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The evolution of the serotonergic nervous system

Anders Hay-Schmidt

Department of Medical Anatomy B, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 N, Denmark (a.hay@server.mai.ku.dk)

The pattern of development of the serotonergic nervous system is described from the larvae of ctenophores, platyhelminths, nemerteans, entoprocts, ectoprocts (bryozoans), molluscs, polychaetes, brachiopods, phoronids, echinoderms, enteropneusts and lampreys. The larval brain (apical ganglion) of spiralian protostomes (except nermerteans) generally has three serotonergic neurons and the lateral pair always innervates the ciliary band of the prototroch. In contrast, brachiopods, phoronids, echinoderms and enteropneusts have numerous serotonergic neurons in the apical ganglion from which the ciliary band is innervated. This pattern of development is much like the pattern seen in lamprey embryos and larvae, which leads the author to conclude that the serotonergic raphe system found in vertebrates originated in the larval brain of deuterostome invertebrates. Further, the neural tube of chordates appears to be derived, at least in part, from the ciliary band of deuterostome invertebrate larvae. The evidence shows no sign of a shift in the dorsal–ventral orientation within the line leading to the chordates.

Keywords: serotonergic nervous system; neurons; invertebrates; phylogeny; development; apical ganglion

1. INTRODUCTION

Serotonin is an ancient neurotransmitter found throughout the animal kingdom, indicating an early origin of a nervous system using this neurotransmitter. More specifically, it suggests that the basic organization of the central part of the vertebrate serotonergic nervous system may already have been established in the line of invertebrate ancestors leading up to vertebrates. The serotonergic neurons in the vertebrate brain are located exclusively in the raphe system, giving rise to both ascending and descending projections, and serotonin is involved in several basal brain functions including the control of sleep, circadian rhythms, feeding and emotional state (Wilkinson & Dourish 1991). In some invertebrates (bivalves) serotonin is involved in the regulation of ciliary activity in the ciliary bands, where it acts to increase the frequency of the ciliary beat (Aiello 1974; Murakami 1983, 1987; Paparo 1986). Several authors have proposed theories about the origin of the vertebrate central nervous system (see the review in Lacalli *et al.* (1994)). The theory of Garstang (1894) is the most relevant one in relation to the evolution of the serotonergic part of the central nervous system. He proposed that the ciliary bands found in echinoderm and enteropneust larvae have been transformed during evolution into the neural tube of vertebrates. Later studies on the neural tube in the cephalochordate amphioxus revealed anatomical and genetic markers which have apparent homologues in both the ciliary bands of non-chordate deuterostomes and in the vertebrate brain (Holland *et al.* 1992; Lacalli *et al.* 1994). According to Nelson (1978) and Bonde (1984), the early embryonic stages in chordates and invertebrates should show the plesiomorphic (primitive) state of a character compared with the apomorphic state appearing later in development. Thus, observations on early free-swimming larval stages in different animal phyla should allow the evolution of the serotonergic nervous system to be followed. In addition, it should be possible to test the hypothesis of Garstang (1894) that ciliary bands have been transformed into the neural tube since serotonergic neurons must then be associated in some way with the ciliary bands.

Invertebrate larvae fall into three rather distinct larval categories: the planula larvae, the trochophore larvae of typical spiralian protostomes and the dipleurula type of some of the non-spiralian invertebrates (deuterostomes) (for a description and literature see Korschelt (1936) and for a review see Nielsen (1995)). Consensus holds that the metazoan phyla can be grouped into more or less natural groups (the diploblastic sponges placozoa, cnidarians and ctenophores and the triploblastic, non-spiralian and spiralian invertebrates and chordates) and that the chordates had their origin within one of the non-spiralian invertebrates. Furthermore, a shift in the orientation of the dorsal–ventral axis is thought to have occurred in the branch leading to the chordates (Arendt & Nübler-Jung 1999). On the basis of the genomic organization of the *Hox* gene cluster, including several protostome and deuterostome groups, De Rosa *et al.* (1999) concluded that the brachiopods belong to the spiralian phyla, in accordance with the conclusions generally drawn from 18S rDNA sequences (Halanych *et al.* 1995; Cohen *et al.* 1998). However, the molecular, phylogenetic evidence from 18S rDNA sequences is equivocal and it certainly does not give any conclusive answers about the protostome–deuterostome relationship (Phillipe *et al.* 1994; Zrzavy *et al.* 1998; Adoutte *et al.* 1999). In the present study, the larvae (i.e. the hatching stage) of ctenophores, flatworms, nemerteans, ectoprocts (bryozoans), entoprocts, annelids, molluscs, phoronids, brachiopods, echinoderms and enteropneusts and pre-hatching embryos and larvae of lampreys were analysed for serotonin immunoreactivity. The pattern of development and structure of the central serotonergic nervous system has been used to compare that specific part of the nervous system throughout the animal kingdom.

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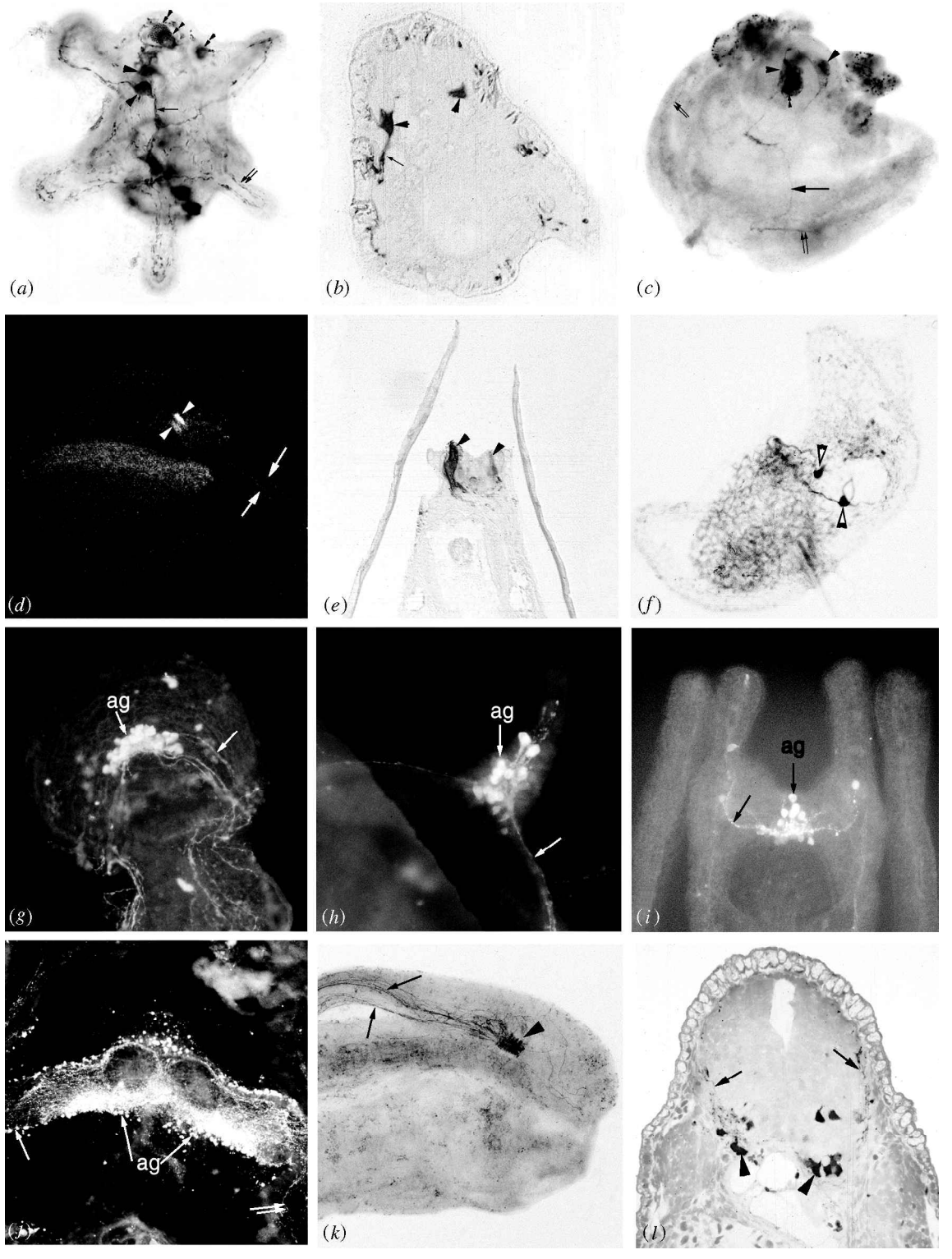

Figure 1. (*a–l*) Serotonin immunoreactivity in the larval nervous system. The arrow heads point to serotonergic cell bodies in the apical ganglion (ag) or neural tube, double arrow heads point to eyes, arrows point to processes from the apical ganglion and double arrows point to ciliary band innervation. (*a*,*b*) Platyhelminths—*S. sanjuana* whole mount (*a*) lateral view and (*b*) 2 µm frontal section. (*c*) Entoproct—*L. pectinaricola* whole mount lateral view. (*d*,*e*) Ectoproct—*Membranipora* sp. detail of apical ganglion in whole mount lateral aspect (*d*) anterior to the right and (*e*) 2 µm frontal section of the apical ganglion. (*f*) Annelid—*O. fusiformis* whole mount lateral view, anterior to the right; the open arrow heads point to the suboesophageal ganglion. (*g*) Phoronid—*P. vancouverensis* whole mount dorsal view; apical ganglion. (*h*) Brachiopod—*Glottidia* sp. whole mount dorsal view; apical ganglion. (*i*) Echinoderm—*D. excentricus* whole mount dorsal view; apical ganglion. (*j*) Enteropneust (tornaria larva) whole mount apical view, ventral side upward; apical ganglion. (*k*,*l*) Vertebrate—*L. fluviatilis* whole mount lateral view (*k*) anterior to the right and (*l*) 2 µm frontal section; the serotonergic cells in the raphe system (arrow head) and the caudal projecting processes located in the intermediolateral part of the spinal cord (arrow) are indicated.

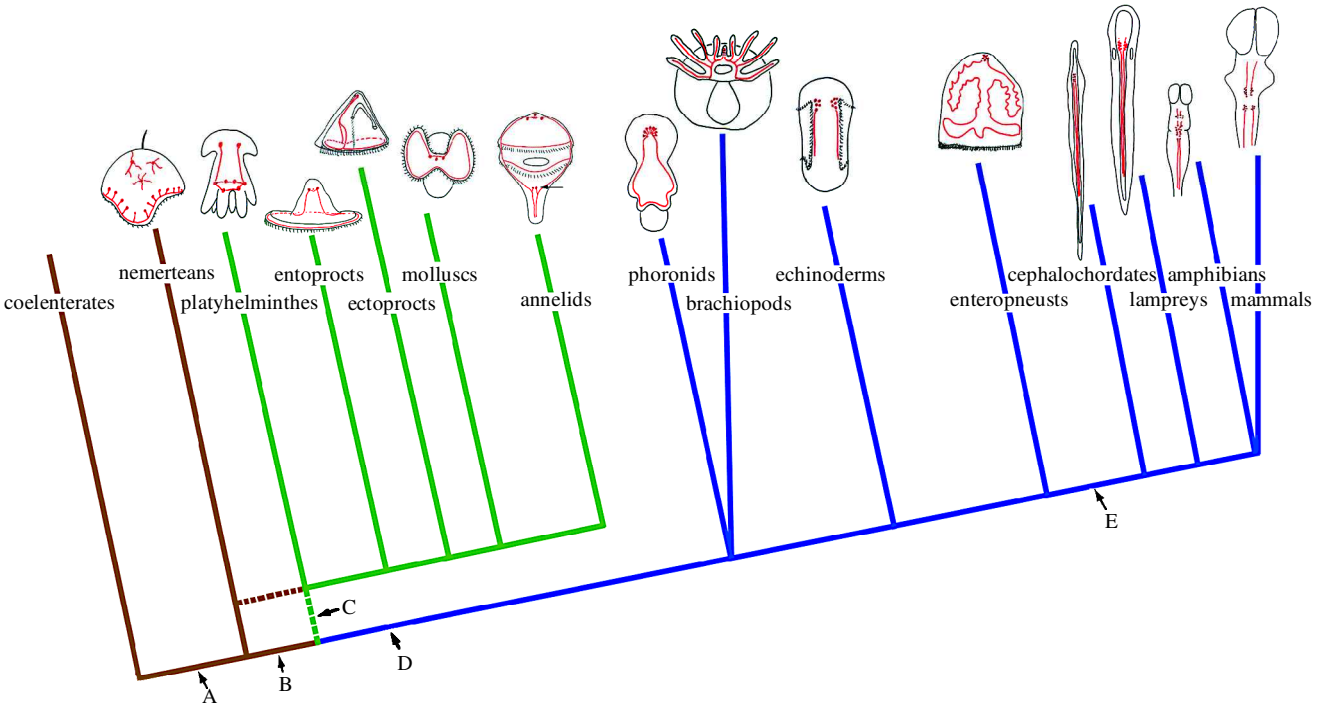

Figure 2. Cladogram showing the evolution of the serotonergic larval nervous system. Each group is represented by a larva or embryo in which the outline of the serotonergic nervous system is shown in red; the suboesophageal ganglion in the annelid trochophore larva is indicated by an arrow, cell bodies are indicated by a dot and neurites by a line. The 'coelenterates' (brown line) are the sister group to all of the other phyla, which are characterized by having a serotonergic innervation of the ciliary band. (*a*) The acquisition of serotonergic processes along a ciliary band. (*b*) The acquisition of an apical ganglion. (*c*) The acquisition of two lateral cells in the apical ganglion and the lateral serotonergic projection to the prototroch. (*d*) The acquisition of many serotonergic cells in the apical ganglion and a caudal serotonergic projection to the ciliary band. (*e*) The loss of the ciliary band and the acquisition of a neural tube and a paired group of serotonergic cells in the midbrain/hindbrain. The nemertean, ectoproct and enteropneust larvae are shown in lateral view. The platyhelminth, entoproct, mollusc, annelid, phoronid and brachiopod larvae are in ventral view and the echinoderm larva and all the chordates are in dorsal view. The cephalochordate is modified from Holland & Holland (1993), the amphibian from Van Mier *et al.* (1986) and the mammal from Wallace & Lauder (1983).

2. MATERIAL AND METHODS

(a) *Larvae and embryos*

Bolinopsis infundibulum (Ctenophora), *Stylostomum sanjuana* (Platyhelminthes), *Membranipora* sp. (Ectoprocta), *Owenia fusiformis* (Polychaeta), *Phoronis vancouverensis* (Phoronida), *Parastichopus californicus* (Holothuroidea), *Pisaster ochraceus* (Asteroidea), *Ophiopholis aculeata* (Ophiuroidea), *Dendraster excentricus* (Echinoidea) and *Florometra serratissima* (Crinoidea) were collected at Friday Harbor Laboratories (WA, USA). *Glottidia* sp. (Brachiopoda) and unknown tornaria larvae (Enteropneusta) were collected at the Smithsonian Marine Station, Fort Pierce (FL, USA). Pilidium larvae (Nemertini), *Loxosoma pectinaricola* (Entoprocta), *Polygordius lacteus* (Polychaeta) and *Phoronis muelleri* (Phoronida) were collected at Kristineberg Marine Biological Station (Sweden). *Philine aperta* (Gastropoda) was collected from Elsinore (Denmark) and *Lampetra fluviatilis* (Cyclostomata) from Ringkoebing Fjord (Denmark).

Larvae and embryos were fixed in 4% paraformaldehyde in 0.2 M Millonig's phosphate buffer, pH = 7.6 (0.2 M NaH_2PO_4 and 0.15 M NaOH) with 0.28 M NaCl at 4 °C for 1–24 h, rinsed twice in 0.2 M Millonig's buffer, pH = 7.6, and transferred to phosphate buffered saline (PBS), pH = 7.4, with 0.1% Triton X-100 (PBST) and 0.1% sodium azide for storage.

(b) *Immunohistochemistry*

One or more species were used for each phylum and, in each case, several stages and specimens (ten to more than 100) were examined. Processing of the specimens for immunohistochemistry was done in accordance with Hay-Schmidt (1995). All antibodies were diluted in PBST with either 2% human serum albumin or 5–10% serum from an appropriate species (goat or swine). All washings were done in PBST. The primary antibody was rabbit anti-serotonin (Incstar, Stillwater, MN, USA) diluted 1:500–1:4000. The secondary antibody was either conjugated goat anti-rabbit (FITC or TRITC) (Sigma) diluted 1:50 or biotinylated goat anti-rabbit (Zymed) diluted 1:500. Embryos which had had biotinylated secondary antibody were incubated in streptavidin Texas red (Amersham, UK) diluted 1:50 or streptavidin biotin horseradish peroxidase complex (Vector Elite, Burlingame, CA, USA) diluted 1:100, followed by the enzymatic peroxidase reaction using diaminobenzidine as a chromogen. As a control for specificity the rabbit anti-serotonin antibody was pre-absorbed with serotonin conjugated to bovine serum albumin before use as primary antibody and no staining was observed. In addition, the specificity of this antibody was tested on rat brain sections and immunoreactivity was observed in the perikarya of the mesencephalic raphe system, whereas no staining was observed in catecholaminergic perikarya (including the hypothalamus, substantia nigra and locus coeruleus). Omission of the primary antibody did not produce any immunoreactivity. Although double immunostaining

was not performed for the larvae used in this study, the serotonergic neurons in several species do not appear to co-localize with either catecholamines or the neuropeptide FMRFamide, since the reactive cell bodies and processes for these different transmitters are not situated in the same place (Hay-Schmidt 1990*a–c*, 1992, 1995).

3. RESULTS

(a) *Descriptions*

The serotonergic part of the central nervous system (CNS) in larvae of the following phyla was investigated. For a more detailed description of individual larval types see Korschelt (1936). The present data show that a serotonergic nervous system is found in the larvae of almost all animal groups.

(i) *'Coelenterates'*

The cnidarians have a planula larva with a nervous system composed of scattered serotonin-immunoreactive cells located in the epidermis (McCauley 1997) forming a nerve net. In contrast, no sign of immunoreactive serotonergic neurons was observed in the comb-jelly cydippid larva of *B. infundibulum*.

(ii) *Plathyhelminths*

The ultrastructure of the nervous system of a Müller's larva was described by Lacalli (1983) and includes an apical ganglion and a set of lateral nerves. The apical ganglion in Müller's larva of *S. sanjuana* houses a pair of serotonergic cell bodies from which an axon extends laterally towards the posterior end where they join a ring of serotonergic processes coming from six serotonergic cell bodies (one for each lateral lobe). Projections from the serotonergic neurons enter the ciliary band (figures 1*a*,*b* and 2).

(iii) *Nemertini*

Lacalli & West (1985) and Hay-Schmidt (1990*a*) described the nervous system of pilidium larvae and juveniles. The pilidium larva does not have a typical apical ganglion, although an apical ciliary tuft (mechanosensory organ) is present. Several serotonin-immunoreactive bipolar cells are found scattered along the ciliary band and their axons extend into the ciliary band nerve (figure 2). In addition, several multipolar serotonin-immunoreactive cells are located in the epidermis of the oral hood.

(iv) *Entoprocta*

Nielsen (1971) described the nervous system of entoproct trochophores in some detail. The entoproct trochophore has an apical ganglion with one medial and a lateral pair of serotonergic perikarya and a weak serotonin-immunoreactive neuropile. An ipsilateral axon projects towards the ciliary band of the prototroch from each lateral neuron (figures 1*c* and 2). No serotonin-immunoreactive cell bodies are found along the ciliary band.

(v) *Ectoprocta* (*Bryozoa*)

A free-swimming planktotrophic cyphonautes larva is found in a few ectoprocts; this larval type neither resembles a spiralian trochophore larva nor any other described larval type. The apical ganglion contains two serotonergic cell bodies each projecting a huge ipsilateral axon (the lateral nerve) towards the anterior side of the locomotory ciliary band (the corona). These axons continue all the way round the corona and innervate it (figures 1*d*,*e* and 2). A second pair of serotonergic cells is located on each side of the pyriform organ and an axon projects from each of these into the lateral nerve. There is no serotonergic innervation of the ciliated ridge, which is the feeding structure.

(vi) *Mollusca*

The apical ganglion of the trochophore (veliger) larva of the opistobranch gastropod *P. aperta* contains a median and a lateral pair of serotonergic neurons. An axon extends from the lateral pair on each side towards and into the prototroch (figure 2). No serotonin-immunoreactive cell bodies are located in the prototroch or elsewhere in the larvae at this early stage. These findings are in accordance with what has been described in other molluscan veliger larvae. At first the apical ganglion has either only the lateral pair, as seen in *Heliosoma* (Diefenbach *et al.* 1995), or three serotonergic cell bodies with an axon projecting from the lateral pair into the prototroch (Marois & Croll 1992; Kempf *et al.* 1997).

(vii) *Annelida*

This group comprises the vast majority of the segmented worms and is here represented by two polychaetes: *P. lacteus* and *O. fusiformis*. The apical ganglion of the trochophore of *P. lacteus* has a median and a lateral pair of serotonergic neurons (figure 2). Axons from the lateral cells project laterally across the episphere to (and along) the prototroch and further caudo-ventrally into what later becomes the suboesophageal ganglion and ventral nerve cord. A single pair of serotonin-immunoreactive cell bodies is observed within the ventral ganglion at this stage. No serotonin-immunoreactive cell bodies were found along any of the ciliary bands including the protrotroch (Hay-Schmidt 1995). In contrast to 'typical' spiralian trochophores, which have a prototroch made of compound multiciliary cells, *Owenia* larvae have a prototroch composed of monociliated cells. In this respect, the ciliary band of *Owenia* larvae superficially resembles that found in phoronid, brachiopod, echinoderm and enteropneust larvae. *Owenia fusiformis* was chosen for this study because it is a true polychaete (D. Eibye-Jacobsen, personal communication), although both the adult and larva (called a mitraria larva) (see Wilson (1932) for a detailed description of this larva) are unique among polychaetes for having only monociliated cells. The apical ganglion is without any serotonergic neurons and there is no serotonergic innervation of the prototroch at any larval stage. The only serotonergic cell bodies seen in the early larva are the pair belonging to the suboesophageal ganglion (figure 1*f*). An axon extends dorsally from each of these neurons around the oesophagus below the apical ganglion. These continue towards the opposite neuron and extend further anteriorly towards the mouth. The juvenile worm, which later develops inside the episphere of the mitraria, possesses a cerebral ganglion containing several serotonergic cell bodies.

(viii) *Phoronida and Brachiopoda*

Actinotroch larvae of *P. vancouverensis* and *P. muelleri* and larvae of the inarticulate brachiopod *Glottidia* sp. have an apical ganglion with numerous serotonergic neurons. Two descending serotonergic tracts arise from these and project into the ciliary band (figures 1*g*,*h* and 2). No serotonergic cell bodies were found along the ciliary band. In later stages, the brachiopod larvae develop a large cluster of serotonergic cell bodies ventral to the oesophagus, which innervates the muscles of the lophophore (Hay-Schmidt 1990*b*,*c*, 1992).

(ix) *Echinodermata*

The echinoderms consist of five separate classes, of which at least four have planktotrophic larvae of the dipleurula type. The larvae have a ciliary band which in the early stages more or less circumscribes the embryo dividing it into a ventral/oral field and a dorsal/aboral field. An inconspicuous apical ganglion is present in *P. californicus*, *P. ochraceus*, *O. aculeata* and *D. excentricus* and serotonergic neurons are observed in either one large cluster or a paired cluster located close to the anterior–dorsal part of the ciliary band (figures 1*i* and 2). In early-stage larvae these serotonergic perikarya send their axons into the antero-dorsal part of the ciliary band. Axon growth cones are seen to continue caudally from these, but they do not reach the posterior–ventral part of the ciliary band (figure 4). In a later-stage embryo axons continue all the way around the band. The present observations are in accordance with what was reported by Bisgrove & Burke (1986) and Nakajima (1988). No other serotonergic perikarya were observed along the ciliary band. In larvae of *F. serratissima*, it was impossible to demonstrate specific immunoreactivity in either the apical ganglion or along the ciliary bands because of background staining/fluorescence from the compact body.

(x) *Enteropneusts*

The tornaria larva of the enteropneusts has well-defined ciliary bands. In the apical ganglion numerous serotonin-immunoreactive perikarya are found grouped into lateral clusters (figures 1*j* and 2). Axons extend from the cells into and along the ciliary band throughout its length. No serotonergic perikarya were observed along the ciliary band.

(xi) *Chordates*

In the cephalochordates, the larval serotonergic system consists of a paired cluster of cells in the neural tube caudal to the 'cerebral vesicle' and serotonergic axons extend caudally from this cluster in the neural tube (Holland & Holland 1993). No serotonergic cell bodies are described from the caudal part of the larval neural tube. I have found no reports on the larval serotonergic nervous system in urochordates. Lampreys have an early pre-hatching embryo (the pre-ammocetes stages) followed by a free-living larval stage (the ammocetes) which superficially resembles the larvae of amphioxus. Both the embryo and larva of *L. fluviatilis* have a single cluster of serotonin-immunoreactive perikarya on each side in the neural tube at the level of the otic placode (figures 1*k* and 2). Axons extend caudally from these neurons on each side of the neural tube, almost reaching the posterior end depending on the larval stage. In later stages a new group of serotonergic perikarya was observed anterior to the otic placode. Ascending serotonergic axons from both groups were observed in the brain. Serotonergic perikarya were never observed caudal of the posterior (first) serotonergic cell group in the early larval stages. Serotonergic cell bodies were not found more caudal in the neural tube in early-stage larvae. The embryonic serotonergic nervous system consists of one (or two) clusters of cell bodies located at the level of the otic placode, with a caudal projection into the spinal cord from the posterior cluster in the embryos and/or larvae of teleosts (Ekström *et al*. 1985), amphibians (Van Mier *et al*. 1986), reptiles (Wallace 1985) and mammals (Wallace & Lauder 1983).

4. DISCUSSION

(a) *Comparison of the embryonic serotonergic nervous systems found in different phyla*

The present data show that a serotonergic nervous system is found in the embryos and/or larvae of almost all animal groups and it is suggested that the serotonergic part of larval nervous systems can be divided into the following types (see also figure 2).

Type 1 is found in cnidarian planula larvae and is formed as a nerve net (reticulum) with the serotonergic cell bodies scattered throughout the epidermis and is without any obvious centralization as seen in the following types.

The next three types (types 2–4) are mostly found in spiralian phyla.

Type 2 is only found in the pilidium larvae of nemerteans; it does not obviously fall into any of the other groups. It is interpreted as being more centralized than type 1 due to the concentration of the serotonergic cell bodies along the nerves supplying the ciliary band. During metamorphosis, none of the serotonergic neurons along the ciliary band are incorporated into the juvenile nervous system. Instead, the adult serotonergic nervous system develops directly within an endo-larva (i.e. a larva within the pilidium larva) which transforms into the juvenile worm (Hay-Schmidt 1990*a*). It is difficult to judge whether the absence of an apical ganglion is primary or represents a secondary loss and, thus, whether the type 2 is a larva nemertean specialization or represents a morphoclade between type 1 and the next two types.

Type 3 is found in the Müller's larva of flatworms; the presence of two serotonergic cell bodies in the apical ganglion is regarded as a synapomorphic characteristic shared with those larvae having the type 4 nervous system. However, type 3 differs from type 4 in having a serotonergic cell body for each lateral lobe supplying the ciliary band of that lobe. The presence of an apical ganglion with two lateral serotonergic cell bodies suggests a close relation between flatworms and other spiralian protostomes, but there are conflicting hypotheses concerning the affinity of these groups (Field *et al*. 1988; Adoutte *et al*. 1999).

Type 4 is found in the trochophore larvae of various spiralian protostome phyla (entoprocts, molluscs and annelids) and in the cyphonautes larva of ectoprocts and appears very conserved. It is characterized by having an apical ganglion with few serotonergic cells of which at least the lateral pair sends a serotonergic projection laterally

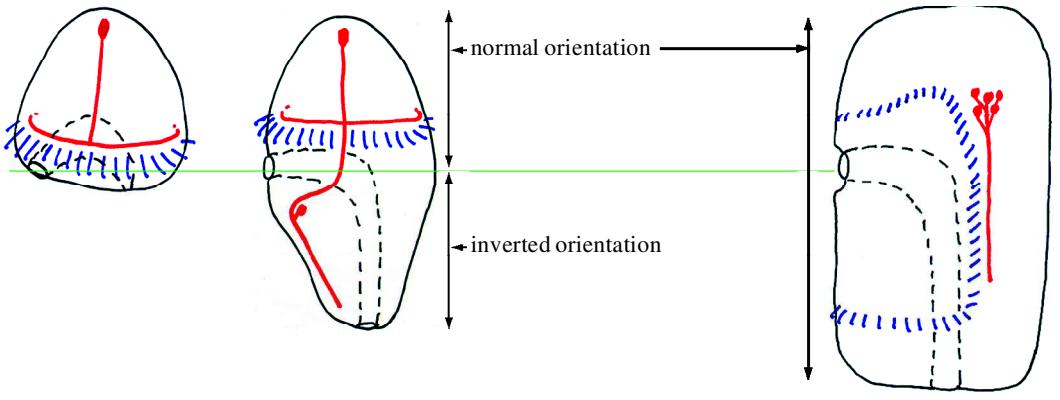

Figure 3. Schematic of the serotonergic nervous system in a non-segmented spiralian larva (i.e. entoproct), a segmented spiralian larva (i.e. annelid) and a dipleurula larva (i.e. echinoderm). The larvae are shown in lateral view and the apical end (anterior) is upwards, the oral side (ventral side) is to the left and the dorsal side is to the right. The mouth has been aligned (green line). There is a normal dorsal–ventral orientation anterior to the mouth in all larvae, whereas the segmented spiralia has an inverted dorsal–ventral orientation posterior to the mouth compared with the dipleurula larva.

into the prototroch, which itself has no serotonergic cell bodies. The fixed number of cells, their order of appearance and their pattern of innervation in the prototroch do indicate a close relationship. Except for the ectoprocts, all the protostomes that have the type 4 system also have a ciliary band which functions as a downstream collecting system (Nielsen 1995).

Type 5, which is found in phoronids, brachiopods (which are sometimes classified as protostomes), echinoderms and enteropneusts also seems very conserved and could indicate a common origin. This type is characterized by having numerous serotonergic cell bodies in the apical ganglion and by a huge serotonergic tract projecting caudally from the apical ganglion into the ciliary band.

Type 6 is found in all chordates, consistent with a common origin of all members of this group. In this type the early embryonic and/or larval part of the serotonergic nervous system consists of a group of serotonergic cell bodies (the future raphe system) located at the level of the otic placode and their caudal projections into the neural tube. That the chordate line must have had a non-chordate deuterostome invertebrate ancestor is beyond doubt (Holland *et al*. 1991) and, therefore, chordates must have inherited their nervous system in some form from their common ancestor with echinoderms and/or hemichordates. Despite the lack of a tripartite division of the coelomic structure in chordate embryos and larvae, the most parsimonious hypothesis regarding the first formed part of the serotonergic nervous system is that it evolved from a type with numerous serotonergic cell bodies located on each side of the midline with descending projections as seen in type 5. Recent studies of the amphioxus larva (Holland *et al*. 1992; Lacalli *et al*. 1994) indicate a very long hindbrain. The serotonergic cell bodies in amphioxus could be homologous with those in higher vertebrates and with those in the ciliary band of non-chordate deuterostomes. The present study on the lamprey embryo and larva tends to support this interpretation.

(b) *Conflict between 18S rDNA and anatomical data?*

The conclusions drawn from 18S rDNA analyses (Mackey *et al*. 1996; Cohen *et al*. 1998), which indicate that phoronids, brachiopods and ectoprocts all cluster within the spiralian invertebrates, is not unambiguously confirmed by this study. Based on structures within the larval nervous system the ectoprocts are found to have synapomorphies with those of mollusc, entoproct and polychaete trochophores, which accords with the molecular data. However, there are other significant differences between phoronids, brachiopods and the spiralian protostome phyla based on the structure of the larval serotonergic nervous system. The type 5 system defines a pattern which obviously groups the phoronids and brachiopods with the deuterostomes and is thus just one more character where anatomical data differ from 18S rDNA sequence data (Nielsen 1995; Cohen *et al*. 1998). Recently, Zrzavy *et al*. (1998) concluded that the affiliation of the brachiopods and phoronids to the spiralian phyla based on rDNA sequences is an out-group artefact and may not reflect a real relationship. The present data are more consistent with the idea that two major groups of animals, typical protostomes (flatworms, nemerteans, entoprocts, ectoprocts, molluscs and annelids) and deuterostomes (phoronids, brachiopods, echinoderms, hemichordates and chordates), separated very early in the evolution of the bilaterians (a first split). Shortly thereafter, the phoronids and brachiopods separated from the deuterostomes *sensu stricto* (a second split) making this separation unqualified for 18S rDNA analysis (Phillipe *et al*. 1994).

(c) *Conflict between genetic and anatomical evidence?*

As shown in this study, the central serotonergic axons occupy a dorsal (aboral) position relative to the digestive tract and oral field (mouth) in the lineage of animals leading towards the chordates (phoronids, brachiopods and all deuterostomes). In larvae belonging to non-segmented spiralian protostomes, the serotonergic larval nervous system initially stops at the prototroch. In segmented protostomes, the serotonergic axons continue to the suboesophageal ganglion and caudally along the ventral midline (Hay-Schmidt 1995). The suboesophageal position of the ventral (i.e. caudal) part of the central serotonergic nervous system of the annelid trochophore

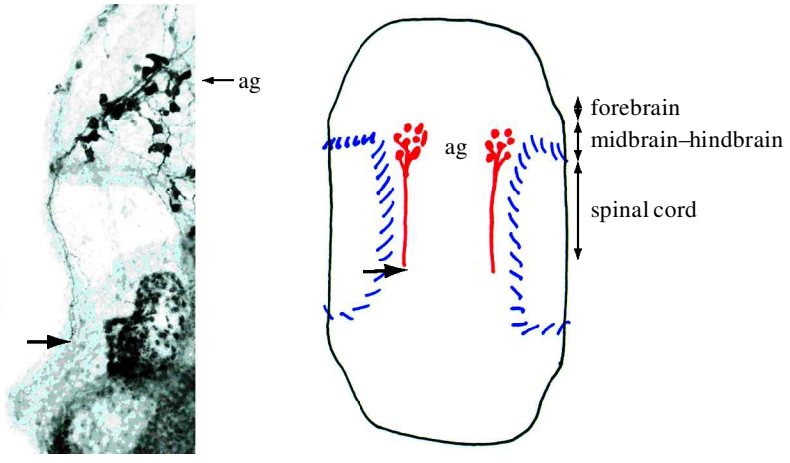

Figure 4. Schematic of the serotonergic nervous system in a dipleurula larva indicating the proposed relations between the apical ganglion and chordate neural tube. The left figure shows serotonin immunoreactivity in the larva of *P. ochraceus* with the left part of the apical ganglion (ag) and the serotonin-immunoreactive processes coursing along the antero-dorsal ciliary band and stopping at the arrow. The right-hand figure is a diagram of the same larva seen from the dorsal side. The relation between the apical ganglion and the chordate neural tube is indicated. The forebrain is anterior to the serotonergic cells of the apical ganglion, the midbrain/hindbrain is at the level of the serotonergic cells and the neural tube is caudal of the apical ganglion at the level of the antero-dorsal ciliary band. The arrow points towards the caudal termination of the serotonergic projection at this larval stage.

larva is important in that it is, by implication, not homologous to any part of the serotonergic nervous system found in the deuterostome lineage. That is not to say that the molecular components involved in axon guidance, cell structure and specification are also necessarily different between these two groups. With regard to the anterior part of the serotonergic system (i.e. the apical ganglion), it is also difficult to find any similarities between the two groups of organisms. It is therefore suggested here that the common ancestor for all of the phyla having a serotonergic larval nervous system of the spiralian trochophore larval type adopted a pair of serotonergic cell bodies in the apical ganglion from which the serotonergic nervous system developed more fully (figure 2). On the basis of the present study there is no sign of a complete shift in the dorso-ventral orientation of the deuterostomes (chordates) compared with protostomes (insects) as proposed by Arendt & Nübler-Jung (1999). The dorso-ventral inversion seems more to be related to the orientation of the postoral part of the nervous system relative to the gastrointestinal tract (figure 3). A ventral nerve cord in the strict sense is found only in annelids and arthropods; more commonly, ventral ganglia are found innervating specific muscle groups. In contrast, the brain in both protostomes and deuterostomes seems to be related to the apical ganglion and is always located dorsal to the gastrointestinal tract. This would imply that the dorsal–ventral orientation of the brain is congruent in protostomes and deuterostomes, whereas the ventral nerve cord found in annelids and arthropods will be 'upside down' (i.e. inverted). The expression patterns of several genes within or in relation to the nervous system have been used to defend the dorsal–ventral inversion hypothesis. The midline marker (*fkh/HNF-3*) is only expressed in the endoderm and/or blastophore lip in larval echinoderms (Harada *et al*. 1996), as in *Drosophila* (Weigel *et al*. 1989) and *Caenorhabditis* (Azzaria *et al*. 1996). Furthermore, in non-chordate deuterostomes the expression of this gene includes the whole of the blastophore lip in contrast to the polarized expression seen in tunicates (Corbo *et al*. 1997; Olsen & Jeffery 1997) and other chordates. The expression patterns of the *Brachyury* (*T*) gene in non-chordate deuterostomes include both the posterior (blastophore) and anterior (secondary mouth) regions of the intestine (Peterson *et al*. 1999). The putative functions of both *fkh* and *Brachyury T* in deuterostomes seem to be related to endoderm–ectoderm and endoderm–mesoderm separation, respectively (i.e. identity specification) as has been proposed for the protostomes (Weigel *et al*. 1989; Kispert *et al*. 1994; Azzaria *et al*. 1996). From the present anatomical data on the embryonic and/or larval serotonergic nervous systems, the expression pattern for *fkh* cannot be used to deduce obvious homologies between the chordate and protostome (i.e. arthropod) nervous systems as proposed by Arendt & Nübler-Jung (1999). Instead, the ability of the *fkh* gene to specify tissue identity was retained when the nervous system shifted from a truly epidermal position to a subepidermal position, whether due to delamination (protostomes) or invagination (chordates). In chordates the function of *Brachyury T* is likely to be separating the notochord from either the gastrointestinal tract and/or the neural tube (i.e. identity specification) (Yasuo & Satoh 1993). Another example is the interaction of the *short of gastrulation* (*sog*)/*chordin* (*chd*) and *decapentaplegic* (*dpp*)/*bone morphogene protein* (*BMP*) seen in arthropods and chordates (Holley *et al*. 1995). These genes are related to 'dorsal–ventral' specification of the segments in segmented animals in which the new segment also needs to be specified along a dorsal–ventral axis. This system need not necessarily specify the dorsal–ventral axis relative to the mouth, but just an axis perpendicular to the longitudinal (antero-posterior) axis. Therefore, dorsal–ventral axis determination in segmented phyla is probably much more a consequence of where the apical ganglion extends its axons to establish the location of the nerve cord than of dorso-ventrality *sensu stricto*. The genetic programme responsible for the organization of the axis perpendicular to the anterior–posterior axis is

probably identical between protostomes and deuterostomes and represents a 'deep homology' as proposed for limb development (Shubin *et al*. 1997). The involvement of the blastophore lips in the specification of the dorsal–ventral axis in chordates will have been required after the loss of the dipleurula larval stage as chordate and non-chordate deuterostomes diverged.

The segmented nature of the neural tube in chordates makes comparisons between the non-chordate deuterostomes and the chordates difficult. Nevertheless, the area rostral to the serotonergic part of the apical ganglion is presumably homologous with the forebrain–midbrain region of the chordate brain, while the serotonergic cell bodies would be midbrain–hindbrain homologues from which the spinal cord would extend caudally (figure 4). It seems most likely that neural segmentation has progressed in two directions, anterior and posterior from a starting point at the larval prosome–mesosome boundary, with *Otx* marking the anterior border and the anterior-most *Hox* genes the caudal border (Williams & Holland 1998; Wada *et al*. 1998).

The present study demonstrates that there are two major types of larval serotonergic nervous system. The first, which is found in spiralian protostomes, has a fixed number of serotonergic neurons. The second, which is found in deuterostome-like invertebrates, has numerous serotonergic neurons in the apical ganglion. In consequence, since the embryonic and/or larval serotonergic system in chordates is very probably derived from a system resembling that found in deuterostome-like invertebrates, I would propose that the serotonergic raphe system of vertebrates can be traced back to the larval serotonergic system of the common ancestor of phoronids, brachiopods and chordates. In general terms, this study also supports the idea that the neural tube of vertebrates may be homologous to a part of the ciliary band (most probably the antero-dorsal part) of a dipleurula larva as proposed by Garstang (1894), but provides no evidence of a dorsal–ventral inversion between protostomes and deuterostomes.

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REFERENCES

Adoutte, A., Balavoine, G., Lartillot, N. & De Rosa, R. 1999 Animal evolution: the end of the intermediate taxa? *Trends Genet.* **15**, 104–108.

Aiello, E. 1974 Control of ciliary activity in metazoa. In *Cilia and flagella* (ed. M. A. Sleigh), pp. 353–375. London: Academic Press.

Arendt, D. & Nübler-Jung, K. 1999 Comparison of early nerve cord development in insects and vertebrates. *Development* **126**, 2309–2325.

Azzaria, M., Goszczynski, B., Chung, M. A., Kalb, J. M. & McGhee, J. D. 1996 A fork head/*HNF*-3 homolog expressed in the pharynx and intestine of the *Caenorhabditis elegans* embryo. *Dev. Biol.* **178**, 289–303.

Bisgrove, B. W. & Burke, R. D. 1986 Development of serotonergic neurons in embryos of the sea urchin, *Strongylocentrotus purpuratus*. *Dev. Growth Differ.* **28**, 569–574.

Bonde, N. 1984 Primitive features and ontogeny in phylogenetic reconstructions. *Vidensk. Meddr. Dansk Naturh. Foren.* **145**, 219–236.

Cohen, B. L., Stark, S., Gawthrop, A. B., Burke, M. E. & Thayer, C. W. 1998 Comparison of articulate brachiopod nuclear and mitochondrial gene trees leads to a clade-based redefinition of protostomes (Protostomozoa) and deuterostomes (Deuterostomozoa). *Proc. R. Soc. Lond.* B **265**, 475–482.

Corbo, J. C., Erives, A., Di Gregorio, A., Chang, A. & Levine, M. 1997 Dorsoventral patterning of the vertebrate neural tube is conserved in a protochordate. *Development* **124**, 2335–2344.

De Rosa, R., Grenier, J. K., Andreeva, T., Cook, C. E., Adoutte, A., Akam, M., Carroll, S. B. & Balavoine, G. 1999 *Hox* genes in brachiopods and priapulids and protostome evolution. *Nature* **399**, 772–776.

Diefenbach, T. J., Sloley, B. D. & Goldberg, J. I. 1995 Neurite branch development of an identified serotonergic neuron from embryonic *Helisoma*: evidence for autoregulation by serotonin. *Dev. Biol.* **167**, 282–293.

Ekström, P., Nyberg, L. & Van Veen, T. 1985 Ontogenetic development of serotonergic neurons in the brain of a teleost, the three-spined stickleback. An immunohistochemical analysis. *Dev. Brain Res.* **17**, 209–224.

Field, K. G., Olsen, G. J., Lane, D. J., Giovannoni, S. J., Ghiselin, M. T., Raff, E. C., Pace, N. R. & Raff, R. A. 1988 Molecular phylogeny of the animal kingdom. *Science* **239**, 748–753.

Garstang, W. 1894 Preliminary note on a new theory of the phylogeny of the chordata. *Zool. Anz.* **17**, 122–125.

Halanych, K. M., Bacheller, J. D., Aguinaldo, A. M., Liva, S. M., Hillis, D. M. & Lake, J. A. 1995 Evidence from 18S ribosomal DNA that the lophophorates are protostome animals. *Science* **267**, 1641–1643.

Harada, Y., Akasaka, K., Shimada, H., Peterson, K. J., Davidson, E. H. & Satoh, N. 1996 Spatial expression of a forkhead homologue in the sea urchin embryo. *Mech. Dev.* **60**, 163–173.

Hay-Schmidt, A. 1990*a* Catecholamine-containing, serotonin-like and neuropeptide FMRFamide-like immunoreactive cells and processes of the nervous system of the pilidium larva (Nemertini). *Zoomorphology* **109**, 231–244.

Hay-Schmidt, A. 1990*b* Distribution of catecholamine-containing, serotonin-like and neuropeptide FMRFamide-like immunoreactive neurons and processes in the nervous system of the actinotroch larva of *Phoronis muelleri* (Phoronidae) *Cell Tissue Res.* **259**, 105–118.

Hay-Schmidt, A. 1990*c* Catecholamine-containing, serotonin-like and neuropeptide FMRFamide-like immunoreactive neurons and processes in the nervous system of the early actinotroch larva of *Phoronis vancouverensis* (Phoronida): distribution and development. *Can. J. Zool.* **68**, 1525–1536.

Hay-Schmidt, A. 1992 Ultrastructure and immunocytochemistry of the nervous system of the larvae of *Lingula anatina* and *Glottidia* sp. (Brachiopoda). *Zoomorphology* **112**, 189–205.

Hay-Schmidt, A. 1995 The larval nervous system of *Polygordius lacteus* Scheinder, 1868 (Polygordiidae, Polychaeta): immunocytochemical data. *Acta Zool.* (*Stockh.*) **76**, 121–140.

Holland, N. D. & Holland, L. Z. 1993 Serotonin-containing cells in the nervous system and other tissues during ontogeny of a lancelet, *Branchiostoma floridae*. *Acta Zool.* (*Stockh.*) **74**, 195–204.

Holland, P. W. H., Hacker, A. M. & Williams, N. A. 1991 A molecular analysis of the phylogenetic affinities of *Saccoglossus cambrensis* Brambell & Cole (Hemichordata). *Phil. Trans. R. Soc. Lond.* B **332**, 185–189.

Holland, P. W. H., Holland, L. Z., Williams, N. A. & Holland, N. D. 1992 An amphioxus homeobox gene: sequence conservation, spatial expression during development and insights into vertebrate evolution. *Development* **116**, 653–661.

Holley, S. A., Jackson, P. D., Sasai, Y., Lu, B., De Robertis, E. M., Hoffmann, F. M. & Ferguson, E. L. 1995 A conserved system for dorsal–ventral patterning in insects and vertebrates involving sog and chordin. *Nature* **376**, 249–253.

Kempf, S. C., Page, L. R. & Pires, A. 1997 Development of serotonin-like immunoreactivity in the embryos and larvae of nudibranch mollusks with emphasis on the structure and possible function of the apical sensory organ. *J. Comp. Neurol.* **386**, 507–528.

Kispert, A., Herrmann, B. G., Leptin, M. & Reuter, R. 1994 Homologs of the mouse Brachyury gene are involved in the specification of posterior terminal structures in *Drosophila*, *Tribolium*, and *Locusta*. *Genes Dev.* **8**, 2137–2150.

Korschelt, E. 1936 In *Korschelt und Heider: Vergleichende Entwicklungsgeschichte der Tiere*. Jena: Von Gustav Fischer.

Lacalli, T. C. 1983 The brain and central nervous system of Müller's larva. *Can. J. Zool.* **61**, 39–51.

Lacalli, T. C. & West, J. E. 1985 The nervous system of a pilidium larva: evidence from electron microscope reconstructions. *J. Can. Zool.* **63**, 1909–1916.

Lacalli, T. C., Holland, N. D. & West, J. E. 1994 Landmarks in the anterior central nervous system of amphioxus larvae. *Phil. Trans. R. Soc. Lond.* B **344**, 165–185.

McCauley, D. W. 1997 Serotonin plays an early role in the metamorphosis of the hydrozoan *Phialidium gregarium*. *Dev. Biol.* **190**, 229–240.

Mackey, L. Y., Winnepenninckx, B., De Wachter, R., Backeljau, T., Emschermann, P. & Garey, J. R. 1996 18S rRNA suggests that entoprocta are protostomes, unrelated to ectoprocta. *J. Mol. Evol.* **42**, 552–559.

Marois, R. & Croll, R. P. 1992 Development of serotonin-like immunoreactivity in the embryonic nervous system of the snail *Lymnaea stagnalis*. *J. Comp. Neurol.* **322**, 255–265.

Murakami, A. 1983 Control of ciliary beat frequency in *Mytilus*. *J. Submicrosc. Cytol.* **15**, 313–316.

Murakami, A. 1987 Control of ciliary beat frequency in the gill of *Mytilus*—I. Activation of the lateral cilia by cyclic AMP. *Comp. Biochem. Physiol.* C **86**, 273–279.

Nakajima, Y. 1988 Serotonergic nerve cells of starfish larvae. In *Echinoderm biology* (ed. R. D. Burke), pp. 235–239. Rotterdam, The Netherlands: Balkema.

Nelson, G. 1978 Ontogeny, phylogeny, paleontology and the biogenetic law. *Syst. Zool.* **27**, 324–345.

Nielsen, C. 1971 Entoproct life-cycles and the entoproct/ectoproct relationship. *Ophelia* **9**, 209–341.

Nielsen, C. 1995 *Animal evolution: interrelationships of the living phyla*. Oxford University Press.

Olsen, C. L. & Jeffery, W. R. 1997 A fork head gene related to *HNF-3b* is required for gastrulation and axis formation in the ascidian embryo. *Development* **124**, 3509–3519.

Paparo, A. A. 1986 Average ciliary beat in the oyster: response to photoperiod, pentylenetetrazole, salyrgan, serotonin and dopamine. *Mar. Behav. Physiol.* **12**, 149–159.

Peterson, K. J., Harada, Y., Cameron, R. A. & Davidson, E. H. 1999 Expression pattern of *Brachyury* and *Not* in the sea urchin: comparative implications for the origins of mesoderm in the basal deuterostomes. *Dev. Biol.* **207**, 419–431.

Phillipe, H., Chenuil, A. & Adoutte, A. 1994 Can Cambrian explosion be inferred through molecular phylogeny? *Development* (Suppl.), 15–25.

Shubin, N., Tabin, C. & Carrol, C. 1997 Fossils, genes and the evolution of animal limbs. *Nature* **388**, 639–648.

Van Mier, P., Joosten, H. W. J., Van Rheden, R. & Ten Donkelaar, H. J. 1986 The development of serotonergic raphe spinal projections in *Xenopus laevis*. *Int. J. Dev. Neurosci.* **4**, 465–475.

Wada, H., Saiga, H., Satoh, N. & Holland, P. W. H. 1998 Tripartite organization of the ancestral chordate brain and the antiquity of placodes: insights from ascidian Pax-2/5/8, Hox and Otx genes. *Development* **125**, 1113–1122.

Wallace, J. A. 1985 An immunocytochemical study of the development of central serotonergic neurons in the chick embryo. *J. Comp. Neurol.* **236**, 443–453.

Wallace, J. A. & Lauder, J. M. 1983 Development of the serotonergic system in the rat embryo: an immunocytochemical study. *Brain Res. Bull.* **10**, 459–479.

Weigel, D., Jurgems, G., Kuttner, F., Seifert, E. & Jackle, H. 1989 The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the *Drosophila* embryo. *Cell* **57**, 645–658.

Wilkinson, L. O. & Dourish, C. T. 1991 Serotonin and animal behavior. Serotonin receptor subtypes: basic and clinical aspects. *Receptor Biochem. Methodol.* **14**, 147–210.

Williams, N. A. & Holland, P. W. H. 1998 Molecular evolution of the brain of chordates. *Brain Behav. Evol.* **52**, 177–185.

Wilson, D. L. 1932 On the mitraria larva of *Owenia fusiformis* Delle Chiaje. *Phil. Trans. R. Soc. Lond.* B **221**, 231–334.

Yasuo, H. & Satoh, N. 1993 Function of vertebrate *T* gene. *Nature* **364**, 582–583.

Zrzavy, J., Mihulka, S., Kepka, P., Bezdek, A. & Tietz, D. 1998 Phylogeny of the metazoa based on morphological and 18S ribosomal DNA evidence. *Cladistics* **14**, 249–285.

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