim1/5* is expressed most strongly in a dorsal patch at the base of the proboscis (Figures 4M and 4N). The most narrowly expressed orthologs are *barH, tll, emx*, and *en*. *tll* is detected in early stages in the anterior prosome, posterior prosome, and anterior mesosome (Figure 4C) and in later stages restricted to the anterior mesosome (Figure 4D). The *emx* domain is a single ring in the anterior mesosome plus an additional domain in the ciliated band in the posterior metasome (Figures 4G and 4H), the only gene of the 25 to be expressed in the band cells. *barH* and *en* are both expressed in narrow ectodermal bands; *barH* in the anterior mesosome (Figures 4E and 4F) and *en* in the anterior metasome (Figures 4S and 4T). A dorsal view of both *en* and *barH* shows a dorsal narrow gap in expression in the midline. Ventrally, no such gap is observed (Figures 6D and 6E). Two additional spots of *en* expression are detected in the ectoderm on either side of the dorsal midline in the proboscis (Figure 6D). In the most posterior ring of *otx* expression in the metasome, a similar gap in expression is observed (Figure 6F). *otp* is expressed predominantly in a punctate pattern in the apical layer of prosome ectoderm and in a

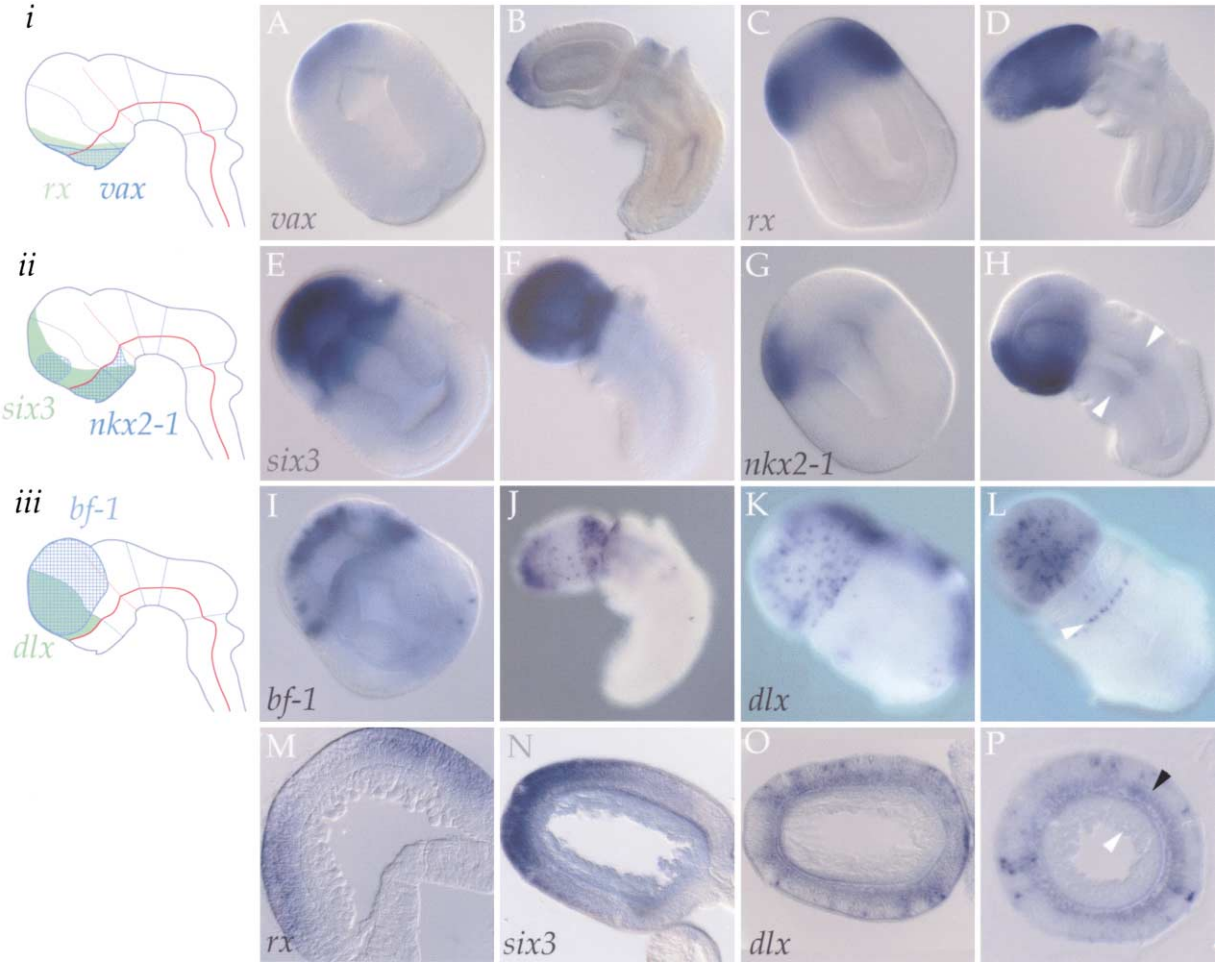

Figure 3. Neural Patterning Genes Expressed in the Chordate Forebrain Are Expressed in the Hemichordate Prosome

Diagrams i–iii (at left) of expression of developmental genes in mouse brain (stage 10.5 [see Rubenstein and Shimamura, 1997]). Solid red line represents division between basal and alar plates. Red dotted line corresponds to the *Zona Limitans Intrathalamica*, and blue dotted lines correspond to prosomere boundaries. (A–L) Whole-mount in situ hybridization (at right) showing expression of orthologous genes in *S. kowalevskii* embryos. Unless otherwise noted, all detectable expression is in ectodermal cells, and panels are optical sections, with anterior to the left. (A) Expression of *vax* in late gastrula in side view and (B) in neurula in side view. (C) Expression of *rx* in late gastrula in side view and (D) at one gill slit stage in side view. (E) Expression of *six3* in late gastrula in side view and (F) in one gill slit stage in side view. (G) Expression of *nkx2.1* in late gastrula side view and (H) at one gill slit stage in side view (arrowheads indicates a second domain of pharyngeal staining). (I) Expression of *bf1* in gastrula in dorsal view and (J) at one gill slit stage in side view, with the ectodermal surface in focus. (K) Expression of *dlx* of gastrula in side view and (L) in late neurula in ventral view, both with ectodermal surface in focus. Arrowhead shows a single line of cells expressing dlx in the anterior metasome. (M) Anterior frontal cryosection of embryo stained in whole mount for *rx*. (N) Sagittal cryosection of whole-mount one gill slit embryo stained for *six3*. (O) Frontal anterior cryosection of one gill slit embryo stained in whole mount for *dlx* through the proboscis. (P) Transverse cryosection through mid proboscis. White arrowhead shows mesoderm, and black arrowhead shows basiepitheilial position of *dlx* staining.

diffuse pattern in the basal layer of prosome ectoderm (Figures 4I and 4J), similar to *dlx*. It is also expressed in a circumferential ring of intermittant ectodermal cells in the posterior mesosome and then in two parallel lines of cells bilateral to the dorsal axon tract of the anterior metasome (Figure 4I). Early *dbx* expression is most strongly detected in an ectodermal ring in the developing mesosome (Figure 4K) overlapping the posterior domain of *tll* (Figure 4C). *dbx* is also expressed in the prosome at low levels throughout the ectoderm and at high levels in scattered individual cells or groups of cells. Later expression is restricted to two ectodermal bands marking the anterior and posterior limits of the mesosome (Figure 4L). An additional endodermal domain of expression is observed predominantly in the ventral anterior pharyngeal endoderm (Figure 4L).

otx, en, and *irx* deserve description in more detail because in chordates, especially vertebrates, the products of these regionally expressed genes are thought to interact in setting up the midbrain-hindbrain boundary and the isthmic organizer (Glavic et al., 2002). Furthermore, the *otx* domain at the midbrain level is the site from which neural crest cells migrate ventrally to the first branchial arch (Suda et al., 1999). In *S. kowalevskii*, *otx* is expressed at low but readily detectable levels in the prosome ectoderm and at high levels in four closely spaced ectodermal rings: one at the base of the prosome, two in the mesosome, and one in the anterior

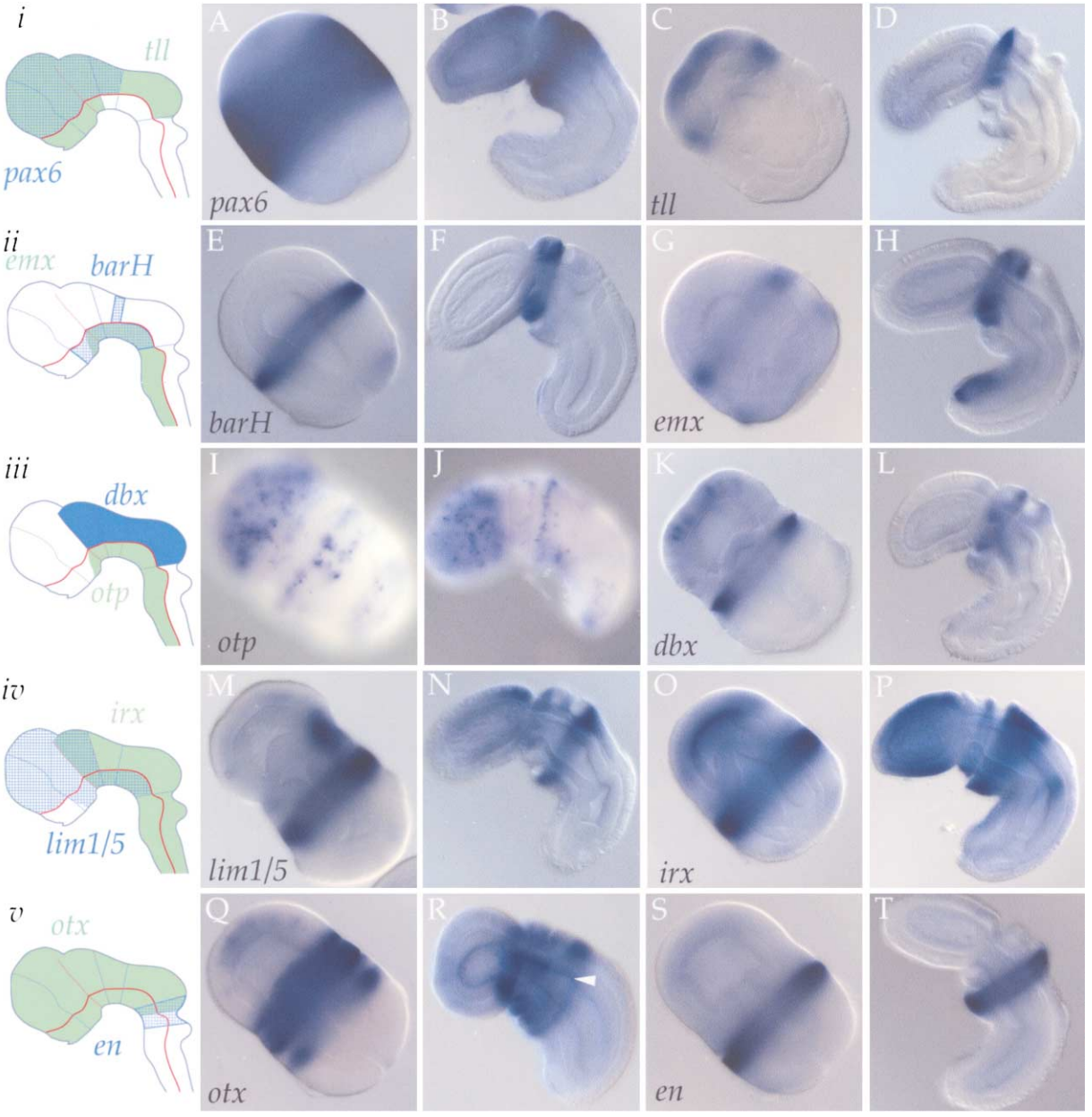

Figure 4. Neural Patterning Genes Expressed in the Chordate Midbrain Are Expressed in the Hemichordate Mesosome and Anterior Metasome

Diagrams i–v (at left) show the domains of expression of ten developmental genes in the mouse brain (stage 10.5 [see Rubenstein and Shimamura, 1997]). Solid red line represents division between basal and alar plates. Red dotted line corresponds to the *Zona Limitans Intrathalamica*, and blue dashed lines correspond to prosomere boundaries. Whole-mount in situ hybridization results of expression of orthologous genes in *S. kowalevskii* embryos are shown at the right. Unless otherwise noted, all detectable expression is in ectodermal cells. (A) Expression of *pax6* in gastrula in side view and (B) at one gill slit stage in side view. (C) Expression of *tll* in late gastrula in side view and (D) at one gill slit stage in side view. (E) Expression of *barH* in late gastrula in side view and (F) at one gill slit stage in side view. (G) Expression of *emx* in gastrula in side view and (H) at one gill slit stage in side view. (I) Expression of *otp* in early neurula in dorsal view (ectodermal, uncleared) and (J) at one gill slit stage in side view (ectodermal uncleared). (K) Expression of *dbx* at early neurula; dorsal view and (L) one gill slit side view. (M) Expression of *lim1/5* in neurula side view and (N) in one gill slit side view. (O) Expression of *irx* in late gastrula dorsal view and (P) in one gill slit side view; endodermal staining apparent in posterior gut. (Q) Expression of *otx* in early neurula side view and (R) in one gill slit; white arrow shows position of first gill slit. (S) Expression of *en* in early neurula in dorsal view and (T) in one gill slit side view.

metasome. This fourth stripe of *otx* expression crosses the site where the first gill slit perforates the ectoderm (Figures 4Q and 4R; enlargement in Figure 6B). As evidence, beyond morphology, that the hemichordate gill slit is homologous to the chordate gill slit/branchial arch, we find that the *pax1/9* ortholog, known to be expressed in chordate gill slits (Neubuser et al., 1995), is expressed in the endoderm of the developing *S. kowalevskii* gill slit (Figure 6A). Ogasawara et al. (1999) have reported gill slit expression of *pax1/9* in the adult of *P. flava*. Thus,

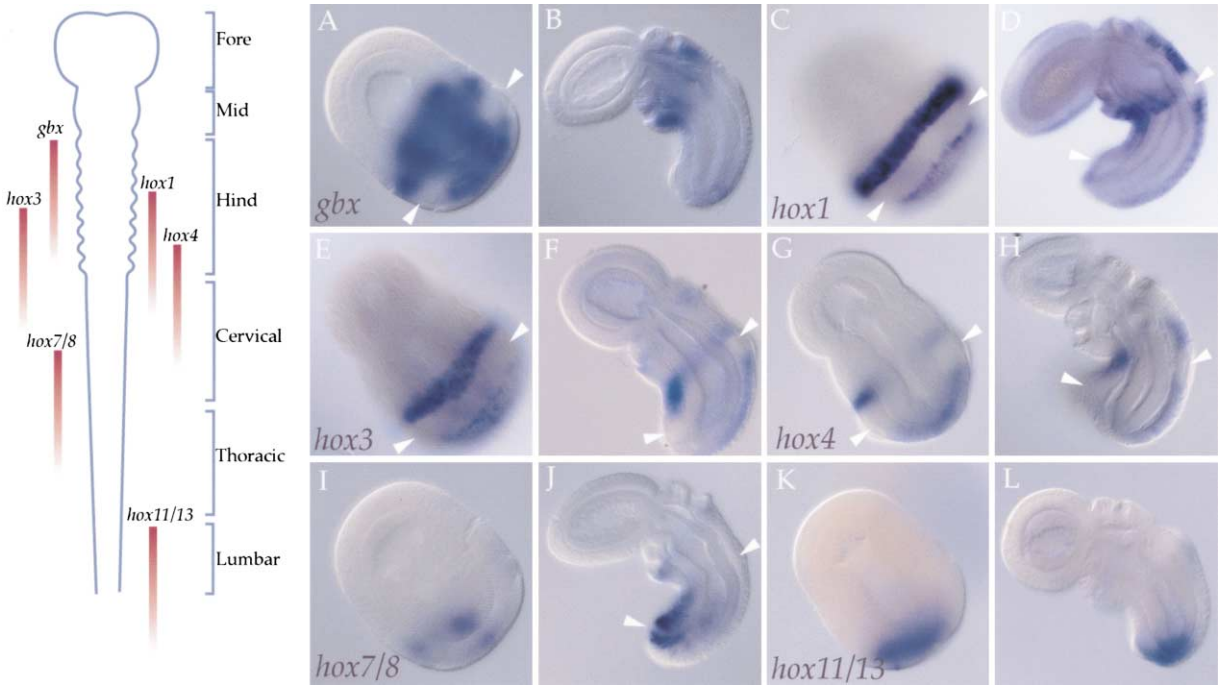

Figure 5. Neural Patterning Genes Expressed in the Hindbrain and Spinal Cord of Chordates Are Expressed in the Posterior Metasome of the Acorn Worm

On the left is a diagram of expression of neural patterning genes in the vertebrate hindbrain and spinal cord. On the right are whole-mount in situ hybridization results for expression of homologous genes in *S. kowalevskii* embryos. Unless otherwise noted, all detectable expression is in ectodermal cells. White arrowheads indicate the ciliary band cells of ectoderm. (A) Expression of *gbx* in gastrula in side view; endodermal expression is also detected at this early stage and (B) at one gill slit stage in side view. (C) *hox1* expression in gastrula in side view with ectodermal surface in focus and (D) at one gill slit stage in side view. (E) Expression of *hox3* in early neurula in ventral view with ectodermal surface in focus and (F) in neurula and one gill slit in side view. (G) Expression of *hox4* in early neurula in side view and (H) at one gill slit in side view. (I) Expression of *hox7/8* in gastrula in side view and (J) at one gill slit stage in side view. (K) Expression (ectodermal and endodermal) of *hox11/13* in gastrula in side view and (L) at one gill slit stage in side view.

chordates and hemichordates have in common the association of the posterior limit of the *otx* domain with the position of the first gill slit or branchial arch.

In hemichordates, the *en* domain overlaps the posterior part of the *otx* domain, and the *irx* domain runs through both of these, as is also the case in chordates (Figures 4O–4T). However, *otx* expression in *S. kowalevskii* extends slightly more posteriorly than does *en*, whereas in chordates the *en* domain extends slightly more posteriorly. The *gbx* gene is also thought to be involved; this will be discussed in the next section on posterior genes.

In summary of this midlevel group of genes, the *S. kowalevskii* orthologs are expressed in the mesosome and anterior metasome (with some domains extending anteriorly into the prosome), that is, more posteriorly than those genes of the anterior group. In general, expression domains that end posteriorly near the midbrain-hindbrain boundary in chordates, end in the anterior metasome in hemichordates. Although the anterior metasome is not the site of an obvious morphological boundary, it is the site of the first gill slit. The first gill slit/branchial arch in chordates is at the same body level as the midbrain-hindbrain boundary.

Posterior Neural Domains

Six genes were examined, namely *gastrulation brain homeobox* (*gbx*), *hox1*, *3*, *4*, *7/8, and 11/13*. As shown in Figure 5, all of these genes are expressed in chordate neurectoderm in major domains entirely within the hindbrain and spinal cord regions of the nervous system. *gbx* was chosen because in chordates, as noted above, it has a role in forming the midbrain-hindbrain boundary and in establishing the site of the isthmic organizer (in vertebrates) by way of a mutual antagonism of *gbx* and *otx* expression (Glavic et al. 2002). Its domain in chordates extends from the midbrain-hindbrain boundary back into the spinal cord (Rubenstein et al., 1998). In *Drosophila*, it may serve an analogous function, delineating a neural boundary and antagonizing otx expression (Hirth et al., 2003). This does not necessarily imply a structural homology between central nervous systems but, merely, a homologous use in anteroposterior patterning. In *S. kowalevskii*, the *gbx* ortholog is initially expressed in the entire metasome except for the ciliated telotroch region (Figure 5A). Later, the anterior metasome becomes the site of strong expression, and posterior expression diminishes (Figure 5B). An additional domain of *gbx* expression is detected only in early stages in the endoderm, with its anterior limit extending into the mesosome, beyond the anterior limit of the ectodermal domain (Figure 5A). The ectodermal domain of *gbx* overlaps anteriorly with both the *en* and *otx* domains (Figures 5A and 5B; Figures 4Q–4T), whereas in chordates *gbx* overlaps *en* partially, but not *otx* (which it antagonizes). *irx* expression overlaps *gbx, en*, and *otx* expression in both chordates and hemichordates. Thus, the contiguity

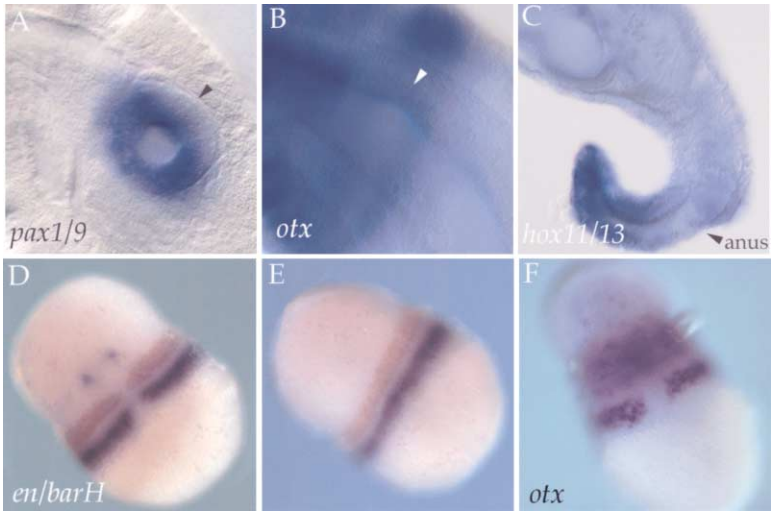

Figure 6. Selected Patterning Gene Expression Domains of *S. kowalevskii*

(A) Detail of expression of *pax1/9* in ring of endoderm cells surrounding the first gill slit.
(B) Ectodermal expression of *otx* ending just posterior to the first gill slit (indicated by white arrowhead).
(C) Expression of *hox11/13* in the ectoderm of the post-anal extension in two gill slit (hatched juvenile) stage.
(D) Expression of *en* (dark blue) and *barH* (brown) of late gastrula dorsal view focused on the ectoderm.
(E) Same specimen as in (D), showing a ventral view.
(F) Expression of *otx*, dorsal view of one gill slit embryo focused on the ectoderm.

of ectodermal domains of *gbx*, *en*, *otx*, and *irx* resembles that in chordates, though with some differences of overlap.

In chordates, the *hox* genes *1–9* are expressed in the hindbrain and spinal cord, but not more anteriorly (Carpenter, 2002). As is well known, their succession of anterior domain boundaries is colinear with the gene order in the chromosomal cluster (McGinnis and Krumlauf, 1992). *hox* genes *10–13* are expressed in the chordate post-anal tail (Figure 5A), whereas *hox* members *1–9* are not (Krumlauf et al., 1993; Carpenter 2002). We have assessed the expression of five *S. kowalevskii hox* genes, namely, *1, 3, 4, 7/8*, and *11/13*, all of which match homeobox sequence fragments found a decade ago in a pcr screen of *S. kowalevskii* (Pendleton et al., 1993). The first four are expressed in the metasome (Figures 5C–5J) posterior to the *otx* domain and posterior to the first gill slit, that is, fully within the *gbx* ectodermal domain. These four *hox* domains are circumferential and exclusively ectodermal. All become strongly expressed during gastrulation. *hox1, 3*, and *4* are expressed anteriorly to the telotroch. Initially, their anterior boundaries are too close together to resolve their relative order (Figures 5C, 5E, and 5G). Slightly later in development, the *hox1* domain can be discerned slightly anterior to the *hox3* and *4* domains and just posterior to the first gill slit (Figures 5D, 5F, and 5H). In still later stages, the *hox1* domain extends forward in a thin streak on the dorsal midline, to the mesosome/metasome boundary. This streak falls within a dorsal midline channel where *otx, en, and barH* are not expressed (Figures 6D–6F). The anterior boundaries of *hox3* and *4* remain very close in later stages. *hox4* is expressed more strongly on the ventral side and *hox3* more broadly on the ventral side than elsewhere in the ring of expression anterior to the telotroch (Figures 5E–5H). The *hox7/8* domain begins clearly posterior to *hox4* and just anterior to the telotroch (Figures 5I and 5J). All four *hox* domains are interrupted by the telotroch (white arrowheads in Figures 5A–5J), the thick band of cilia appearing in the midgastrula embryo and persisting through juvenile stages, and all four domains resume expression posterior to the telotroch, ending at the anus.

We have identified our most posterior *hox* gene as a *hox11/13* gene. It is expressed in a domain posterior to all other *hox* genes in this study (Figures 5K and 5L). The sequence is most similar to the sea urchin genes *HeHbox7* and *SpHbox7* (*hox11/13b*) and to the sea cucumber gene *HgHbox12* (Méndez et al., 2000), all of which are posterior members of the echinoderm Hox cluster. There remains uncertainty about whether the posterior group genes diversified before or after the divergence of the three deuterostome phyla (Popodi et al. 1996), so orthology relationships between the echinoderm and hemichordate clusters and chordates remain uncertain. In *S. kowalevskii* neurula stage embryos, the anterior boundary of *hox11/13* expression is entirely posterior to the telotroch and close to the blastopore in both ectoderm and endoderm (Figures 5K and 5L). In hatched juveniles, it is expressed exclusively within the ventral post-anal posterior sucker, also called the tail (Figure 6C). Thus, like the chordate *hox11/13* members of the Hox cluster, it is expressed in a post-anal territory of the body axis.

Thus, these six genes, which are orthologs of genes expressed in the chordate hindbrain and spinal cord, have prominent ectodermal expression domains in the metasome posterior to the first gill slit, that is, the most posterior body region of this hemichordate. Their expression is posterior to that of members of the anterior and midlevel groups of neural patterning genes. The five *S. kowalevskii hox* genes appear to be expressed in a domain order colinear with their *hox* numerical identity. We have not yet established their gene order in a Hox cluster.

Discussion

Conservation of the Domain Map between Chordates and Hemichordates

The 22 expression domains of orthologs of chordate neural patterning genes of *S. kowalevskii* correspond strikingly to those in chordates, as summarized in Figures 7B and 7D. There are differences such as the extent of overlap of edges of domains of *otx*, *en*, and *gbx* and other midlevel genes that are critical for forming

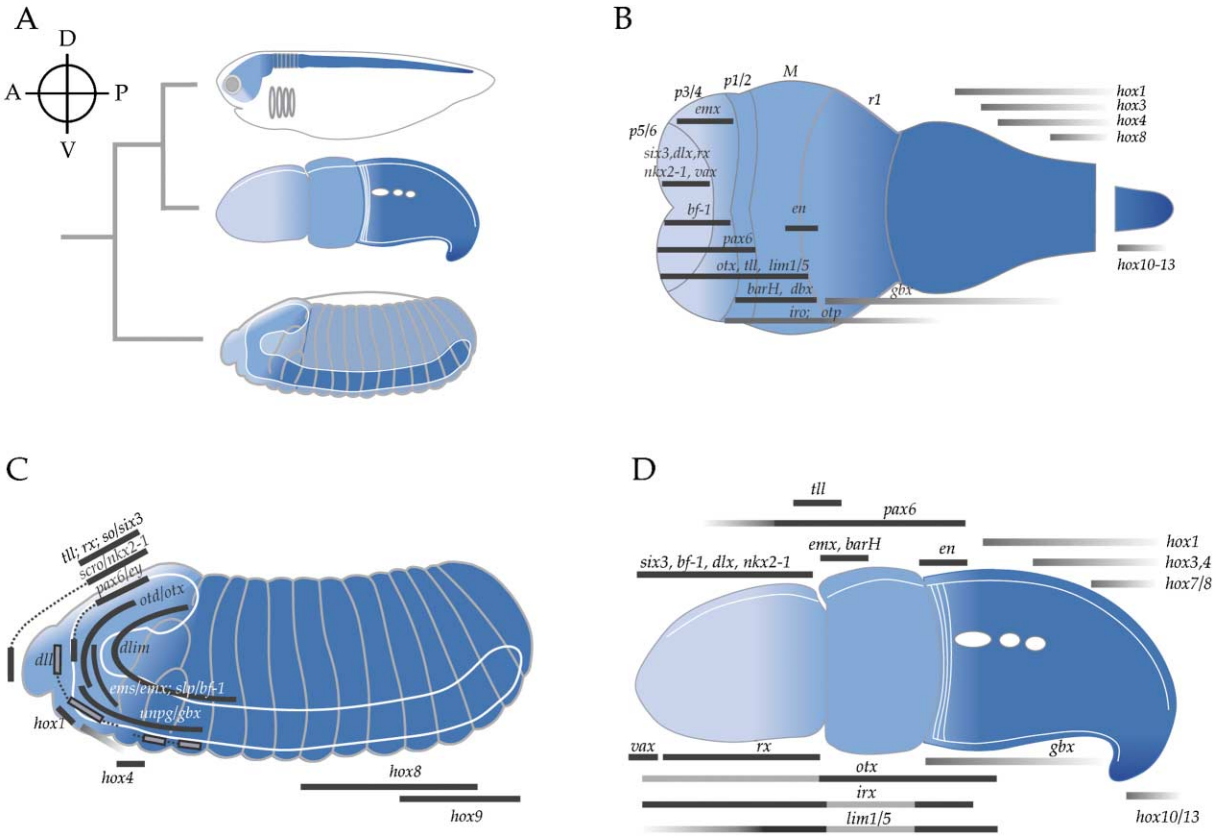

Figure 7. Comparison of the Neural Gene Domain Maps of Hemichordates, Chordates, and *Drosophila*

In addition to the individual gene domains, the blue color gradient in each panel is meant to indicate the general similarities when the gene expression domains are considered as a group. (A) Representation of the general organizational features of the central nervous systems of chordates and arthropods, and the diffuse nervous system of hemichordates arranged on a phylogram. The compass indicates the axial orientation of each model. (B) Representation of a dorsal view of a vertebrate neural plate (see Rubenstein and Shimamura, 1997). Abbreviations: p1/2, prosomeres 1 and 2; p3/4, prosomeres 3 and 4; p5/6, prosomeres 5 and 6; M, midbrain; and r1/2, rhobomeres 1 and 2. The discontinuous domain represents the post-anal territory of the nerve cord. All 22 expression domains are shown. (C) *Drosophila* late stage 12 embryo model with 14 expression domains shown (lateral view, postgermband retraction, before head involution). All models are positioned with anterior to the left. (D) The acorn worm (lateral view), with its diffuse nervous system, is shown with a blue color gradient of expression in the ectoderm; the anterior domains, the midlevel domains, the posterior domains, and the post-anal territory are color matched to the anteroposterior dimension of the chordate model.

boundaries within the chordate brain, but the relative domain locations are nonetheless very similar. This similar topography of domains is most parsimoniously explained by conservation in both lineages of a domain arrangement (a map) already present in the common ancestor, the ancestor of deuterostomes. An alternate explanation of the similar maps as due to the multiple cooption of 22 individual genes into a convergent topology is not likely, though convergence might be possible for a few genes.

At least 14 of the 22 conserved domains have similar locations in one or more protostome groups (Supplemental Table S1). Such similarities are most parsimoniously explained as a conservation of domains from the ancestral bilaterian. In the case of the *hox* genes, *otx*, *emx*, *pax6*, *six3*, *gbx*, and *tll*, there is strong evidence for such conservation (Reichert and Simeone, 2001), but less so for the others (*barH* and *rx*). At least four of the chordate-hemichordate conserved domains may not be shared by protostomes. Namely, three of these genes (*dbx*, *vax*, and *hox11/13*) are absent from the *Drosophila* genome and have not been cloned from other protostome groups. Also, one gene, *engrailed*, has no clear corresponding domain of expression known in protostomes. In *Drosophila*, *en* is expressed in the posterior compartments of 14 body segments (Karr et al., 1989) and at three or more sites in the head that probably derive from ancient preoral segments (Schmidt-Ott and Technau, 1992). This pattern for *en* appears very different from the single ectodermal band in deuterostomes (Joyner, 1996).

The remaining four genes of uncertain conservation between deuterostomes and protostomes (Supplemental Table S1) are ambiguous regarding their shared expression. In the case of *dlx*, although several of its developmental roles may have been conserved between arthropods and chordates, it remains unclear if its role in anterior central nervous system development has been conserved (Panganiban and Rubenstein, 2002). *otp* is unusual in chordates because it is locally expressed in the anterior central nervous system, whereas in *Drosophila*, it is expressed widely in the gut and central

nervous system without anterior restriction (Simeone et al., 1994). *lim1/5* is expressed throughout the ventral nervous system and developing brain in *Drosophila* (Lilly et al., 1999) rather than an anterior specific domain. Finally, *iroquois* genes in vertebrates are expressed early in the neurectoderm, but in *Drosophila* their expression occurs much later during imaginal disc formation (Cavodeassi et al., 2001). In addition, vertebrate and *Drosophila* clusters of *iroquois* genes may have evolved independently (Gomez-Skarmeta and Modolell, 2002). Thus, some of these domains shared between hemichordates and chordates, but only ambiguously by protostomes, may represent real deuterostome-specific patterns of expression. Alternatively, some of the domains may yet be found in more basal protostomes.

Although the domain maps between hemichordates and chordates are similar in the anteroposterior dimension, they differ in the dorsoventral dimension. Most hemichordate domains encircle the ectoderm of the body as bands and rings with little or no dorsoventral difference, similar to the early expression in *Drosophila*, whereas the chordate domains are restricted to a dorsal subregion of ectoderm, the neural plate and tube, within which they display further dorsoventral differences. These differences of dorsoventral extent reflect the differences of the nervous systems themselves. The hemichordate system is a body-encircling basiepithelial nerve net, while the chordate central nervous system arises from a neurogenic subregion of ectoderm. Arthropods such as *Drosophila* present an intermediate situation (Jan and Jan, 1993). Within the ectoderm are two major regions: (1) the ventral neurogenic ectoderm, from which derive not only motoneurons and interneurons of the ventral nerve cord, but also from which derive ventral epidermal ectoderm cells, and (2) the dorsal nonneurogenic ectoderm, from which derives not only dorsal epidermal ectoderm, but also all the sensory neurons (e.g., those of the bristles and sensilla). Thus, arthropods don't have a fully nonneurogenic region of ectoderm as do chordates, and they express many of these genes in body-encircling bands, like hemichordates.

Nonconservation of Nervous System Morphology

The hemichordate body, while innervated by a diffuse nervous system of rather little apparent morphological complexity, has a chordate-like array of expression domains of 22 orthologs of neural patterning genes (all those tested so far), which in chordates are involved in the anteroposterior patterning of the morphologically complex central nervous system. The organization of gene expression domains such as these (the organism's second anatomy [Slack et al., 1993]) is much more conserved across bilateria than is overt morphology. This conclusion was already inescapable from the previous work of others comparing distantly related and morphologically diverse organisms possessing the same *hox* expression patterns.

The nervous systems of hemichordates and chordates are so different morphologically that it has been difficult to make valid comparisons. This study provides a rational basis for investigating the possibility of structural homologies between the two groups by restricting direct morphological comparisons to regions that develop from the same expression domains of the two maps, as shown in Figure 7. At this stage in our analysis, we do not suggest any structural homologies of the respective nervous systems of the two groups but do call attention to corresponding parts that evolved from the same domains of the deuterostome ancestor. Thus, from the body region of the anterior group of neural domains of the deuterostome ancestor, *S. kowalevskii* has evolved the nerve net of the prosome, whereas chordates have evolved the forebrain, particularly the ventral forebrain (subpallium). From the body region of the midlevel group of domains of the ancestor, the hemichordate line evolved the nerve net and epidermis of the mesosome and anterior metasome through the first gill slit. Chordates, on the other hand, evolved the dorsal forebrain and midbrain from this same midlevel domain region (Figure 7). From the posterior domains of the ancestor, the hemichordate line evolved the diffuse nervous system of the posterior metasome, whereas in chordates these domains evolved to the hindbrain (rhombomeres 2–8) (Tallafuss and Bally-Cuif, 2002) and spinal cord, as well as adjacent neural crest cells in vertebrates. The *hox11/13* domain is particularly interesting. In the hemichordate line, this domain of the ancestor evolved to a ventral post-anal tail-like extension, sometimes called the posterior sucker, which is contractile, ciliated, and mucous secreting. In chordates, the *hox10–13* domains of the ancestor evolved into the dorsal post-anal tail, a defining trait of the chordate phylum.

The Ancestral Nervous System and Chordate Origins

The nerve net of hemichordates could represent the basal condition of the deuterostome ancestor, or it could represent the secondary loss of a central nervous system from an ancestor. Was the complex map of the ancestor associated with a complex diffuse nerve net or a central nervous system in the ancestor? We suggest that the deuterostome ancestor may have had a diffuse basiepithelial nervous system with a complex map of expression domains, though not necessarily a diffuse net exactly like that of extant hemichordates. Hemichordates would then have retained a diffuse system in their lineage and early in the chordate lineage, centralization would have taken place. In this proposal, the domain map predates centralization and is carried into the nervous system. In this respect, the core questions of nervous system evolution would concern the modes of centralization utilized by the ancestor's various descendents rather than a dorsoventral inversion, per se. Thus, we would propose that in chordates, especially vertebrates, the major innovation may have been the formation of a large contiguous nonneural (epidermogenic) region. In this view, both the Bateson hypothesis, which assumed the persistence of a centralized nervous system in hemichordates, and the inversion hypothesis, which assumed the persistence of an inverted centralized nervous system, would be invalid.

Several arguments support a deuterostome ancestor with a diffuse basiepithelial nerve net. (1) The phyla that are potentially relevant outgroups to the bilateria, namely the ctenophores and cnidarians, have diffuse nervous systems, both ectodermal and endodermal (Brusca and

Brusca 1990); hence the stem bilateria presumably started from this diffuse condition. (2) Bilateral animals that are considered possible sister groups of extant deuterostomes and protostomes also have diffuse nerve nets. These are the acoels, nemertodermatids (Reuter et al., 1998; Ruiz-Trillo et al., 2002), xenoturbellids (Raikova et al., 2000), and chaetognaths (Papillon et al., 2003). These are all free-living animals, not ones for which a secondary loss of a central nervous system can be attributed to the animal's parasitic or sessile lifestyle. (3) Bullock (1965) considered that the hemichordate nervous system is hardly more complex morphologically than the nets of radial organisms. (4) Echinoderms, the other deuterostome phylum, also have a basiepithelial plexus. (5) From molecular phylogeny, the deuterostomes have been proposed as the most basal bilaterian lineage (Peterson and Eernisse, 2001), and if true, deuterostome traits, not protostome traits, may be basal to the bilateria. (6) Chordates have a basiepithelial central nervous system in which neuron cell bodies remain in the neurectoderm of the neural tube. Thus, it is rather different from the subepithelial central nervous system of many protostomes in which neuron precursors migrate out of the ectoderm into the space between ectoderm and mesoderm (reviewed in Arendt and Nübler-Jung, 1996). Although we raise the possibility of a diffuse nerve net in the deuterostome ancestor, evidence is still equivocal. Identification and characterization of relevant outgroups are of paramount importance in resolving this issue. The analysis of dorsoventral neural gene expression domains may also be required since centralization and also decentralization seem mostly to be modifications in this dimension.

Our data also do not support the Garstang (1894, 1928) hypothesis, which proposed that the chordate central nervous system evolved from the ciliated band and associated nerve cells of an ancestral deuterostome larva. By contrast, we find an extensive domain map in the adult and no evidence from the work of others for such a domain map in larvae. For example, most *hox* genes are not expressed during the formation of the larval body plan of sea urchins, and those that are expressed do not exhibit colinearity along the anteroposterior axis of the larva (Arenas-Mena et al., 2000).

The deuterostome ancestor we propose, with its complex anteroposterior organization but diffuse nervous system, may already have had some other differentiated characteristics of the chordate lineage, including a post-anal extension and gill slits, under the regulation of a conserved set of patterning genes. Our findings lend support to the use of certain morphological criteria, such as gill slits and, perhaps, post-anal body parts, to identify deuterostome ancestors in recently discovered Early Cambrian deposits (Jefferies et al., 1996; Shu et al., 2003). In general though, the conserved domain map appears very weakly linked to the particular morphologies of different evolutionary lines. Although the ancestor of bilateral animals probably had complex anteroposterior organization based on many of these domains, this organization set few limits on morphology and cytodifferentiation in subsequent evolution. The existence of a modern hemichordate with a highly patterned but diffuse nerve net suggests that the nervous system may be very plastic in its evolutionary possibilities and that its exact neuroanatomy may tell us little about the early branching of metazoan phyla.

Experimental Procedures

Eggs, Embryos, and Juveniles

Adult *S. kowalevskii* were collected intertidally in September near Woods Hole, MA. Ovulation and fertilization were achieved in the laboratory by the methods of Colwin and Colwin (1950, 1962), with several modifications (Lowe et al., in press). Embryos were staged by the normal tables of Bateson (1884, 1885, 1886a) and Colwin and Colwin (1953).

Library Construction

Two libraries were used in this study, one from mixed blastula and gastrula stages and another from mixed gastrula and neurula stages. For each, 800–1000 embryos were rapidly frozen with liquid nitrogen. Total RNA was isolated using TRIzol reagent (Invitrogen). Poly(A)$^+$ RNA was then isolated by two rounds of oligo(dT) selection with oligo(dT)-coated magnetic particles (Seradyn, Inc.). The SuperScript Plasmid System (Invitrogen) was used to generate cDNA libraries from this mRNA.

The gastrula/neurula library (22×10^6 primary clones) and the blastula/gastrula library (50×10^6 primary clones) were each produced from single bulk ligations. The cDNA inserts from 23 randomly picked clones from each of the libraries were sized using PCR and SP6 and T7 primers. The average insert was 1.9 kb (blastula/gastrula) and 1.7 kb (gastrula/neurula).

Cloning of Orthologs

Three strategies were used. (1) We surveyed 30,000 EST clones from the two libraries. (2) We screened cDNA libraries at low stringency using short probes complementary to highly conserved regions of orthologs from other deuterostomes. Alternatively, end-labeled degenerate oligo probes were used. (3) We designed degenerate primers (Codehop) used for PCR assay of ortholog sequences in arrayed aliquots of the cDNA libraries.

In Situ Hybridization

The whole-mount in situ hybridization protocol was based on Salic et al. (1997) and is outlined in the Supplemental Data and in Lowe et al. (in press). For the preparation of sections, heavily stained embryos were embedded in 2.5% agarose-5%sucrose and blocks were soaked in 30% sucrose overnight. The blocks were mounted with tissue freezing medium (Triangle Biomedical) in a cryostat and sectioned at 25 μm.

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References

Adoutte, A., Balavoine, G., Lartillot, N., Lespinet, O., Prud'homme. B., and de Rosa, R. (2000). The new animal phylogeny: reliability and implications. Proc. Nat. Acad. Sci. USA *97*, 4453–4456.

Arenas-Mena, C., Cameron, A.R., and Davidson, E.H. (2000). Spatial expression of Hox cluster genes in the ontogeny of a sea urchin. Development *127*, 4631–4643.

Arendt, D., and Nübler-Jung, K. (1996). Common ground plans in early brain development in mice and flies. Bioessays *18*, 255–259.

Balser, E.J., and Ruppert, E.E. (1990). Ultrastructure and function of the preoral heart-kidney in *Saccoglossus kowalevskii* (Hemichordate; Enteropneusta) including new data on the stomochord. Acta Zool. *71*, 235–249.

Bateson, W. (1884). The early stages in the development of *Balanoglossus* (sp. Incert.). Quart. J. Microscop. Sci. *24*, 208–236.

Bateson, W. (1885). The later stages in the development of *Balanoglossus kowalevskii*, with a suggestion as to the affinities of the enteropneusta. Quart. J. Microscop. Sci. *25*, 81–128.

Bateson, W. (1886a). Continued account of the later stages in the development of *Balanoglossus kowalevskii*, and of the morphology of the enteropneusta. Quart. J. Microscop. Sci. *26*, 511–534.

Bateson, W. (1886b). The ancestry of the chordata. Quart. J. Microscop. Sci. *26*, 535–571.

Brusca, R.C., and Brusca, G.J. 1990. Invertebrates (Sunderland, MA: Sinauer).

Bullock, T.H. (1946). The anatomical organization of the nervous system of enteropneusta. Quart. J. Microscop. Sci. *86*, 55–112.

Bullock, T.H. (1965). The nervous system of hemichordates. In Structure and Function in the Nervous Systems of Invertebrates, T.H. Bullock and G.A. Horridge, eds. (San Francisco: WH Freeman and Co), pp.1567–1577.

Burdon-Jones, C. (1952). Development and biology of the larva of *Saccoglossus horsti* (enteropneusta). Proc. R. Soc. Lond. B Biol. Sci. *236*, 553–589.

Cameron, C.B., and Mackie, G.O. (1996). Conduction pathways in the nervous system of Saccoglossus sp. (Enteropneusta). Can. J. Zool. *74*, 15–19.

Cameron, C.B., Garey, J.R., and Swalla, B.J. (2000). Evolution of the chordate body plan: new insights from phylogenetic analyses of deuterostome phyla. Proc. Natl. Acad. Sci. USA *97*, 4469–4474.

Carpenter, E.M. (2002). Hox genes and spinal cord development. Dev. Neurosci. *24*, 24–34.

Cavodeassi, F., Modolell, J., and Gomez-Skarmeta, J.L. (2001). The Iroquois family of genes: from body building to neural patterning. Development *128*, 2847–2855.

Colwin, A.L., and Colwin, L.H. (1950). The developmental capacities of separated early blastomeres of an enteropneust, *Saccoglossus kowalevskii*. J. Exp. Zool. *115*, 263–296.

Colwin, A.L., and Colwin, L.H. (1953). The normal embryology of *Saccoglossus kowalevskii*. J. Morphol. *92*, 401–453.

Colwin, L.H., and Colwin, A.L. (1962). Induction of spawning in *Saccoglossus kowalevskii* (Enteropneusta) at Woods Hole. Biol. Bull. *123*, 493.

De Robertis, E.M., and Sasai, Y. (1996). A common plan for dorsoventral patterning in bilateria. Nature *380*, 37–40.

Garstang, W. (1894). Preliminary note on a new theory of the phylogeny of the chordata. Zool. Anzeiger *22*, 122–125.

Garstang, W. (1928). The morphology of the Tunicata. Quart. J. Microscop. Sci. *72*, 51–189.

Gee, H. (1996). Before the Backbone: Views on the Origin of the Vertebrates (London: Chapman & Hall).

Geoffroy-St. Hilaire, E. (1822). Considérations générales sur les vertebrés. Mem. Hist. Nat. *9*, 89–119.

Glavic, A., Gomez-Skarmeta, J.L., and Mayor, R. (2002). The homeoprotein Xiro1 is required for midbrain-hindbrain boundary formation. Development *129*, 1609–1621.

Gomez-Skarmeta, J.L., and Modolell, J. (2002). *Iroquois* genes: genomic organization and function in vertebrate neural development. Curr. Opin. Genet. Dev. *12*, 403–408.

Hirth, F., Kammermeier, L., Frei, E., Walldorf, U., Noll, M., and Reichert, H. (2003). An urbilaterian origin of the tripartite brain: developmental genetic insights from Drosophila. Development *130*, 2365–2373.

Jan, Y.N., and Jan, L.Y. (1993). The peripheral nervous system. In The Development of *Drosophila melanogaster*. M. Bate and A.M. Arias, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 1207–1244.

Jefferies, R.P.S., Brown, N.A., and Daley, P.E.J. (1996). The early phylogeny of chordates and echinoderms and the origin of chordate left-right asymmetry and bilateral symmetry. Acta Zool. *77*, 101–122.

Joyner, A.L. (1996). Engrailed, Wnt and Pax genes regulate midbrain-hindbrain development. Trends Genet. *12*, 15–20.

Kaneko, Y., Sakakibara, S., Imai, T., Suzuki, A., Nakamura, Y., Sawamoto, K., Ogawa, Y., Toyama, Y., Miyata, T., and Okano, H. (2000). *Musashi1*: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev. Neurosci. *22*, 139–153.

Karr, T.L., Weir, M.P., Ali, Z., and Kornberg, T. (1989). Patterns of engrailed protein in early Drosophila embryos. Development *105*, 605–612.

Kim, Y.J., and Baker, B.S. (1993). The Drosophila gene rbp9 encodes a protein that is a member of a conserved group of putative RNA binding proteins that are nervous system-specific in both flies and humans. J. Neurosci. *13*, 1045–1056.

Knight-Jones, E. (1952). On the nervous system of *Saccoglossus cambriensis* (Enteropneusta). Philos. Trans. R. Soc. Lond. B Biol. Sci. *236*, 315–354.

Krumlauf, R., Marshall, H., Studer, M., Nonchev, S., Sham, M.H., and Lumsden, A. (1993). Hox homeobox genes and regionalization of the nervous system. J. Neurobiol. *24*, 1328–1340.

Lacalli, T.C. (1994). Apical organs, epithelial domains, and the origin of the chordate central nervous system. Am. Zool. *34*, 533–541.

Lilly, B., O'Keefe, D.D., Thomas, J.B., and Botas, J. (1999). The LIM homeodomain protein dLim1 defines a subclass of neurons within the embryonic ventral nerve cord of *Drosophila*. Mech. Dev. *88*, 195–205.

Lowe, C.J., Tagawa, K., and Humphreys, T. Kirschner, M., and Gerhart, J. (in press). Hemichordate embryos: procurement, culture, and basic methods. In Methods in Cell Biology, G.A. Wray, C. Ettensohn, and G. Wessel, eds. (San Diego: Elsevier Press).

McGinnis, W., and Krumlauf, R. (1992). Homeobox genes and axial patterning. Cell *68*, 283–302.

Méndez, A.T., Roig-Lopez, J.L., Santiago, P., Santiago, C., and Garcia-Arraras, J.E. (2000). Identification of hox gene sequences in the sea cucumber *Holothuria glaberrima* Selenka (Holothuroidea: Echinodermata). Mar. Biotechnol. *2*, 231–240.

Morgan, T.H. (1894). Development of *Balanoglossus*. J. Morphol. *9*, 1–86.

Neubuser, A., Koseki, H., and Balling, R. (1995). Characterization and developmental expression of Pax9, a paired-box-containing gene related to Pax1. Dev. Biol. *170*, 701–716.

Nielsen, C. (1999). Origin of the chordate central nervous system - and the origin of chordates. Dev. Genes Evol. *209*, 198–205.

Nübler-Jung, K., and Arendt, D. (1999). Dorsoventral axis inversion: Enteropneust anatomy links invertebrates to chordates turned upside down. J. Zool. Systematics & Evol. Res. *37*, 93–100.

Ogasawara, M., Wada, H., Peters, H., and Satoh, N. (1999). Developmental expression of *pax1/9* genes in urochordate and hemichordate gills: insight into function and evolution of the pharyngeal epithelium. Development *126*, 2539–2550.

Panganiban, G., and Rubenstein, J.L.R. (2002). Developmental functions of the Distal-less/Dlx homeobox genes. Development *129*, 4371–4386.

Papillon, D., Perez, Y., Fasano, L., Le Parco, Y., and Caubit, X. (2003). *Hox* gene survey in the chaetognath *Spadella cephaloptera*: evolutionary implications. Dev. Genes Evol. *213*, 142–148.

Pendleton, J.W., Nagai, B.K., Murtha, M.T., and Ruddle, F.H. (1993). Expansion of the Hox gene family and the evolution of chordates Proc. Natl. Acad. Sci. USA *90*, 6300–6304.

Peterson, K.J., and Eernisse, D.J. (2001). Animal phylogeny and the ancestry of bilaterians: inferences from morphology and 18S rDNA gene sequences. Evol. Dev. *3*, 170–205.

Popodi, E., Kissinger, J.C., Andrews, M.E., and Raff, R.A. (1996). Sea urchin Hox genes: insights into the ancestral Hox cluster. Mol. Biol. Evol. *13*, 1078–1086.

Raikova, O.I., Reuter, M., Jondelius, U., and Gustafsson, M.K.S. (2000). An immunocytochemical and ultrastructural study of the nervous and muscular systems of *Xenoturbella westbladi* (Bilateria inc. sed.) Zoomorphology *120*, 107–118.

Reichert, H., and Simeone, A. (2001). Developmental genetic evidence for a monophyletic origin of the bilaterian brain. Philos. Trans. R. Soc. Lond. B Biol. Sci. *356*, 1533–1544.

Reuter, M., Raikova, O.I., and Gustafsson, M.K.S. (1998). An endocrine brain? The pattern of FMRF-amide immunoreactivity in Acoela (Plathelminthes) Tissue Cell *30*, 57–63.

Rubenstein, J.L.R., and Shimamura, K. (1997). Regulation of patterning and differentiation in the embryonic vertebrate forebrain. In Molecular and Cellular Approaches to Neural Development, W.M. Cowan, T.M. Jessell, and S.L. Zipursky eds. (Cambridge: Oxford University Press), pp. 356–390.

Rubenstein, J.L.R., Shimamura, K., Martinez, S., and Puelles, L. (1998). Regionalization of the prosencephalic neural plate. Annu. Rev. Neurosci. *21*, 445–477.

Ruiz-Trillo, I., Paps, J., Loukota, M., Ribera, C., Jondelius, U., Baguna, J., and Riutort, M. (2002). A phylogenetic analysis of myosin heavy chain type II sequences corroborates that Acoela and Nemertodermatida are basal bilaterians. Proc. Natl. Acad. Sci. USA *99*, 11246–11251.

Salic, A.N., Kroll, K.L., Evans, L.M., and Kirschner, M.W. (1997). *Sizzled*: a secreted *Xwnt8* antagonist expressed in the ventral marginal zone of *Xenopus* embryos. Development *124*, 4739–4748.

Sasai, Y. (2001). Roles of Sox factors in neural determination: conserved function signaling in evolution? Int. J. Dev. Biol. *45*, 321–326.

Schmidt-Ott, U., and Technau, G.M. (1992). Expression of *en* and *wg* in the embryonic head and brain of Drosophila indicatres a refolded band of seven segment remnants. Development *116*, 111–125.

Shimamura, K., Hartigan, D.J., Martinez, S., Puelles, L., and Rubenstein, J.L.R. (1995). Longitudinal organization of the anterior neural plate and neural tube. Development *121*, 3923–3933.

Shu, D.G., Conway Morris, S., Han, J., Zhang, Z.-F., Yasui, K., Janvier, P., Chen, L., Zhang, X.-L., Liu, J.-N., and Liu, H.-Q. (2003). Head and backbone of the early Cambrian vertebrate *Haikouichthys*. Nature *421*, 526–529.

Simeone, A., D'Apice, M.R., Nigro, V., Casanova, J., Graziani, F., Acampora, D., and Avantaggiato, V. (1994). *Orthopedia*, a novel homeobox-containing gene expressed in the developing CNS of both mouse and Drosophila. Neuron *13*, 83–101.

Slack, J.M.W., Holland, P.W., and Graham, C.F. (1993). The zootype and the phylotypic stage. Nature *361*, 490–492.

Suda, Y., Nakabayashi, J., Matsuo, I., and Aizawa, S. (1999). Functional equivalency between *Otx2* and *Otx1* in development of the rostral head. Development *126*, 743–757.

Sussel, L., Marin, O., Kimura, S., and Rubenstein, J.L.R. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. Development *126*, 3359–3370.

Takacs, C.M., Moy, V.N., and Peterson, K.J. (2002). Testing putative hemichordate homologues of the chordate dorsal nervous system and endostyle: expression of NK2.1 (TTF-1) in the acorn worm *Ptychodera flava* (Hemichordata, Ptychoderidae). Evol. Dev. *4*, 405–417.

Tallafuss, A., and Bally-Cuif, L. (2002). Formation of the head-trunk boundary in the animal body plan: an evolutionary perspective. Gene *287*, 23–32.

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