In Amphioxus Embryos, Some Neural Tube Cells Resemble Differentiating Coronet Cells of Fishes and Tunicates

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Abstract

For neurula embryos of amphioxus (chordate subphylum Cephalochordata), the anterior region of the neural tube was studied with transmission electron microscopy. This survey demonstrated previously unreported cells, each characterized by a cilium bearing on its shaft a protruding lateral bubble packed with vesicles. Such cilia resemble those known from immature coronet cells in other chordates—namely, fishes in the Vertebrata and ascidians and appendicularians in the Tunicata. This wide occurrence of coronet-like cells raises questions about their possible homologies within the phylum Chordata. When considered at the level of the whole cell, such homology is not well supported. For example, the fish cells are generally thought to be glia, while the tunicate cells are considered to be neurons; moreover, cytoplasmic smooth endoplasmic reticulum, which is predominant in the former, is undetectable in the latter. In contrast, a more convincing case for homology can be made by limiting comparisons to the cell apices with their modified cilia. In addition to the fine-structural similarities between fishes and tunicates already mentioned, nonvisual opsins have been found associated with the vesicles in the modified cilia of both groups. Such opsins are thought to link photoreception to endocrine output controlling behavior. Further work would be needed to test the idea that the amphioxus diencephalic cells with lateral bubble cilia might similarly be opsin rich and could provide insights into the evolutionary history of the coronet cells within the phylum Chordata.

Introduction

The story of coronet cells in chordates has been unfolding slowly for a little over a century following the pioneering work of Dammerman (1910) and has taken some unexpected turns along the way. Therefore, it is useful to begin the present contribution with a condensed history of the subject unencumbered by excessive literature citations. Dammerman (1910) used light microscopy (LM) to study a part of the fish brain called the saccus vasculosus—a region alof neuroepithelium located ventrally in the diencephalon and fronting on the third ventricle (Fig. 1A). For the most prevalent cell type there, he demonstrated that the apical end projected a cluster of swollen protuberances into the ventricular lumen (Fig. 1B). The cell apex reminded him of a piece of aristocratic headgear he called a Krönchen, which translates from German to English as "coronet," specifically, the baronial model, which is a metal headband bearing a palisade of pearl-tipped spikes. In addition to describing the structure of the coronet cells, Dammerman speculated (without any experimental data) that they function to sense The ventricular fluid pressure. During the next half century, there were several more LM studies focused on the same piscine cell type, but these added little to what had already been discovered. Bargmann and Knoop (1955) were the first to use transmission electron microscopy (TEM) to study fish coronet cells. Their images were unaesthetic but quite informative. They showed that each apical projection (Fig. 1C) was a greatly modified cilium (now called a globule) that arose from a basal body and included a very short 9+0 axoneme.

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Received 16 November 2022; Accepted 7 February 2023; Published online 8 March 2023.
Abbreviations: CNS, central nervous system; LB, lateral bubble.
The Biological Bulletin, February 2023, volume 244, number 1: 1–8. https://doi.org/10.1086/724581

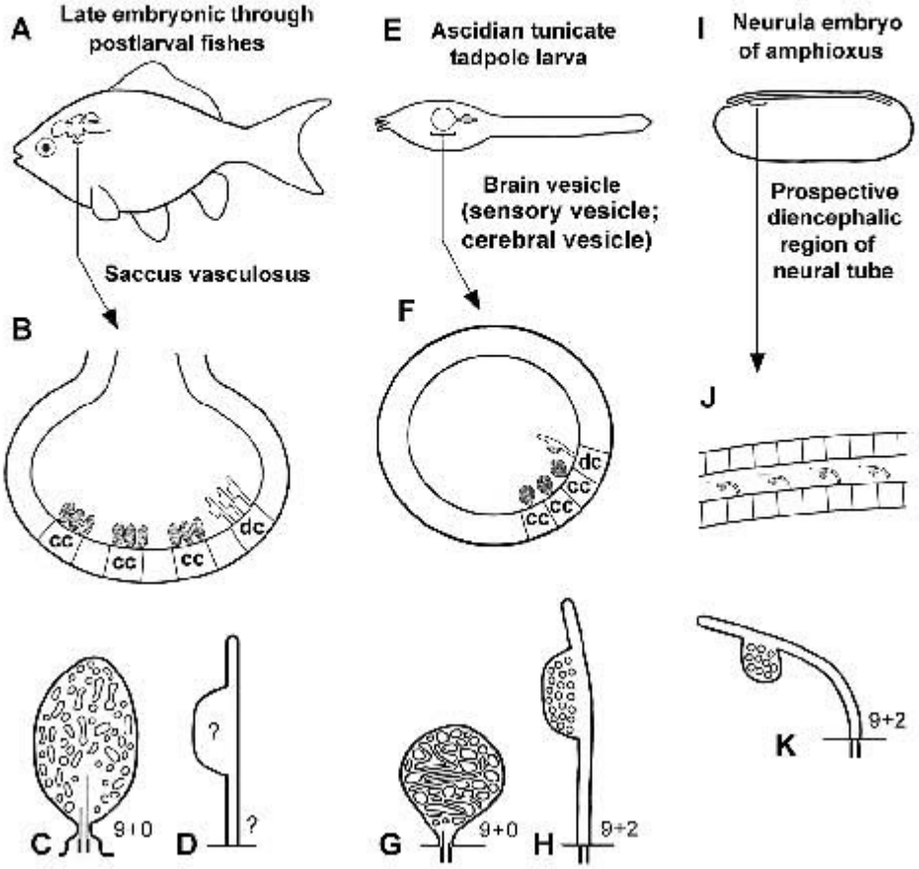

Figure 1. Coronet-like cells in vertebrates, tunicates, and cephalochordates. (A) In chordate subphylum Vertebrata (specifically fishes), coronet cells occur in the neuroepithelium of the saccus vasculosus, a region on the ventral side of the third ventricle. (B) Each coronet cell (cc) projects several modified cilia (termed "globules") into the ventricular lumen. The neuroepithelium also includes a few possibly differentiating coronet cells (dc) characterized by cilia distended by a lateral bubble (LB) of cytoplasm. (C) Globule of a mature coronet cell characterized by a very short 9+0 axoneme and an aggregation of vesicles in the cytoplasmic matrix. (D) Differentiating coronet cell globule (question marks indicate internal features of the flagellum not yet studied with transmission electron microscopy). (E) In subphylum Tunicata, proposed homologs of fish coronet cells occur in the larval brain. (F) The tunicate neuroepithelium includes mature coronet cells (cc) bearing only a single globule; diagrammed near them is a possibly differentiating coronet cell (dc). (G) Detail of a globule on a differentiated coronet cell of a tunicate; there is a short 9+0 axoneme, and the cytoplasmic matrix is filled with vesicles. (H) Detail of a differentiating tunicate coronet cell with a cilium characterized by a 9+2 axoneme (not shown) and associated with a LB of vesicle-filled cytoplasm. (I, J) In subphylum Cephalochordata (amphioxus), the prospective diencephalic region of the neural tube of the neurula embryo includes some cells with a LB cilium thus resembling immature coronet cells of other chordates. (K) Detail of a LB cilium on one of the foregoing cells with 9+2 axoneme (not shown) associated and a LB of vesicle-filled cytoplasm.

basally. In addition, they established that the abundant cytoplasmic matrix filling the globule was not a mass of secretion as formerly believed but represented a packed array of vesicles (the term "vesicle" will be used consistently here, although with the understanding that these structures represent cross sections through a mass of contorted tubules). Over the next 15 years, fish coronet cells were frequently studied by TEM (well reviewed by Zimmerman and Altner, 1970); however, such work added little to an understanding of their structure and led to no consensus about their function.

Dilly (1969) broadened the scope of coronet cell biology by demonstrating that such cells are not limited to piscine vertebrates but are also found in the chordate subphylum Tunicata. More exactly, he demonstrated coronet cells in the wall of the brain vesicle of the tadpole larva of an ascidian tunicate (Fig. 1E). The tunicate cells, as compared to their counterparts in the fish saccus vasculosus, are orders of magnitude less abundant, are clustered together in the neuroepithelium, and project only a single apical globule into the neurocoel (Fig. 1F). Although a cell with only a single protuberance looks nothing at all like a coronet, the name coined for fishes was applied to the tunicates without any discussion. In tunicates, as in fishes, a coronet cell globule (detailed in Fig. 1G) is a modified cilium. It arises from a basal body, includes a very short 9+0 axoneme, and is packed with vesicles. By now, such cells have been demonstrated in two major tunicate groups — in the larvae of many ascidians (Burighel and Cloney, 1997) and throughout the postlarval life of one appendicularian (Olsson, 1975).

The presence of a similar cell type in two different chordate subphyla raised potentially interesting questions about evolution. Even so, ichthyologists long ignored this new development and continued to focus exclusively on the piscine version of the coronet cell. On the positive side, their studies were increasingly augmented with fine structural histochemistry and data from environmental salinity manipulation, although the results were not clear enough to produce a broad consensus about cell function(s). In contrast to fish biologists, tunicate biologists often called attention to the possible homology between their coronet cells and those of fishes (as summarized in Table 1).

Up to now, it was presumed that the coronet cells found in tunicates and fishes lack any counterparts in the chordate subphylum Cephalochordata (commonly called amphioxus). Here we report the unexpected discovery of coronet-like cells in neurula embryos of amphioxus. These cells occur near the anterior end of the central nervous system (CNS) (prospective diencephalic region), where they are ventrally located in the neural tube (Fig. 1I, J). Each such cell (Fig. 1K) bears a cilium characterized by what we are calling a "lateral bubble" (LB) that resembles a similar protuberance on cilia of partly differentiated coronet cells in fishes (Fig. 1D) and tunicates (Fig. 1H). The discussion compares and contrasts these structural features for the three chordate subphyla and also considers proposed functions for chordate coronet cells generally, with special attention to their recently appreciated importance for nonvisual photoreception.

Materials and Methods

Ripe males and females of the Florida amphioxus, *Branchiostoma floridae* Hubbs, 1922, were obtained from a laboratory culture maintained at Scripps Institution of Oceanography (appropriately enough, in Hubbs Hall). Spawning was induced by temperature shock (Holland and Yu, 2004), the eggs were fertilized by addition of sperm, and the embryos were raised at 27 °C. After 17.5 h, the culture comprised relatively late neurula embryos (the N4 stage in the staging system of Carvalho *et al.*, 2021). At this point, the embryos were fixed and processed further for TEM as well as for scanning electron microscopy (SEM) by methods given in detail in Holland (2018).

The TEM illustrations show the specimen best oriented for longitudinal sectioning. In addition, cross sections of the anterior neural tube of two other neurulae at the same stage confirmed the general findings for the longitudinal section, although in a less inclusive context. Because our TEM study was not based on serial fine sections, we could neither determine such details as the exact total number of the cells with LB cilia nor characterize the fine structure of the neural tube in three dimensions.

Results

In the neurula embryo of the Florida amphioxus, we found a dozen or so examples of the neuroepithelial cell type described below in the bracketed zone at the base of the arrow in Figure 1I. Such cells were definitely not present anterior to this zone and probably not posterior to it, although the proposed absence there is based on TEM cross sections (*e.g.*, fig. 3A, B in Mansfield *et al.*, 2015), which are less likely than sagittal sections to detect the diagnostic fine structure. In our Figure 1, the cells of interest are shown progressively enlarged in panels J and K.

The neurula embryo mentioned above is shown by SEM in Figure 2A. This stage is characterized by an epidermis of monociliated cells and a neuropore located dorsally near the anterior end. Figure 2B is a low power TEM of a sagittal section through the anterior end of a comparable neurula.

Table 1

Comparing and contrasting some salient features of coronet cells of fishes, tunicates, and amphioxus neuroepithelial cells with lateral bubble (LB) cilia

	Fish coronet cells[a]	Tunicate coronet cells[a]	Amphioxus LB cilia cells
Neuronal identity	Undecided[b]	Yes[c]	Neurons preaxonogenic[d]
Smooth endoplasmic reticulum	Predominant[e]	Not detectable	Not detectable
Source of vesicles	Globule membrane[f]	Globule membrane[g]	Insufficient sample
Nonvisual opsins	In vesicles[h]	In cell as a whole[i]	No data

[a] The possible homology of coronet cells between fishes and tunicates has sometimes been tentatively suggested on the basis of some commonalities in cytochemistry and developmental genetics (Moret *et al.*, 2005; Razy-Krajka *et al.*, 2012; Oonuma and Kusakabe, 2021).

[b] These fish cells, which have never definitively been identified as neurons, are sometimes called glia (Sueiro *et al.*, 2007; Anadón *et al.*, 2013). Even so, some cytochemical data, if not conclusive, do suggest a neuronal identity (Razy-Krajka *et al.*, 2012; Cid *et al.*, 2015), and the issue remains unsettled.

[c] The axon at the base is very short, but cytochemistry (Moret *et al.*, 2005), synaptic fine structure (Ryan *et al.*, 2016), and developmental genetics (Horie *et al.*, 2018; Cao *et al.*, 2019) strongly indicate neural identity.

[d] Even if cells with LB cilia were neurons, it would be too early in development to tell.

[e] Not source of vesicles within the globule as once claimed.

[f] By invagination (Corujo *et al.*, 1990; their fig. 10).

[g] By invagination (Eakin and Kuda, 1971; their fig. 13).

[h] Melanopsin and short-wavelength sensitive opsin localized in vesicle membranes, a possible link between photoreception and endocrine output controlling reproduction (Nakane and Yoshimura, 2019).

[i] Melanopsin found in cornet cell as a whole, including the globule, perhaps playing a key role in translating fading light level at dusk into the start of metamorphosis (Lemaire *et al.*, 2021).

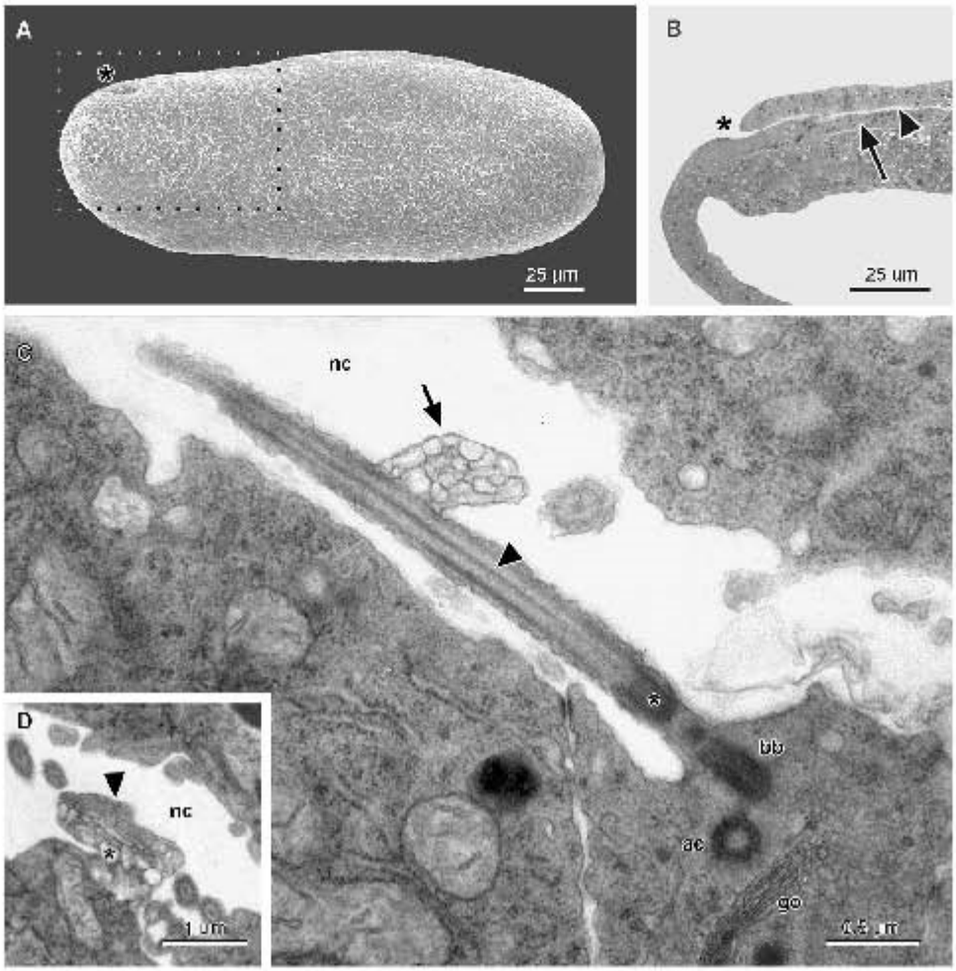

Figure 2. Moderately advanced neurula embryo of amphioxus. (A) Scanning electron microscopy of the whole embryo in left-side view. The neuropore (asterisk) opens on the dorsal side near the anterior end. (B) Low-power transmission electron microscopy (TEM) of the region outlined with dots; this midsagittal section shows the neuropore (asterisk) opening into the neurocoel (arrowhead), running middorsally within the tubular neuroepithelium; a few cells resembling immature coronet cells of other vertebrates are located ventrally (arrow) in the neuroepithelium. (C) TEM of apical parts of three neuroepithelial cells fronting on the neurocoel (nc), one that (lower right) has a lateral bubble (LB) cilium (arrow) similar to the sort characterizing immature coronet cells in other chordates. Other cilium-associated structures are a 9+2 arrangement of microtubules (the arrowhead indicates the central pair), a dense basal transition zone (asterisk), and a basal body (bb) near an accessory centriole (ac) and a Golgi complex (go). (D) TEM of the apex of a neuroepithelial cell (evidently not ciliated) with a cytoplasmic protrusion (arrowhead) bulging into the neurocoel (nc) and containing some vesicles (one marked by an asterisk).

The neuropore opens into the neurocoel, which runs down the center of the neural tube. The arrow in Figure 2B indicates the ventral side of the neural tube, where the neuroepithelium includes the dozen or so cells that are reminiscent of the developing coronet cells of other chordates.

Figure 2C is a higher magnification TEM within the neuroepithelial region arrowed in Figure 2B; the anterior is toward the left. The apical region of one of these neuroepithelial cells (bottom right) projects its distinctive cilium into the neurocoel. The shaft of the cilium is associated with a lateral, bubble-like excrescence (arrowed). The bubble is packed with vesicles ranging in diameter from about 50 to 200 nm. The shaft of the cilium is characterized by a 9+2 axoneme (the central microtubule pair is conspicuous; arrowhead) and a basal, relatively dense region (asterisk) that may correspond to a transition zone (*sensu* Avidor-Reiss *et al.*, 2017). In the apical cytoplasm, the cilium arises from a basal body that has neither a basal foot nor a ciliary rootlet but is adjacent to an accessory centriole associated with a Golgi complex. Strikingly, no smooth endoplasmic reticulum can be demonstrated anywhere in the cytoplasm of these neuroepithelial cells. In this respect, the amphioxus cells resemble coronet cells in larval tunicates (Eakin and Kuda, 1971) but differ strikingly from fish coronet cells, where smooth endoplasmic reticulum is superabundant.

Figure 2D shows the apex of a nonciliated neuroepithelial cell in the prospective diencephalic region of the neurula embryo of amphioxus. Protruding slightly into the neurocoel, there is a bleb of apical cytoplasm (arrowhead) containing a cluster of vesicles. These vesicles are evidently unrelated to smooth endoplasmic reticulum, because the latter organelle is undetectable anywhere in such cells. It is

not known whether the apical cluster of vesicles is somehow related (*e.g.*, as a very early developmental stage) to the vesicle cluster in the LB cilia. For the saccus vasculosus of fishes, similar vesicle clusters have been described in the apical cytoplasm of some nonciliated cells in the neuroepithelial lining by Jansen and Flight (1969), who were unsure of their function and fate.

Discussion

Immature stages of fish and tunicate coronet cell globules compared to amphioxus neuroepithelial cells bearing a lateral bubble cilium

The present study suggests that the amphioxus cells with an LB cilium might be comparable to immature coronet cells in other chordates. It is therefore important to review what is known about coronet cell differentiation in fishes and tunicates. For fishes, the work of Rodríguez Moldes and Anadón (1988) indicates that such cells comprise a slowly growing population. Consequently, at a given time, fully differentiated cells predominate in the neuroepithelium of the saccus vasculosus, while their developing precursors are rare and widely scattered in the tissue. One such differentiating cell seen by SEM (illustrated in fig. 4 of Rodríguez Moldes and Anadón, 1988) bore on its apex numerous cilia, each characterized by a conspicuous lateral "bubble" of cytoplasm that the authors suggested might be gliding along the ciliary shaft. Unfortunately, this rare cytodifferentiative stage could not be found during a subsequent TEM study of the fish saccus vasculosus by Corujo *et al.* (1990). The chances of finding a rare cell stage in a large tissue volume with conventional TEM are vanishingly small (TEM based on serial sectioning or serial block face scanning electron microscopy would be much more likely to succeed). In Figure 1D of this paper, the lack of information on internal structure of the presumably differentiating ciliary globule is indicated by question marks.

Tunicates, unlike fishes, develop according to a fixed cell lineage. Consequently, the differentiation of tunicate coronet cells can be followed from two progenitor blastomeres situated in the neural plate of the embryo (Cao *et al.*, 2019; Hartenstein *et al.*, 2021) to a clone of about a dozen definitive cells in the wall of the brain vesicle of the swimming larva. Unfortunately, in spite of all the work done on this developing system, there are no data showing the coronet cells caught in the act of transitioning *en masse* to their distinctive morphology. Such a transition is doubtlessly quite rapid, because, in organisms generally, ciliogenesis can occur in just a few minutes (Dingle and Fulton, 1966). It would be interesting to sample the development of tunicate larvae with TEM on a temporal schedule finely spaced enough to capture a sample of coronet cell precursors entering the early stages of differentiation into the definitive cell type.

Importantly, even during normal tunicate development, not every coronet cell in the clone reaches full differentiation before the end of the brief larval stage. Eakin and Kuda (1971) found a late differentiation stage (their fig. 12) with a relatively long axoneme and a lateral, vesicle filled bubble near the fully differentiated cells (their fig. 11). Moreover, Konno *et al.* (2010) illustrated a mature coronet cell next to what seems to be an early differentiation stage (their fig. 4D) with a cilium bearing a lateral cytoplasmic bubble containing vesicles and closely resembling the amphioxus LB cilium described in this paper.

The question of homologies among coronet-like cells of the three chordate subphyla

Here homology is considered from the viewpoint of the conventional historical concept and judged by the recognition criteria of relative position and special quality (Remane, 1956) and with special attention to levels of biological organization (Striedter and Northcutt, 1991). It should be noted that the proposed homologies discussed here are not being used to help determine phylogenetic tree topography. The tree shape is already well established for the chordate subphyla, with cephalochordates sister group of the tunicates plus vertebrates (Delsuc *et al.*, 2006). Thus, one has the luxury of mapping characters onto a known tree to propose whether they are innovations of a particular subphylum or indicators of a common ancestry.

Table 1 is a synoptic comparison of some prominent characters among fish and tunicate coronet cells as well as amphioxus cells with LB cilia. By relative position, the gross anatomical position of the cells in question—ventrally within the diencephalic region of the CNS—is consistent with their proposed homology across all three chordate subphyla. In contrast, by the criterion of special quality, the homology between fish and tunicate coronet cells, considered at the level of the cell as a whole, is poorly supported. First, the tunicate cells are dopaminergic neurons (Moret *et al.*, 2005; Horie *et al.*, 2018; Cao *et al.*, 2019), while the fish cells may well be glia, based on their reported expression of glial markers (Sueiro *et al.* 2007). However, there are conflicting reports about whether fish coronet cells have axons (Dammerman, 1910; Sueiro *et al.*, 2007; discussed in Ryan *et al.*, 2016), but they do express melanopsin, suggesting neuronal function. Resolving this question remains a goal of future research.

Establishing whether amphioxus cells with LB cilia are neuronal is another unanswered question. However, at the relatively early developmental time studied here, no cell anywhere in the CNS has yet produced neurites. At present, the later developmental fate of the neuroepithelial cells with LB cilia is unknown. Disconcertingly, a thorough fine structural study of the amphioxus diencephalon (Lacalli and Kelly, 2000) at a somewhat later developmental stage (the early larva) did not clearly demonstrate any cell type(s) obviously descended from cells with LB cilia. At the late neurula stage examined here, fine structural criteria alone are inadequate for distinguishing between most cell types in the CNS. However, the problem might be alleviated by

studying gene expression known to be cell type specific very early in development, even before advent of morphologically detectable cytodifferentiation. For instance, the *spondin* gene transcribed early in the larval subcommissural organ (Bozzo *et al.*, 2021) might be expressed even earlier in cells before they show morphological signs of becoming definitive cell types. Such gene expression in closely spaced developmental stages might throw light on the later development of cells with LB cilia and indicate their possible neuronal fate. If they do prove to be neurons, this feature could be considered plesiomorphic for chordates, and it could be concluded that fish coronet cells have lost their neuronal identity during the course of evolution.

A second striking difference between fish coronet cells and those of tunicates is the great abundance of smooth endoplasmic reticulum in the cell body of the former and its virtual absence in the latter. This discrepancy has not been commented upon in the literature, and available data throw no light on the role (or roles) the organelle plays in fish coronet cells. The predominant smooth endoplasmic reticulum appears to be an apomorphy of fish coronet cells because it is found neither in their tunicate counterparts nor in amphioxus cells with LB cilia.

In comparison to the lack of support for homology of chordate coronet cells when considered at the level of the whole cell, the evidence (Table 1) for homology is more compelling when considered at the organellar level—that is, with the focus limited to the cell apices. In fishes and tunicates, the globules are obviously modified cilia, as are the LB cilia of the amphioxus cells described in this study. Moreover, in both fishes and tunicates, the membrane bounding the coronet cell globule invaginates to form the vesicles within (Eakin and Kuda, 1971; Corujo *et al.*, 1990). A comparable origin was not detected on the LB of the cilia of the amphioxus cells but could easily have been missed because invagination appears to be a rare event and the number of sampled amphioxus cells was small.

The foregoing criterion of structural special quality was strikingly strengthened by that of functional special quality—namely, that the membrane system represented by the vesicles in both fishes and tunicates is characterized by opsins such as melanopsin (Lemaire *et al.*, 2021) that receive non-visual light and link it to endocrine output controlling behavior and reproduction. One yet unaddressed problem here is that vertebrate and tunicate coronet cells, although clearly ciliary photoreceptors, are characterized by opsins that are otherwise known to occur only in rhabdomeric photoreceptors. There is a parallel problem in amphioxus, where photoreceptors known to be melanopsin-rich are rhabdomeric (Konagaya *et al.*, 2005; Ferrer *et al.*, 2012), while the cells with LB cilia (if in fact photoreceptors) would be of the ciliary variety.

In the cells with LB cilia described here, the possible presence of opsins in the vesicle membranes has not been investigated. It is also unknown whether light stimulation of the cells with LB cilia impacts the behavior of the neurula embryos. The LB cilia might be involved in light reception influencing hormone output and subsequent behavior, for example, by influencing ciliary swimming of amphioxus neurulae, which are known to be phototropic well before the differentiation of the first obvious light-receptive organ of Hesse. In sum, the demonstration of amphioxus cells with LB cilia has raised a number of specific questions that should be resolved to help address the broader issues of homology and evolution of chordate coronet cells.

Acknowledgments

Amphioxus laboratory culture at Scripps Institution of Oceanography is supported by National Science Foundation grant IOS-1952567 to Linda Z. Holland and N.D.H. Transmission electron microscopy (TEM) support for J.H.M. was supplied by a Presidential Research Award from Barnard College. Theresa Fassel and Malcolm R. Wood of the Scripps Research Institute microscopy core provided skillful help with scanning electron microscopy and TEM preparation and visualization. Our manuscript was improved by the critical comments of Linda Z. Holland.

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