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The ontogeny of the notochord of *Branchiostoma lanceolatum*

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Abstract

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Anatomical details of the ontogeny of the notochord of the european lancelet (= amphioxus), *Branchiostoma lanceolatum*, are described, based on complete series of alternating thin and semithin sections for light and transmission electron microscopy of early neurula and larval stages, as well as observation of living specimens. The notochord is formed by the outgrowth of dorsal medial cells of the archenteron. A gradient along the anterio-posterior axis is discerned, the more anterior part of the notochord being further developed. The differentiation of the central notochordal cells to muscle plates with a spacious intracellular vacuole is demonstrated. The arrangement of these cells as a 'stack of coins' is established early during ontogeny and is shown to be a plesiomorphy within the Chordata. These cells are capable of contraction in one gill slit larvae. The existence of dorsal and ventral Müller cells is proven. These cells are interconnected by desmosomes, form extracellular spaces between them, and show signs of high transcriptional activity.

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Introduction

The synapomorphic characters of the phylum Chordata include amongst others, a dorsal hollow nerve cord, a pharynx forming a branchial basket, and a locomotory system built up by the lateral musculature, and an antagonistic skeletal element the Chorda dorsalis or notochord. The name of this monophyletic group, which is comprised of the Tunicata (= Urochordata), the Cephalochordata (= Acrania), and the Craniota (= Vertebrata) is derived from the latter of these synapomorphic characters and many phylogenetic considerations focused on the variation of the notochord amongst the different chordate groups (e.g. Flood et al. 1969; Welsch and Storch 1969; Ruppert 1997a).

Adults of the Cephalochordata possess a notochord made up of special muscle cells arranged like a stack of coins and bordered by Müller cells dorsally and ventrally (Flood 1975; Welsch and Storch 1976). Both extracellular spaces and intracellular vacuoles are numerous. The extracellular spaces form a conspicuous dorsal canal and a less evident ventral one (Flood 1975). There is some uncertainty regarding the nature of the Müller cells. Welsch (1968) and Welsch and Storch (1973) described them as the somata of the notochordal muscle lamellae but later these authors adopted the view of Flood that the Müller cells might be separate cells (Flood 1975; Welsch and Storch 1976).

Electron microscopical observations of the ontogeny of the notochord in cephalochordates are sparse. Hirakow and Kajita (1994) furnished electron micrographs of early stages of *Branchiostoma belcheri*. These authors were concerned with the general description of the anatomy of the developmental stages and did not follow the ontogeny of the notochord specifically. Ruppert (1997b) furnished electron microscopical aspects of the notochord of a three-gill-slit-stage larva of *Branchiostoma virginiae*. Details of the earlier ontogenetic stages of the notochord among cephalo-

chordates were thus mainly studied by light microscopy (Cerfontaine 1906; Conklin 1932).

The notochord of cephalochordates occupies still a prominent position in phylogenetic considerations concerning the Chordata. Only recently Ruppert proposed an alternative interpretation of the phylogenetic relationship within the chordates based on notochord characters (Ruppert 1997a). In contrast to the predominant current opinion (e.g. Maisey 1986; Nielsen 1995) he did not unite the cephalochordates with craniates but argued that cephalochordates could form a monophylum together with the tunicates.

A detailed anatomical description of the early ontogeny of the notochord of *Branchiostoma lanceolatum* based on electron microscopy should render some insights into these questions.

Materials and Methods

Sexually mature specimens of *Branchiostoma lanceolatum* (Pallas) were provided by the Biologische Anstalt Helgoland and kept constantly at 10 °C. Spawning was induced by raising the temperature to 18 °C. Developmental stages were observed alive by means of light microscopy (Leitz, Axioplan) and recorded on videotape (Sony, KSP – 60, U – matic). The entire fixation procedure was performed at 0 °C.

The younger embryonic stages were fixed in 2.5% glutardialdehyde – cacodylate (0.2 m) solution at pH 7.4 for 40 min. Rinsing in cacodylate buffer was followed by post-fixation with OsO_4 (2%) for 3 h and 30 min. Embedding was in Epon 812. Sections of these stages were stained with uranyl acetate (5 min) and lead citrate (1 min) and with toluidine blue for light microscopy.

The larvae with one primary gill slit were fixed for 30 min in a glutardialdehyde (8%) – sea water mixture (1 : 2). Rinsing in sea water was followed by post-fixation for 3 h in an OsO_4 (4%) – sea water mixture (1 : 1). The animals were stained with uranyl acetate en bloc prior to embedding in Epon 812. Sections were stained with lead citrate (1 min) for TEM and with toluidine blue for light microscopy.

Serial sections of different developmental stages for light and transmission electron microscopy (TEM) were prepared on a LKB Ultratome 1 as follows: a survey series of about 20 semithin-sections 0.5 μm thick was made, followed by a series of ultra-thin- sections 0.05 μm in thickness. This pattern was repeated for the length, respeçtively, height of the entire animals.

TEM micrographs were prepared with a Siemens Elmiskop 102.

Results

Figure 1 summarizes the development of the body proportions of ontogenetic stages investigated for the present account. It also indicates the various planes of the sections reproduced in this study.

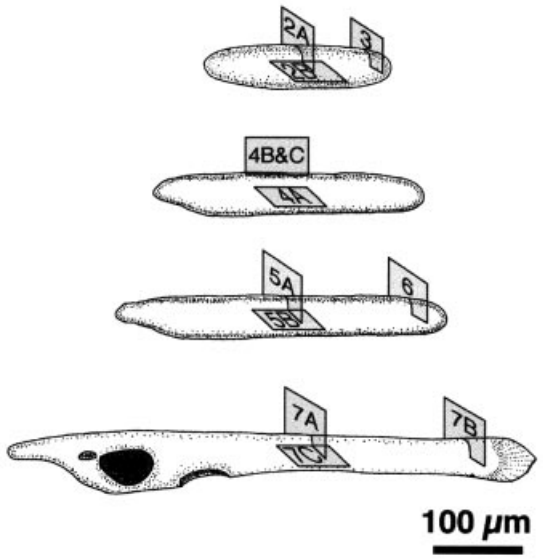

Fig. 1.—Schematic line drawings of developmental stages of *Branchiostoma lanceolatum*. Age: 22 h, 30 h, 35 h, 110 h post-fertilization at 18 °C. Planes indicate planes of sections reproduced in Figs 2,3,4,5,6,7. Labelling refers to these Figures.

Early neurula stage (22 h post-fertilization, 18 °C)

The anlage of the notochord of these neurula stages is not separated from the epithelium of the archenteron by any extracellular matrix (ecm). The prospective notochordal tissue is thus continuous with the endoderm of the archenteron (Fig. 2A). Nevertheless the horizontal sections reveal that the prospective notochordal cells are already typically arranged in single file, the well known arrangement as a 'stack of coins' (Fig. 2B), where each cell is situated behind another and spans the entire breadth of the future notochord. Approximately 7 prospective notochordal cells border the side of a single myomere. The myomeres from both sides of the body are still bilaterally symmetrical. A gradient along the antero-posterior axis of the animals is recognizable. In the posterior part the outgrowth of the prospective notochord in the dorsal medial line of the archenteron is less accentuated than in the anterior part (Fig. 3).

The prospective notochordal cells of this early developmental stage are fairly undifferentiated and resemble the cells of the archenteron at this age (Fig. 2A, B). There are no spacious intracellular vacuoles other than the numerous yolk granules. The number of yolk granules seen in one section of a cell is about 10 thus resembling the number found in archenteron cells. Profiles of rough endoplasmic reticulum are frequently encountered. No myofilaments are visible.

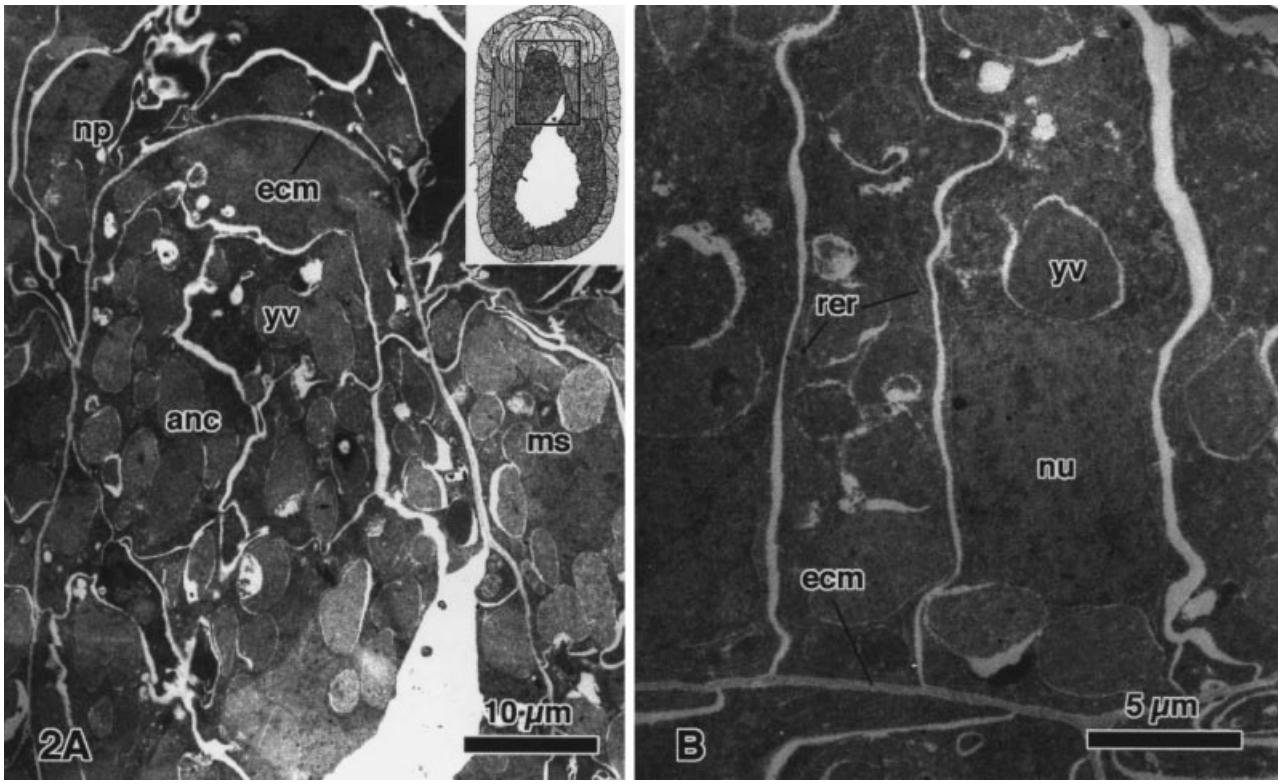

Fig. 2.—Transmission electron micrographs of an early neurula of *Branchiostoma lanceolatum*.—**A,** cross section. —**B,** horizontal section; see Fig. 1 for exact planes of sections. Inset: line drawing of the entire cross section with the area enlarged in A indicated by rectangle. **anc,** anlage of the notochord; **ecm,** extracellular matrix; **ms,** mesoderm; **np,** neural plate; **nu,** nucleus; **rer,** rough endoplasmic reticulum; **yv,** yolk granules.

Neurula stage (30 h post fertilization, 18 °C)

The notochord is now entirely separated by an ecm from the epithelium of the archenteron in the anterior and the major part of the trunk region. Only at the posterior part the notochordal tissue is still continuous with the endodermal archenteron. Like in the ontogenetic stage described above the horizontal section reveals that the cells are arranged in single file, spanning the entire breadth of the notochord (Fig. 4A). There are ≈15 central notochordal cells per myomere, considerably more than in the previous stage. The myomeres from either side of the body are asymmetrically arranged.

On the subcellular level several changes occurred (Fig. 4). The number of yolk granules decreased. Profiles of rough endoplasmic reticulum are very numerous indicating a high transcriptional activity. The high number of mitochondria seen in these cells agrees well with that finding. Most obviously the central notochordal cells develops a prominent intracellular vacuole. This vacuole appears empty in TEM preparations most of the time (but see Fig. 4A). The larger central vacuoles seem to be formed by the confluence of numerous smaller vesicles. These smaller vesicles display the same electron lucent impression in TEM preparations and are encountered often in association with the larger vacuoles (Fig. 4A). Small extracellular vacuole-like spaces are formed on this stage between adjacent central notochordal cells (Fig. 4A, B, C). Sometimes two neighbouring central cells can be seen to be interconnected by cell junctions, which are possibly desmosomes of the adherens type.

The sagittal sections (Fig. 4B &C) clearly show that the central notochordal cells, which are arranged as a 'stack of coins' are bordered dorsally and ventrally by distinctively different cells. These rest on the dorsal, respectively, ventral ecm of the notochord and are of a more or less cuboidal shape. Each of these cells lies above, respectively, below 3–4 central notochordal cells. The dorsal and ventral cells are likewise well equipped with rough endoplasmic reticulum, mitochondria, and yolk granules. They seem not to produce intracellular vacuoles like the central vacuoles of the central notochordal cells on this ontogenetic stage. On the other hand they seem to be a preferential site for the formation of vacuole-like spaces between neighbouring cells (Fig. 4C). Like the central

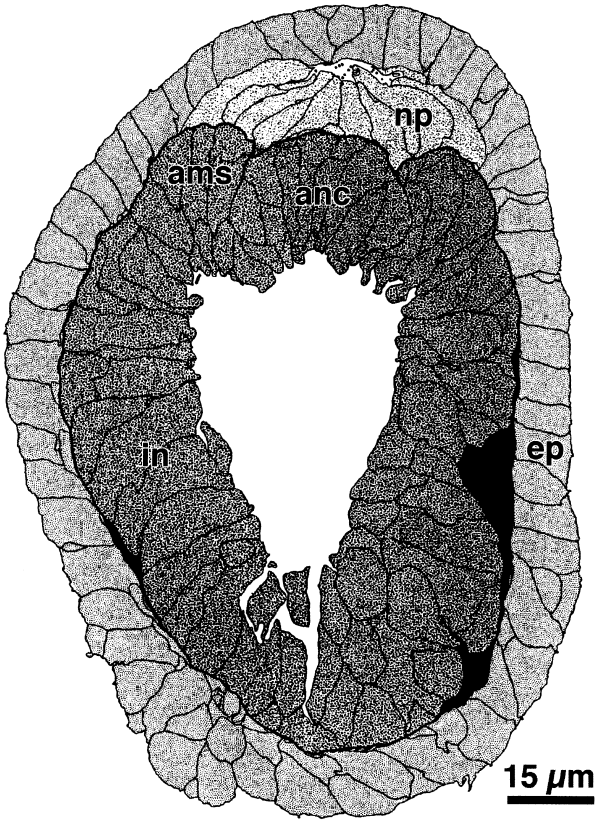

Fig. 3.—Schematic line drawing of an entire cross section through the posterior part of an early neurula of *Branchiostoma lanceolatum*. See Fig. 1 for plane of section. **ams,** anlage of the mesoderm; **anc,** anlage of the notochord; **ep,** epidermis; **in,** intestine; **np,** neural plate.

notochordal cells the dorsal and ventral cells are interconnected by desmosomes mostly basally close to the ecm (Fig. 4C inset). Rarely interconnections of the same type between central and dorsal or ventral notochordal cells are found.

Later neurula stage (35 h post fertilization, 18°C)

The neurula stages of this age are considerably longer and the rostrum at the anterior tip became clearly pronounced. The notochord stretches from the anteriormost tip of the rostrum where it is covered by a thin epidermis to the posterior end of the archenteron. Again the notochord is separated from the intestinal archenteron throughout the greater part of the embryonic body, leaving only the posterior most part of the notochord in continuity with the archenteron (Figs 5A, 6). The number of notochordal cells per myomere is even higher than in the neurula stage described above. About 25 notochordal cells border the medial side of a lateral myomere now (the myomeres themselves are more than twice as long as in the earliest neurula stage described here).

The horizontal sections show that the overall shape of the central notochordal cells considerably changed compared to the previous stage. Although they are still arranged behind each other in single file, the cells as a whole, show a sinusoidal appearance (Fig. 5B). This change in shape makes it difficult in cross sections to discern cell boundaries unambiguously as always several cells are cut and one cell is probably cut several times in one cross-section (Fig. 5A). The central notochordal cells are otherwise comparable in ultrastructure to the ones described from the previous stage. Nevertheless two major changes have to be noted. First, the empty appearing vacuoles within the cells increased their size. Second, some of the cells developed small amounts of myofilaments on this stage (Fig. 5A, lower inset). Due to the difficulties stemming from the altered shape of the central notochordal cells nothing definitive can be said about the dorsal and ventral notochordal cell.

Larva with 1 primary gill slit (110 h post-fertilization, 18 C)

The notochord of this early larval stages is now entirely separated from the intestine throughout the larval body (Fig. 7A, B). The central notochordal cells are still sinusoidal in their outline on horizontal sections (Fig. 7C) leading to the difficulties for the interpretation of cross sections mentioned for the previous stage. The central notochordal cells are now of a very specialized ultrastructural appearance. The empty appearing vacuoles occupy the major part of the cell bodies. The nucleus is situated lateral to this prominent vacuole in the medial part of the notochord and bulges into the vacuole (Fig. 7A, C). Anterior and posterior to the central vacuole a sheath of myofilaments is found within each central notochordal cell. Thick and thin myofilaments are present, the thick ones measuring ≈20 nm in diameter. A pattern of cross striation is not clearly marked in these larvae. Judging from horizontal sections, only intracellular vacuoles are present among the central notochordal cells. Dorsal and ventral to the central notochordal cells nuclei of the dorsal and ventral notochordal cells can be found on cross sections (Fig. 7A). The ventral notochordal cells have obviously acquired a spacious intracellular, empty appearing vacuole, too (Fig. 7A). Although the entire notochord is now clearly separated from the intestine, there is still a gradient of ontogenetic differentiation within the notochord along the antero-posterior axis of the animals. The central cells in the most posterior part of the notochord possess smaller vacuoles and a smaller amount of myofilaments (Fig. 7B). Even few yolk granules are still present in these posterior notochordal cells.

Light microscopic observations of living specimens of this larval stage reveal that the notochordal myofilaments observed by TEM are well capable of contraction. Contraction of the myofilaments results in a noticeable

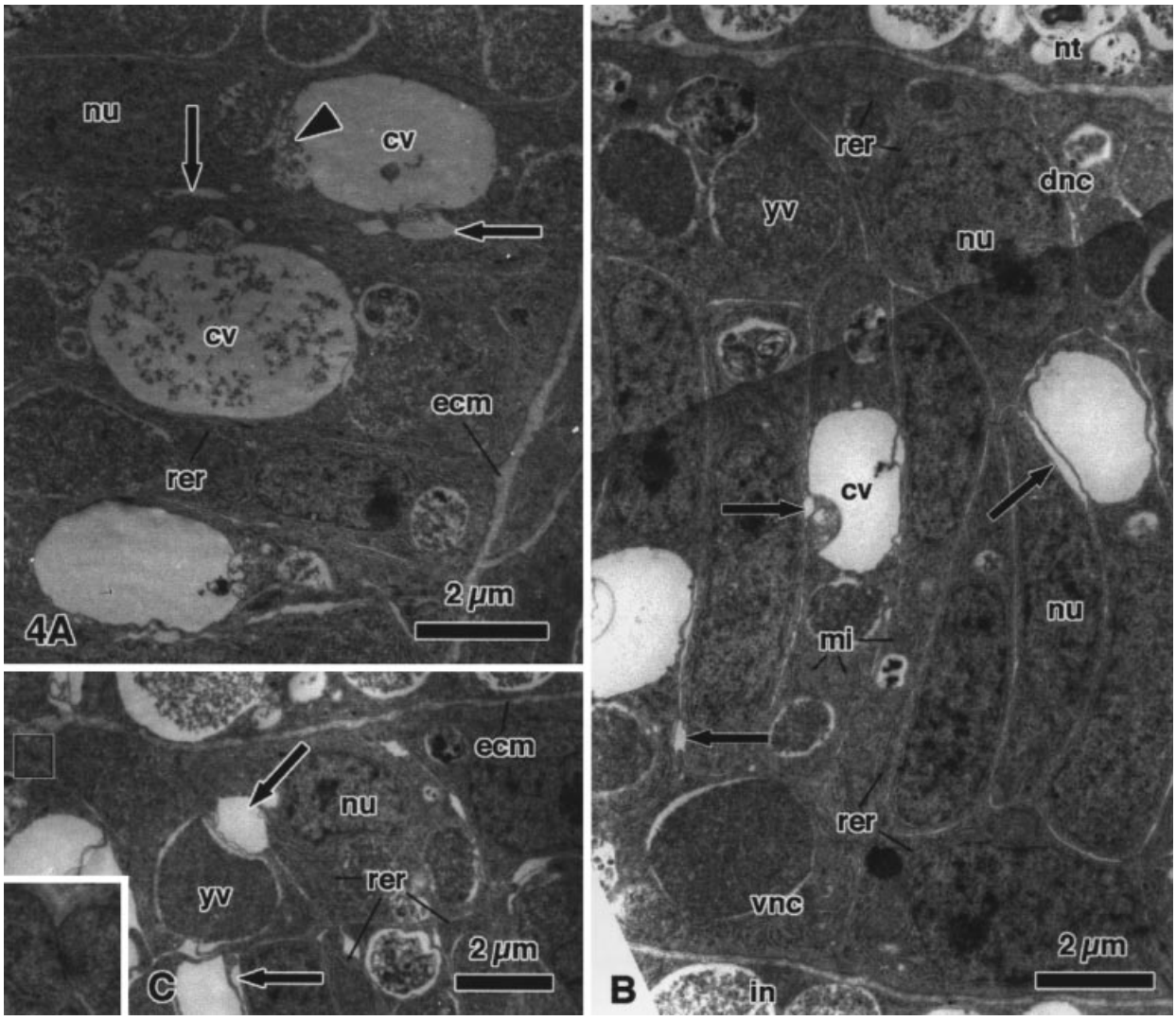

Fig. 4.—Transmission electron micrographs of a neurula stage of *Branchiostoma lanceolatum*.—**A,** horizontal section. —**B, C,** sagittal sections; see Fig. 1 for exact planes of sections. Inset: desmosome junction of the adherens type, enlarged area of the rectangle in C. **cv,** central vacuole; **dnc,** dorsal notochordal cells; **ecm,** extracellular matrix; **in,** intestine; **mi,** mitochondrion; **nt,** neural tube; **nu,** nucleus; **rer,** rough endoplasmic reticulum; **vnc,** ventral notochordal cells; **yv,** yolk granules. Arrows point towards extracellular vacuoles, arrowhead shows numerous small vesicles adjacent to a central vacuole, note that one of the central vacuole in A contains flocculate material.

change of the shape of the central notochordal cells. Such a change of shape during an activation cycle is documented in lateral aspect in Fig. 8. The width of the central vacuole is decreased by about 8% (approx. 0.3 μm) of its usual width of about 3 μm during a cycle of myofilamentous activity.

Discussion

The ontogenetic origin of the notochord from the roof of the archenteron is well known from embryological studies based on light microscopical observations (Hatschek 1881; Cerfontaine 1906; Conklin 1932). Anatomical data from electron microscopic studies are sparse. Flood (1975) and Ruppert (1997b) reported TEM observations of later larval and juvenile stages; Hirakow and Kajita (1994) depicted few cross sections of early neurula stages but found it difficult to discern 'the boundaries between the notochord, the mesoderm and the endoderm'. However, concerned with a general description of the early development of *Branchiostoma belcheri*, these latter authors did not follow the ontogeny of the notochord in detail.

A noticeable difference compared to the reports of light microscopists is seen in the mechanism of the

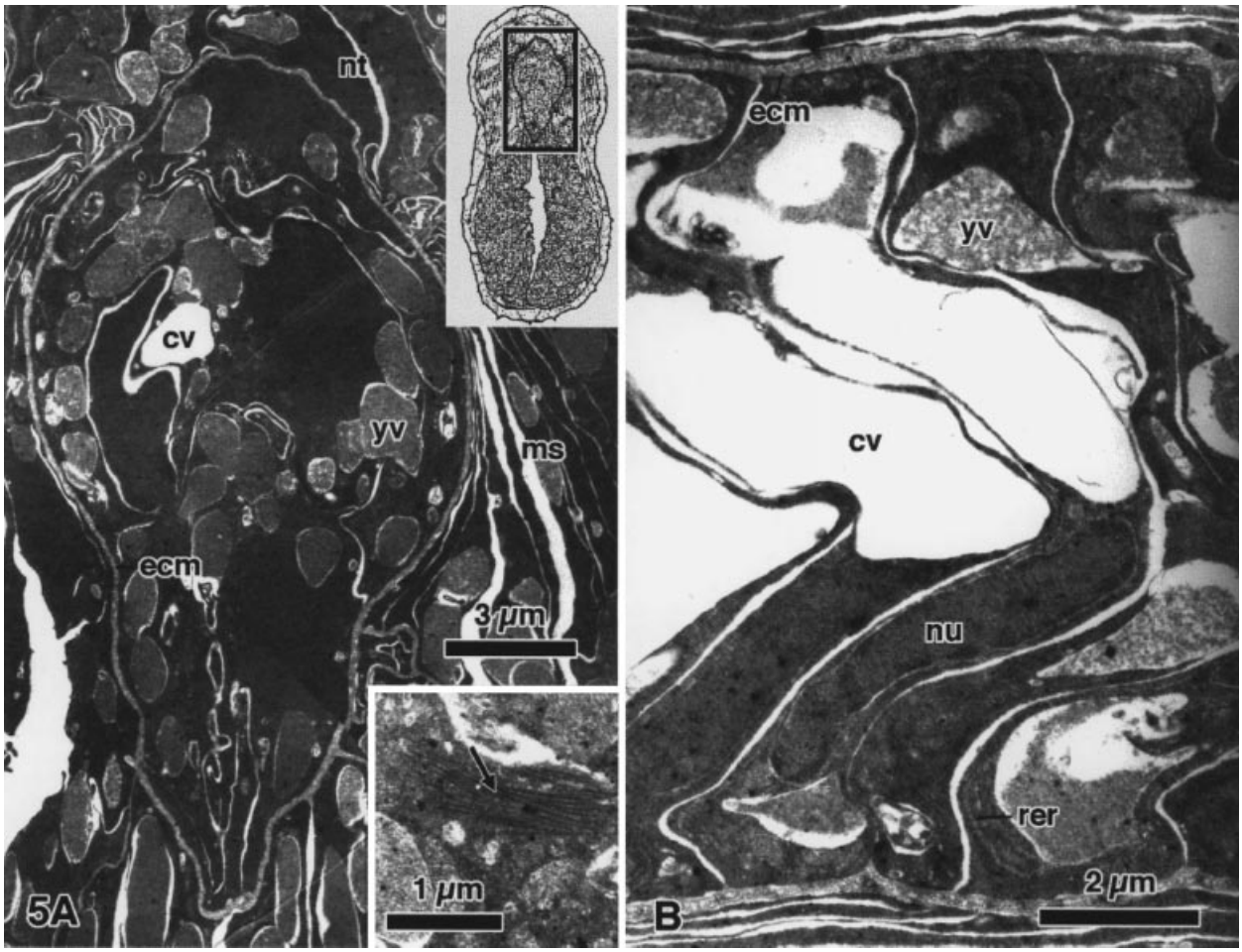

Fig. 5.—Transmission electron micrographs of a later neurula of *Branchiostoma lanceolatum*.—**A,** cross section. —**B,** horizontal section; see Fig. 1 for exact planes of sections. Upper inset: line drawing of the entire cross section with the area enlarged in A indicated by rectangle. Lower inset: enlargement of another cross section of the same individual. **cv,** central vacuole; **ecm,** extracellular matrix; **nt,** neural tube; **nu,** nucleus; **rer,** rough endoplasmic reticulum; **yv,** yolk granules. Arrow points towards myofilaments.

notochord formation. Rather than developing from an outfold of the roof of the archenteron and a subsequent process of rearrangement of the cells (Hatschek 1881; Conklin 1932) it seems that the notochordal cells continuously migrate out of the roof of the archenteron. In particular the prospective notochordal cells do not pinch off from the archenteron epithelially arranged around a central lumen, which later on obliterates. Nevertheless the notochordal tissue is ontogenetically derived from the epithelium of the archenteron and it may be that the cell junctions (probably desmosomes of the adherens type) seen in the notochordal cells are relics of the earlier ontogenetic epithelial organization. It is of interest, that this type of cell junction is to be found in the mesodermal compartments of amphioxus larvae as well (Ruppert 1996; Stach and Eisler 1998).

It was demonstrated in this study that the arrangement of the notochordal cells in a 'stack of coin'-like fashion is established very early during ontogeny, when the notochordal tissue is still in continuity with the epithelium of the archenteron (Fig. 2A). The notochordal cells of tunicates show a similar arrangement during ontogeny although the final adult organization of the notochord may differ profoundly in this taxon (Burighel and Cloney 1997). The similarity in the cellular arrangement of notochordal tissue in single file and the occurrence of extracellular spaces led Ruppert (1997a) to the phylogenetic hypothesis that the Tunicata and the Cephalochordata may be monophyletic sister groups. A survey of literature available about the ontogeny of major craniate taxa, the third group of the Chordata, unambiguously describes an arrangement of notochordal cells in single file during the ontogeny of Hyperotreta (= Myxinoidea, Pasteels 1958), Hyperoartia (= Petromyzonoidea, ibid.), Chondrichthyes (Boeke 1908), Actinopterygii (Boeke 1908), and Amphibia (Hausen and Riebesell 1991). A 'Geldrollenstadium', the

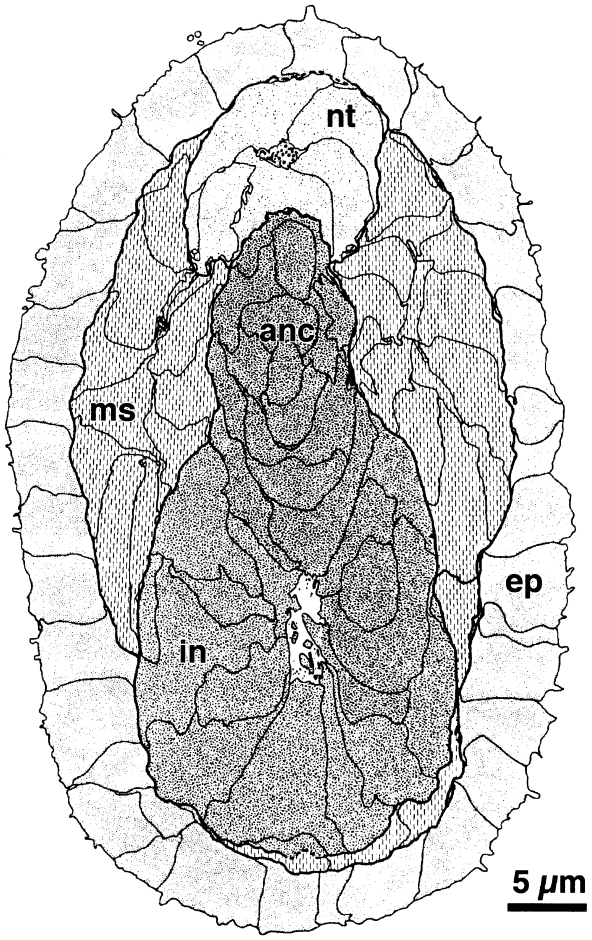

Fig. 6.—Schematic line drawing of an entire cross section through the posterior part of a late neurula of *Branchiostoma lanceolatum*. See Fig. 1 for plane of section. anc, anlage of the notochord; **ep,** epidermis; **in,** intestine; **ms,** mesoderm; **nt,** neural tube.

transitory arrangement of the notochordal cells in single file, is thus present in the ontogeny of the notochord in all craniate groups except the Amniota (see also Starck 1979). The arrangement of the notochordal cells in single file thus has to be interpreted as a plesiomorphy within the Chordata. The occurrence of a transitory 'Geldrollenstadium' in some species of the Tunicata and Craniota may then be termed an ontogenetic recapitulation. The second character in the hypothesis of Ruppert (1997a), the occurrence of extracellular spaces between notochordal cells was also observed in the ontogenetic stages of the cephalochordates in the present study. Whether this character is plesiomorphic within the Chordata or apomorphic for the Tunicata + Cephalochordata, cannot be decided based on current knowledge, mainly because outgroup comparison remains ambiguous.

The nature of Müller's tissue is not yet clearly established (Ruppert 1997b). Welsch (1968) and Welsch and Storch (1973) thought the dorsal Müller cells to be the nucleus-bearing somata of the central muscle plates but adopted in a later account (Welsch and Storch 1976) the alternative interpretation of Flood (1975), that Müller's tissue consisted of entirely distinct cells, not in continuity with the central notochordal muscle plates. With the finding of a group of central notochordal cells bordered dorsally and ventrally by distinctively different cell types (Fig. 4B, C) the present study favours the latter interpretation. The differentiation of the central notochordal cells from nearly undifferentiated embryonic cells into functional muscle lamellae has been demonstrated. The differentiation of the dorsal and ventral notochordal cells, i.e. Müller cells, remains more dubious. Although their distinct cellular nature could clearly be proven, they show little ultrastructural specializations to allow clear-cut functional interpretations. The establishment of microfilaments, and dorsal and ventral cytoplasmic processes to the notochordal horns and the muscular lamellae, respectively, as described for the dorsal Müller cells of adult cephalochordates (Flood 1975; Ruppert 1997b), must occur later during ontogeny. The same holds true for the development of a wide extracellular canal between the dorsal Müller cells. Smaller extracellular spaces demonstrated in this study may represent the anlagen of this canal. The ventral notochordal cells demonstrated for early neurula stages in the present study form large intracellular vacuoles by the early larval stage. They do not however, possess microfilaments, as reported for the ventral Müller cells of the adults (Flood 1975). Both, the dorsal and ventral cells of the ontogenetic stages examined here, lie adjacent to the dorsal and ventral ecm. They show many profiles of rough endoplasmic reticulum and are interconnected by desmosomes. The transcriptional products of this cells may play a role in the generation of the canal system of the adults and its fluid content, but the production of factors that influence the development of other tissues, as demonstrated for the notochord of some craniates (see e.g. Hirano et al. 1995), is also a possible function of Müller cells at this stage. For the adults Welsch (1968) suggested that the dorsal Müller cell may play an important role in the mediation of electrical stimulation from the central nervous system to the muscular plate cells. Flood (1975) renders this hypothesis improbable as he could not find junctional specializations between the dorsal Müller cell and the notochordal muscle cells. In the serial TEM sections prepared for this study no connections between notochordal and neuronal cells, corresponding to the dorsal horns of the adults, could be detected. As the ecm, that covers the entire notochord, is extremely narrow (about 0.2 μm), specialized zones of contact may not be necessary, as diffusion may suffice as transmissionary mode for nervous excitations. It could also be that the notochordal function in these early developmental stages

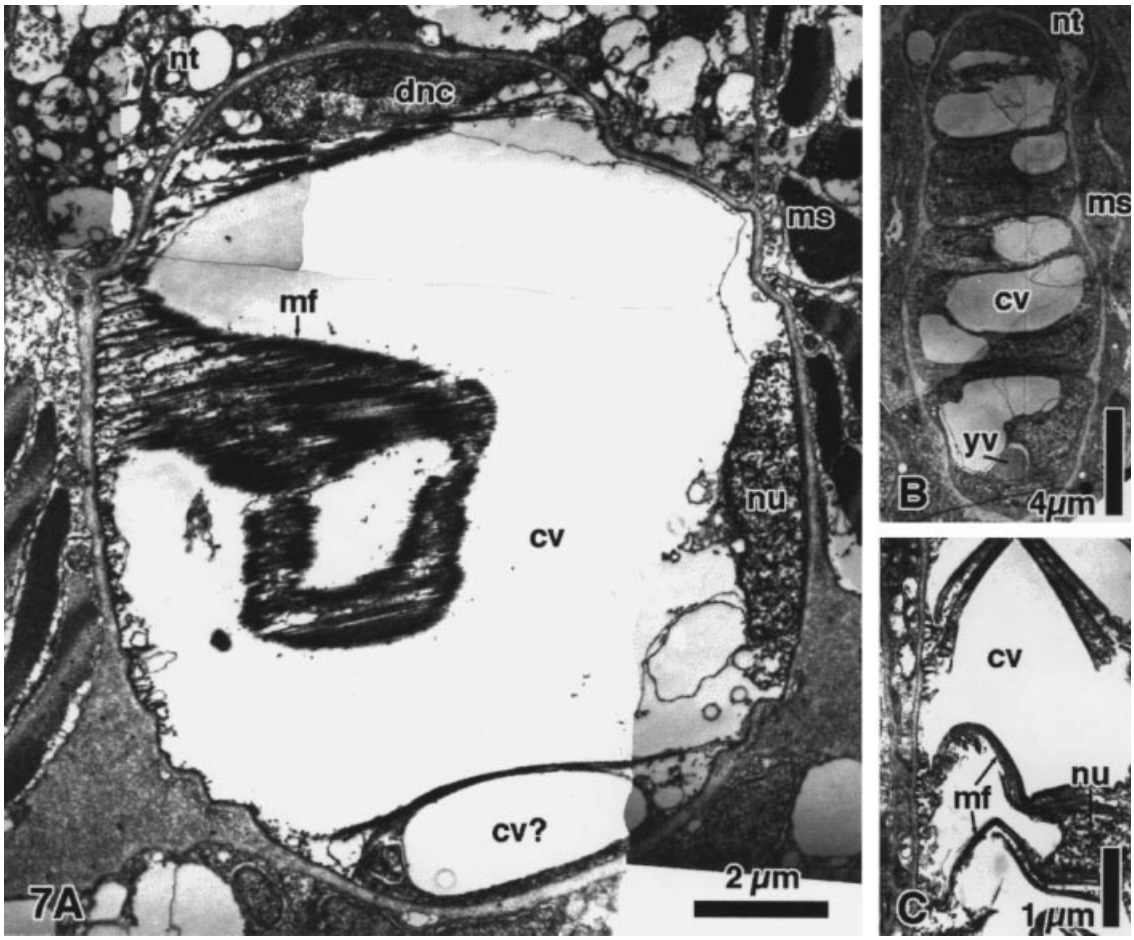

Fig. 7.—Transmission electron micrographs of a larva of *Branchiostoma lanceolatum*.—**A, B,** cross section. —**C,** horizontal section; see Fig. 1 for exact planes of sections. **cv,** central vacuole; **cv?,** possible central vacuole of a ventral notochordal cell; **ecm,** extracellular matrix; **mf,** myofilaments; **ms,** mesoderm; **nt,** neural tube; **nu,** nucleus; **yv,** yolk granules.

does not depend on nervous control at all. Nevertheless the muscular components of the notochord are clearly functional (see Fig. 8) and the larvae of this age are well capable of the same complex locomotory performances as the adults (Stokes 1997). If this is taken as an indication that the function of the larval notochord depends likewise on neuronal control as in adults (Guthrie and Banks 1970), then the dorsal notochordal cells (dorsal Müller cells) have to play a role in the mediation of the nervous signal to the central notochordal cells as already hypothesized by Welsch (1968).

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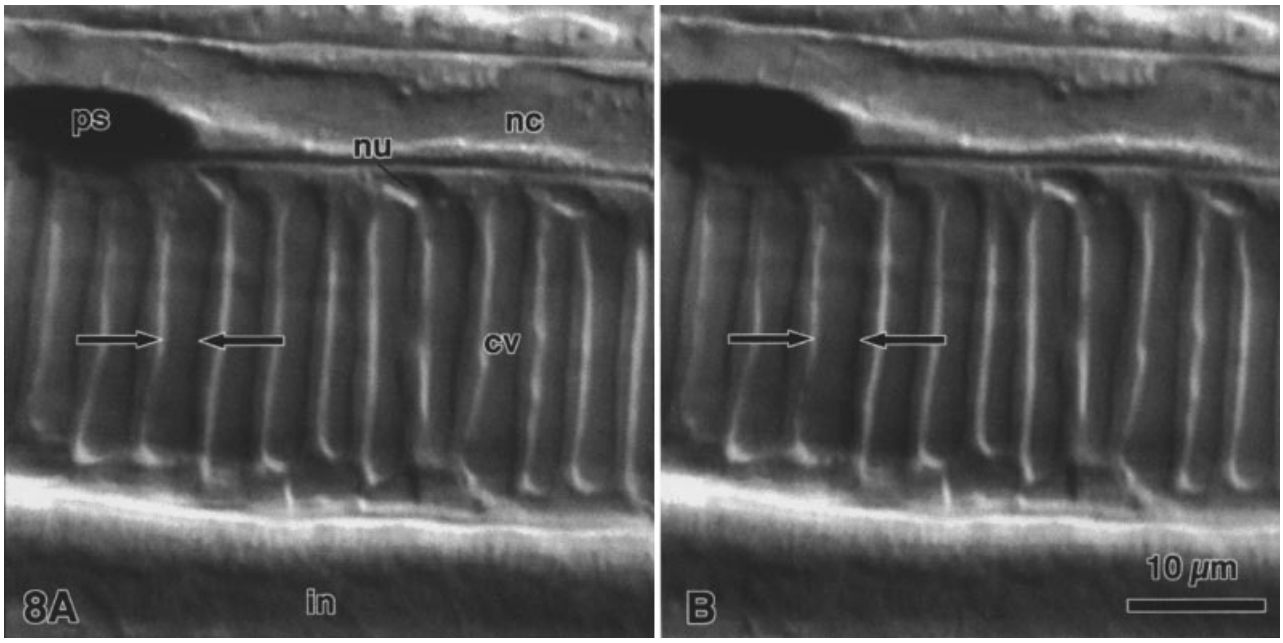

Fig. 8.—Light microscopical observation of a living larva of *Branchiostoma lanceolatum*. Lateral aspect, anterior is to the left. **cv,** central vacuole; **in,** intestine; **nc,** neural canal; **nu,** nucleus of a dorsal notochordal cell; **ps,** pigment spot (first one appearing in ontogeny), arrows demarcate the breadth of the same central vacuole separated by one video frame (= 20 ms) between the two pictures.

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