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Anatomy of the lamprey ear: morphological evidence for occurrence of horizontal semicircular ducts in the labyrinth of *Petromyzon marinus*

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Abstract

In jawed (gnathostome) vertebrates, the inner ears have three semicircular canals arranged orthogonally in the three Cartesian planes: one horizontal (lateral) and two vertical canals. They function as detectors for angular acceleration in their respective planes. Living jawless craniates, cyclostomes (hagfish and lamprey) and their fossil records seemingly lack a lateral horizontal canal. The jawless vertebrate hagfish inner ear is described as a torus or doughnut, having one vertical canal, and the jawless vertebrate lamprey having two. These observations on the anatomy of the cyclostome (jawless vertebrate) inner ear have been unchallenged for over a century, and the question of how these jawless vertebrates perceive angular acceleration in the yaw (horizontal) planes has remained open. To provide an answer to this open question we reevaluated the anatomy of the inner ear in the lamprey, using stereoscopic dissection and scanning electron microscopy. The present study reveals a novel observation: the lamprey has two horizontal semicircular ducts in each labyrinth. Furthermore, the horizontal ducts in the lamprey, in contrast to those of jawed vertebrates, are located on the medial surface in the labyrinth rather than on the lateral surface. Our data on the lamprey horizontal duct suggest that the appearance of the horizontal canal characteristic of gnathostomes (lateral) and lampreys (medial) are mutually exclusive and indicate a parallel evolution of both systems, one in cyclostomes and one in gnathostome ancestors.

Key words: horizontal ducts; jawless vertebrates; lamprey; parallel evolution.

Introduction

In the late 19th century, Retzius (1881) provided an anatomical description of the cyclostome (Myxines and Petromyzon) ear in a series of comparative anatomical studies of the ear of different classes of vertebrates. In Myxines (hagfish), he described a labyrinth with one torus comprising a single vertical canal ending in one ampulla at each end with a common macula in between. In Petromyzon (lamprey), he described a more elaborate labyrinth with two canals, three maculae with a common otolith on top, and two ciliated chambers. The ciliated chambers are unique features of the lamprey among all other vertebrates. Retzius' observations were confirmed in a later study by de Burlet & Versteegh (1930) and others. Lowenstein et al. (1968) performed a thorough analysis on the lamprey ear using classical histological and TEM methods. Although they provided a detailed account on the cristae and maculae morphology, as well as the hair cell morphological polarity, they agreed that the lamprey ear has two canals. They considered them functional homologues of the anterior and posterior vertical canals of higher vertebrates, and therefore concluded that the horizontal canal is missing in the lamprey. Similar conclusions were made by Hagelin (1974) in a detailed histological investigation of the developing labyrinth in larval lampreys.

To date, these early descriptions and suggestions of horizontal movement detections have remained unchallenged, and the lamprey ear has often been cited in subsequent studies dealing with physiological, molecular and evolutionary analyses of the inner ear in vertebrates as having no horizontal canal (of the gnathostome kind). In fact, the lamprey eye muscle orientation and innervation is unusual in that two muscles are innervated by the abducens nerve instead of one and only three muscles (instead of four) are

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innervated by the oculomotor nerve (Fritzsch et al. 1990). However, the lamprey, a lateral-eyed vertebrate, has rostral and caudal recti muscles with a line of pull in the horizontal planes, making them functional homologues for the lateral and medial recti in frontal-eyed vertebrate (Rovainen, 1976; Fritzsch et al. 1990). This raises the question of how the lamprey perceives angular horizontal movement and how any horizontal movement detection is related to the different extraocular muscle and a different ocular motor innervation (Fritzsch, 1998). It is, in principle, possible that the lamprey has no need to perceive horizontal angular movement due to peculiarities in its locomotor behavior. However, the lamprey swims using rhythmic lateral undulation of the body, generated by a central pattern generator in the spinal cord. This movement entails rhythmic sinusoidal movement of the head in the horizontal plane previously described by Hagelin (1974). Thus, perception of horizontal angular movement is theoretically an essential component for this locomotor behavior to provide the appropriate postural and ocular reflexes mediated by vestibular nuclei onto ocular and spinal motor neurons.

Electrophysiological recordings from the VIII nerve in the lamprey have revealed responses to natural angular stimuli in all planes, including the horizontal (Lowenstein, 1970). More interestingly, Lowenstein (1970) reported that the anterior branch of the canal crista responded with deactivation on ipsilateral turns and activation on contralateral turns in the horizontal (yaw) plane. The latter responses differ from that of the horizontal canal responses in higher vertebrates. In contrast, recordings from the posterior branch revealed activation on ipsilateral horizontal angular stimuli, and deactivation on contralateral horizontal angular stimuli. This response pattern is similar to that of the horizontal canals in higher vertebrates. Thus, the lamprey vestibular system does indeed respond to horizontal movement, although it responds with a different pattern than that observed in gnathostome vertebrates. This intriguing observation raises the question: where do these antagonistic responses to angular horizontal stimuli originate, recognizing the apparent absence of a horizontal canal?

Lowenstein (1970) attributed these responses to horizontal angular acceleration to the elaborate shape of the cristae in the vertical canals. The cristae of the two vertical canals are unique among vertebrates. The two cristae are trifid (three-armed) with vertical, horizontal and basal arms (Lowenstein et al. 1968). He speculated that the vertical deployment of some parts of the trifid cristae render them sensitive to endolymph displacement in the horizontal plane. However, behavioral studies suggested that ocular and postural responses to the movement in the yaw plane were as robust as those obtained in the movement in the roll and a pitch planes (Rovainen, 1976; Wannier et al. 1998; Zelenin et al. 2003; Pflieger & Dubuc, 2004; Karayannidou et al. 2007). This raises the possibility of the presence of anatomical substrate, i.e. a duct in the horizontal plane to provide an effective and discrete endolymph flow. Hagelin (1974) considered previous hypothesis of involvement of the unusual two ciliated chambers of lampreys and suggests that endolymph from ciliated chambers can, on rapid horizontal movement, contribute to endolymph movement of the canals through existing connections, despite the fact that previous work showed no such endolymph exchange using carbon particles. In essence, none of these suggestions is straightforward and can explain how endolymph movement occurs in a horizontal plane to be perceived by at least a part of the canal cristae as recorded by Lowenstein (1970).

This unresolved question prompted us to reevaluate the morphology of the adult lamprey labyrinth in a quest for parts of the duct system associated with the unusual trifid canal end organs that may be geometrically capable of translating horizontal angular stimuli into an endolymph flow capable of cupular deflection and hair cell mechanotransduction in the horizontal plane. Here, we provide morphological evidence that the lamprey ear has two horizontal semicircular ducts in each labyrinth, allowing directions of endolymph flow in a horizontal (yaw) plane. We propose a model that explains the agile response of the lamprey to lateral undulation movement and dual antagonist electrophysiological responses. Lampreys, apparently in parallel with jawed vertebrates, have evolved a genome size and complexity comparable to jawed vertebrates (Smith et al. 2013). In addition, we show here that lampreys have also evolved in parallel an anatomically distinct horizontal duct system. Part of this study has been published as an abstract (Trotter et al. 2013).

Materials and methods

Twenty-five adult lampreys, *Petromyzon marinus*, were used for the current study. Petromyzon were obtained from Hammond Bay Biological Station (Millersburg, MI). They were euthanized by tricaine (200 mg L^{-1}). The animals were perfused through the heart with 4% paraformaldehyde in phosphate-buffered saline (PBS). They were then decapitated and their heads were postfixed in 4% paraformaldehyde in PBS overnight. The otic capsules were exposed by removing the muscle on the lateral side of the head, and then extracted from the head. The lateral wall of each cartilaginous otic capsule was removed to reveal the membranous labyrinth, which was dissected out and cleaned to expose the duct system surrounding the ciliated chambers. The furrows and groove between the ducts and ciliated chambers are filled with a delicate connective tissue strands forming a net-like structure that is loaded with a sandy-looking ground substance and perilymph. With the exception of the ducts on the lateral surface of the ciliated chambers, which were obvious without any cleaning, the remaining ducts were buried in this mesh of connective tissue and ground substance and required meticulous cleaning to excavate the entire outline of the semicircular duct system. The labyrinths were dissected under a dissecting microscope (Wild, M420) connected to a camera (Leica, EC3). Images were taken from all angles to reveal the entire extent of the complex duct system and the two bipartite ciliated chambers, and were used to make a three-dimensional rendition of the whole labyrinth using MAXON CINEMA 4D software. All experiments were conducted in accordance with NIH guidelines for animal care and use, and approved by the University of Mississippi Animal Care and Use Committee.

For scanning electron microscopy, dissected labyrinths were processed as described elsewhere (Popper & Hoxter, 1987). In summary, dissected labyrinths were post-stained using the O-T-O-T-O (1% osmium tetroxide and 1% thiocarbohydrazide) method for building electron conductivity in the samples so that charging of the samples in the SEM would be minimized. After fixation, the samples were run through a graded series of acetone at 10-min intervals and transitioned into tetramethylsilane, before being air-dried. The tetramethylsilane is of such low surface tension that it is possible to use air drying as an alternative to critical point drying, thus producing a minimal amount of drying artifacts. After the samples were dried, they were mounted on SEM stubs and coated with 15 nm gold in a Hummer × sputter coater, and examined by a scanning electron microscope (JEOL JSM T-300).

Results

Stereoscopic dissection of the lamprey ear revealed that the labyrinth was enclosed in an oval cartilaginous otic capsule located behind the eye. Viewed laterally from above, the long axis of the otic capsule (Fig. 1A; dotted line a) lay in the horizontal plane and passed approximately through the center of the eye, 1 cm below the dorsal midline (Fig. 1A; dotted line c), which was marked by the opening of the

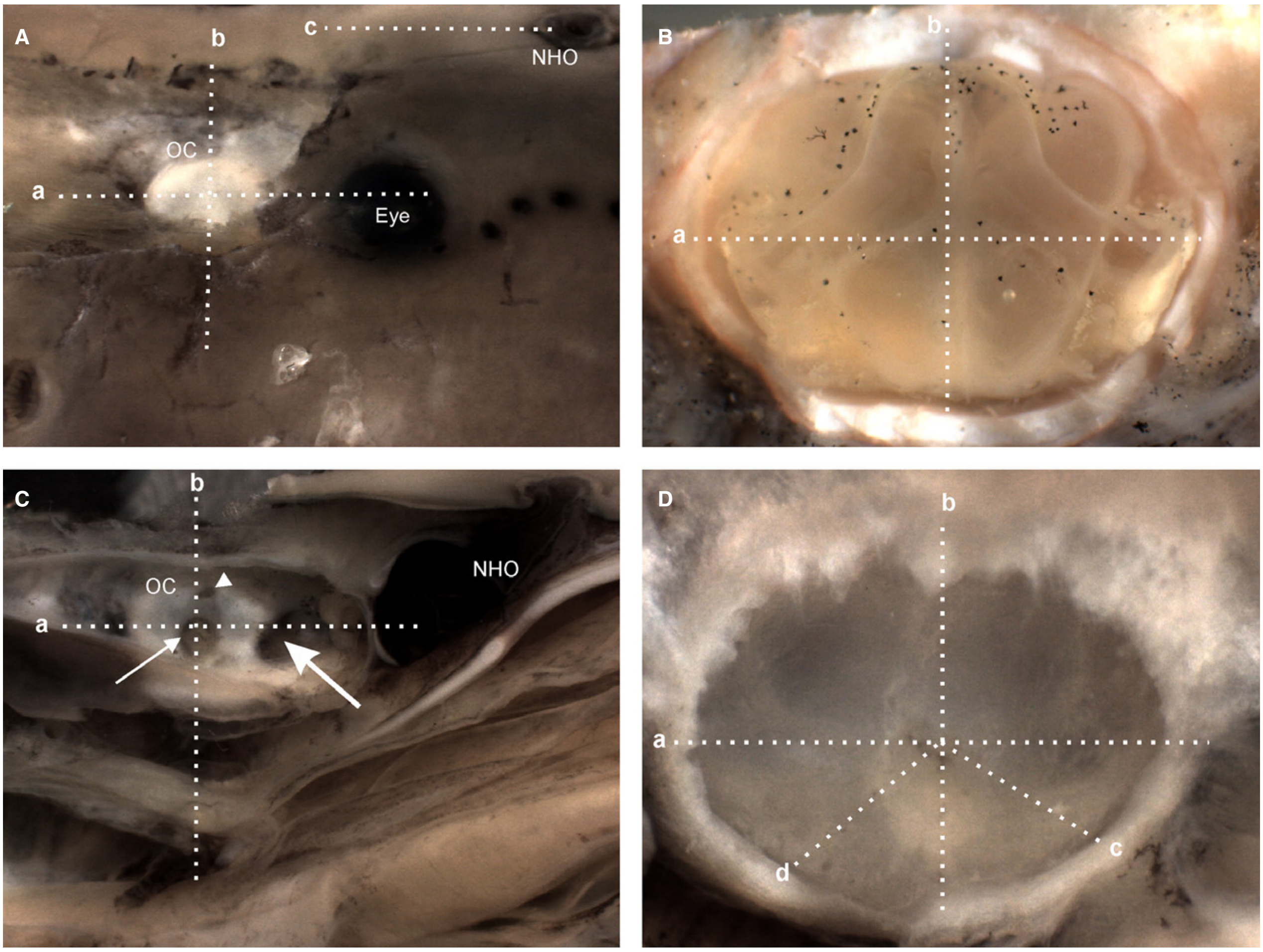


Fig. 1 Location and orientation of the labyrinth in lamprey. (A) Lateral surface of the skull, with otic capsule exposed, showing the oval shape of the otic capsule (OC) with the long, horizontal axis (dotted line a) passing 1 cm below the dorsal midline (dotted line c), which runs through the dorsal opening of nasohypophyseal organ (NHO). The short vertical axis (dotted line b) passes approximately 7 mm behind the center of the eye. (B) Lateral surface of the membranous labyrinth after removing the lateral wall of the otic capsule. Note that the membranous labyrinth fills the entire otic capsule. (C) Medial surface of the otic capsule (OC) viewed from the interior of skull. The horizontal axis passes through the upper margin of the optic canal (thick arrow), which is located at the anterior pole of otic capsule. The opening for the VIII cranial nerve (thin arrow) is located at the intersection of horizontal and vertical axes (dotted lines a and b). The opening for the endolymphatic duct (arrowhead) lies at the upper margin of the otic capsule at the vertical axis. (D) The medial surface of the membranous labyrinth after removal of the medial wall of the otic capsule. On the medial wall, the mean plane of two horizontal ducts is located at the horizontal axis, the equator (dotted line a). The mean planes for the two vertical ducts (dotted lines c and d) are located on the medial surface on diagonal planes midway between the horizontal and vertical axes (line a and b).

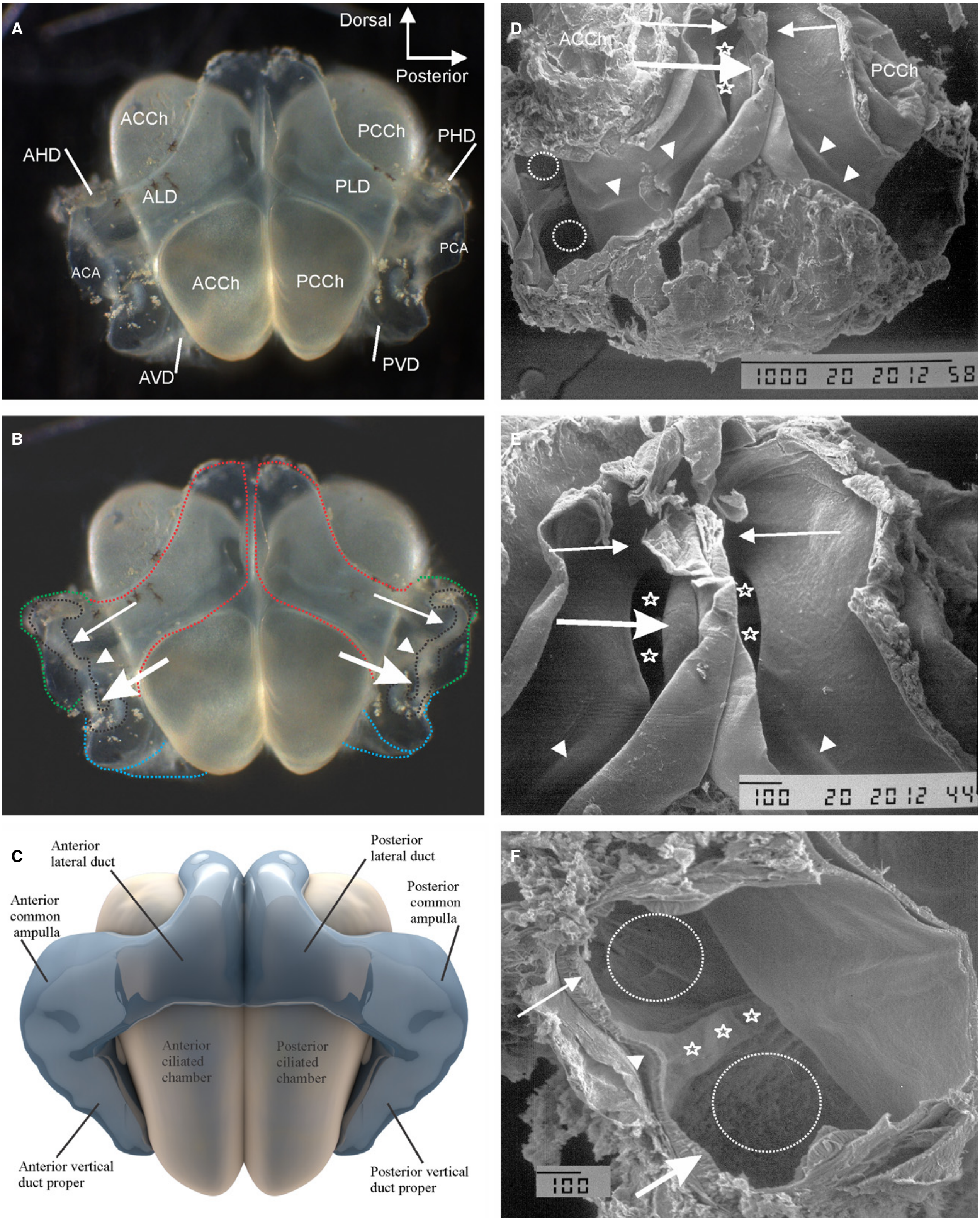
nasohypophyseal organ (Fig. 1A; NHO). The short axis lay in the vertical plane, sitting 4 mm behind the center of the eye (Fig. 1A; dotted line b). Inside the otic capsule the membranous labyrinth was formed by two ciliated chambers (de Burlet & Versteegh, 1930; Hagelin, 1974; Popper & Hoxter, 1987) and was surrounded by an elaborate system of semicircular ducts (Fig. 1B). The long axis (the equator) divided the membranous labyrinth into asymmetrical dorsal and ventral halves and was in the approximate mean plane of the horizontal ducts (Fig. 1B; dotted line a). The short, vertical axis divided the labyrinth into symmetrical anterior and posterior halves and lay at the divide between the two ciliated chambers (Fig. 1B; dotted line b). The medial surface of the otic capsule, OC (Fig. 1C), as it appeared from the inside of the skull, was also oval. The long, horizontal axis (Fig. 1C; dotted line a) passed close to the upper margin of the optic canal (Fig. 1C; thick arrow). The short, vertical axis (Fig. 1C; dotted line b) passed just posterior to the oral cavity. The opening for the VIII cranial nerve (Fig. 1C; thin arrow) was at the intersection of the two axes, whereas the opening of the endolymphatic duct was at the dorsal margin of the otic capsule at the short axis (Fig. 1C; arrowhead). The medial surface of the membranous labyrinth showed the ciliated chambers and the medial extensions of the semicircular duct. The short axis (Fig. 1D; dotted line b) separated the two ciliated chambers. The long horizontal axis (Fig. 1D; dotted line a) marked the mean plane of the horizontal ducts, whereas the mean planes of the anterior and posterior vertical ducts were located in diagonal planes (Fig. 1D; dotted line c and d, respectively). For convenience, we will describe the anatomical details of the membranous labyrinth of the lamprey from four views which cover the whole structure: lateral, medial, dorsal and ventral views. We will use the term ‘duct’ when we refer to the lamprey labyrinth, reserving the term ‘canal’ for the bone- or cartilage-surrounded canals of jawed vertebrates.

Lateral view

In the lateral view, the anterior and posterior ciliated chambers looked like inverted cones and filled most of the cavity inside the otic capsule (Fig. 2A; ACCh and PCCh, respectively). Two ducts, the anterior and posterior lateral ducts (Fig. 2A; ALD and PLD; respectively; traced in red in Fig. 2B), lay on the lateral surface of the anterior and posterior ciliated chambers. These two ducts were formerly known as the anterior and posterior vertical canals (Retzius, 1881). Each lateral duct had a short vertical arm which started at the upper surface of the ciliated chamber and ran vertically downward, close to the median furrow between the two ciliated chambers. At the equator of the labyrinth, the vertical segments of the anterior and posterior lateral duct turned anteriorly and posteriorly, respectively, running an approximately horizontal course at about the equator of the labyrinth and forming the horizontal segments of the lateral duct (Fig. 2A, ALD, PLD, respectively; traced in red in Fig. 2B). In the middle of the anterior and posterior edges of the anterior and posterior ciliated chambers, each duct widened to form the anterior and posterior common ampullae (Fig. 2A; ACA and PCA, respectively). Each common ampulla contained a common bifid crista which was clearly visible through the transparent wall of the ampulla (Fig. 2B; traced in black dotted line). Each crista was formed of two arms: a vertical and a horizontal arm (Fig. 2B, thin and thick arrows, respectively). The two arms of the crista sat at right angles to each other, and at this angle there was a small elevation, the cruciate eminence (Fig. 2B; arrowheads). A summary of the findings on the lateral view is illustrated in a three-dimensional reconstruction in Fig. 2C, and in Supporting Information, Figs S1 and S2.

In SEM preparations, the lateral walls of the anterior and posterior lateral ducts were removed to examine the interior of these ducts. In these preparations, the vertical

Fig. 2 The anatomical features on the lateral wall of the membranous labyrinth in the lamprey. (A, B) Lateral views of the membranous labyrinth of the left ear showing the anterior and posterior ciliated chambers (ACCh and PCCh, respectively, in A). The anterior and the posterior lateral ducts (ALD and PLD, respectively in A; traced in red dotted lines in B) run on the lateral surfaces of the anterior and posterior ciliated chambers, roughly at the equator. At the anterior pole, the anterior lateral duct widens to form the anterior common ampulla (ACA in A) from which two anterior ducts start: the anterior horizontal duct and anterior vertical duct (AHD and AVD, respectively, in A). Their openings are traced in green and blue, respectively, in B. In the ampulla, there is a two-armed crista (traced in black in B); the vertical arm (thin arrow) sits at the opening of the horizontal duct and the horizontal arm (thick arrow) at the opening of the vertical duct. At the divide between the two ducts is the cruciate eminence (arrowhead in B). Similarly, at the posterior pole, the posterior lateral duct widens to form the posterior common ampulla (PCA in A) from which the posterior horizontal and posterior vertical ducts start (PHD and PVD, respectively, in A). (C) Lateral surface of the labyrinth in a 3D reconstruction of the labyrinth. (D) SEM image of the lateral surface of the labyrinth after removing the lateral wall of the ALD and PLD. The upper parts of the lateral ducts are adjacent to each other but are separated by a wall (thin arrows) which is reinforced medially by the spindle organ (thick arrow). The two lateral ducts open superiorly via a single common opening (stars) into the ciliated chambers. Inferiorly, each lateral duct bifurcates at the ampulla into two ducts: the horizontal and vertical ducts. The lateral openings of the ducts into the ampullae are indicated by dotted circles. The interior of the lateral ducts shows a prominent ridge on the medial wall (arrowheads). (E) A higher magnification of the upper area in (D). (F) A higher magnification image of the lower, ampullary region in (D), showing the lateral openings of the horizontal and vertical ducts into the ampullae (dotted circles). The vertical crista (thin arrow) sits at the horizontal duct opening and the horizontal crista (thick arrow) at the opening of the vertical duct. The two cristae are separated by the cruciate eminence (arrowhead), which extends medially to form the partition between the horizontal and vertical ducts proper (stars).



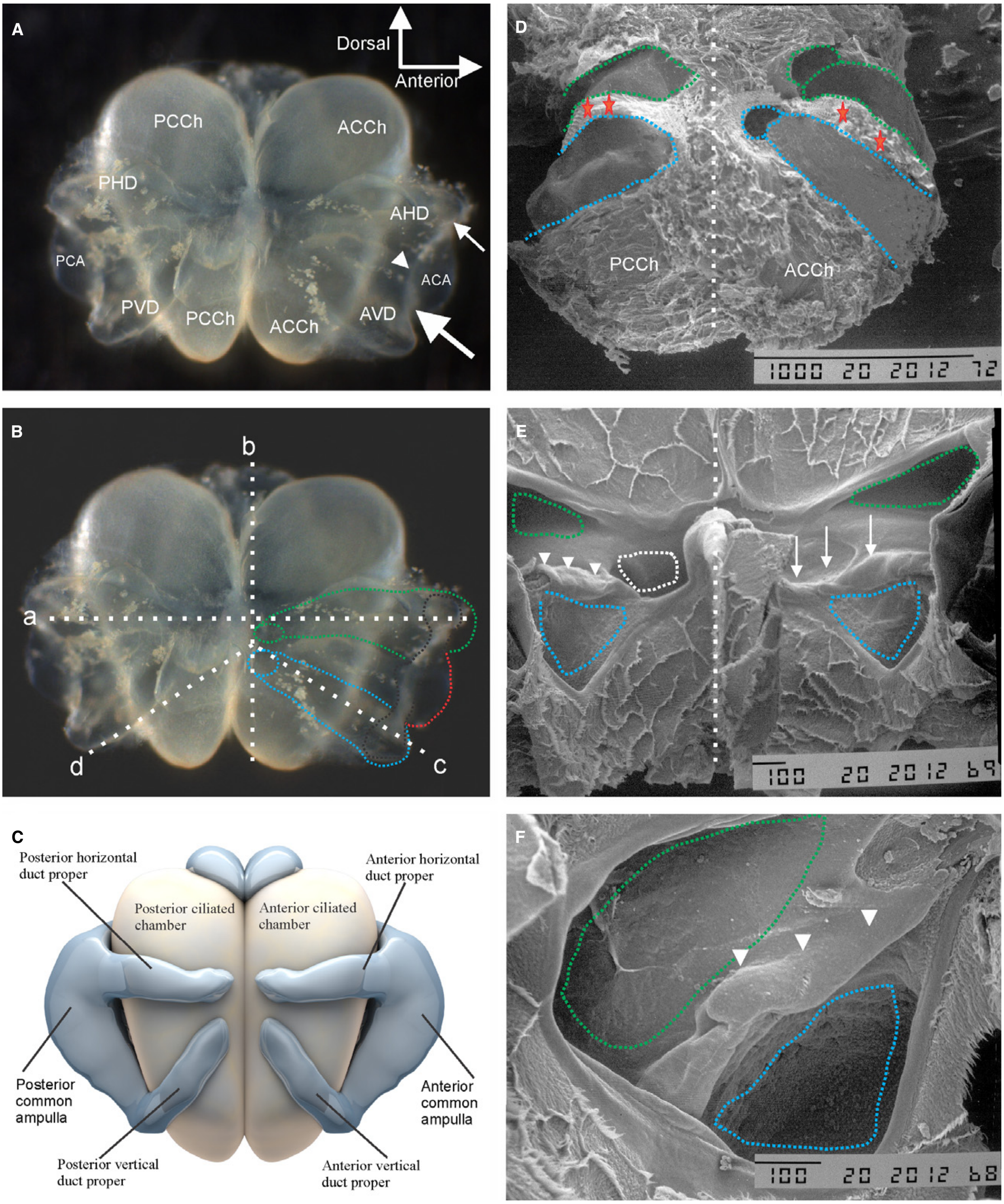
segments of the anterior and posterior lateral ducts abutted each other at the median furrow between the ciliated chambers and were separated by a double-layered septum (Fig. 2D,E; thin arrows). The posterior part of this septum was reinforced by a spindle-shaped structure (Fig. 2D,E; thick arrow), referred to by Lowenstein et al. (1968) as the spindle organ. Behind the spindle organ, the two ducts communicated with each other and opened into the ciliated chambers at a heart-shaped opening (Fig. 2D,E; stars). The inside of the horizontal segments of the lateral ducts was smooth, except for a prominent ridge on the medial walls of the lateral ducts (Fig. 2D,E; arrowheads). Each ampulla bifurcated into two separate ducts where their ampullary openings appeared in the lateral view after removing the lateral wall of the ampulla (Fig. 2D,F; circles). The vertical arm of the crista (Fig. 2B; thin arrow) sat at the opening of the superior duct which formed the horizontal duct proper on the medial wall, whereas the horizontal crista (Fig. 2F; thick arrow) sat at the opening of the inferior duct which formed the vertical duct proper on the medial wall. The cruciate eminence was located at the right angle between the two arms of the crista (Fig. 2F; arrowhead) and extended medially to form the partition separating the horizontal and vertical ducts proper (Fig. 2F; stars). The cruciate eminence along with it is a medial extension, separating the two ducts, and was referred to by Lowenstein et al. (1968) as the third basal arm of the crista.

Medial view

In the medial view, the two ducts emerging from the ampulla diverged and assumed separate courses on the medial walls of the ciliated chambers. On the medial wall of the anterior ciliated chamber, the superior duct started at the anterior common ampulla (Fig. 3A; ACA; traced in red in B) and ran a horizontal course (outlined in green dotted line in Fig. 3B) at the equator of the ciliated chamber (Fig. 3B, dotted line a), following the curvature of the medial wall of the chamber, and ended close to the middle of the posterior border of the anterior ciliated chamber, at the median furrow separating the two ciliated chambers (Fig. 3A; dotted line b). The vertical arm of the crista sat in the common ampulla at the ampullary end of this duct (Fig. 3A; thin arrow, traced in black dotted line in B). Here, we refer to this horizontal medial extension as the anterior horizontal duct proper (Fig. 3A; AHD; traced in green in B). The inferior duct (Fig. 3A; AVD) started from the anterior common ampulla (ACA) and ran vertically in the diagonal plane (Fig. 3B, dotted line c) between the *zx* and *zy* planes on the inferomedial wall of the anterior ciliated chamber (Fig. 3B; traced in blue). Here, we refer to this duct as the anterior vertical duct proper. The horizontal arm of the crista sat at the ampullary opening of the anterior vertical duct proper (Fig. 3A; thick arrow, traced in black dotted line in B). The cruciate eminence separated the vertical and horizontal arms of the crista (Fig. 3A; arrowhead). Similarly, at the posterior ciliated chamber (PCCh), the posterior common ampulla (PCA) bifurcated to form the posterior horizontal and vertical ducts proper (Fig. 3A). The mean plane of the horizontal ducts is marked by dotted line a, and the mean planes of the two vertical ducts by dotted lines c and d in Fig. 3B. A three-dimensional rendition of the medial view is illustrated in Figs 3C, S1 and S2.

SEM examination of the interior of the horizontal and vertical ducts proper, after removing their medial walls, revealed that they were thin-walled, arcuate-shaped, tubular structures (Fig. 3D; lumens are traced in green and blue, respectively). The luminal surfaces of these ducts were covered by smooth, non-ciliated epithelium (Fig. 3D). The two ducts were separated by thick, shelf-like partitions which formed the floor of the horizontal duct proper and the roof of the vertical duct proper (Fig. 3D; red stars). In the medial wall of the anterior ciliated chamber, the anterior horizontal and anterior vertical ducts opened into the anterior ciliated chamber at two triangular openings (Fig. 3E; traced in green and blue dotted lines, respectively) close to the midline partition separating the two ciliated chambers (Fig. 3E; dotted vertical line). Likewise, the posterior horizontal and posterior vertical ducts opened in the posterior ciliated chambers at two triangular openings close to the midline (Fig. 3E,F; traced in green and blue dotted lines, respectively). Between the latter two openings there was a third opening in the medial wall of the posterior ciliated chamber (Fig. 3E; traced in white dotted line),

Fig. 3 The anatomical features on the medial wall of the membranous labyrinth in the lamprey. (A, B) Stereoscopic dissection images of the medial wall of labyrinth, showing anterior and posterior ciliated chambers (ACCh and PCCh in A). The anterior and posterior horizontal ducts proper (AHD and PHD in A; traced in green dotted lines in B) are shown passing in the plane of the equator (dotted horizontal line in B). The medial wall also has the two vertical duct pairs: the anterior and posterior vertical ducts proper (AVD and PVD in A; traced in blue dotted lines in B). The vertical ducts proper pass in the diagonal planes (dotted diagonal line). A bifid crista (traced in black dotted line in B) sits at the ampullary ends of the ducts. The vertical crista (thin arrow in A) sits at the ampullary end of the horizontal duct, whereas the horizontal crista (thick arrow in A) sits at the ampullary ends of the vertical duct. The cruciate eminence between the two cristae (arrowhead in A) is level with the partition between the two ducts. (C) A 3D reconstruction of the medial wall of the labyrinth. (D) SEM image of a medial view of the medial wall of the labyrinth that shows the horizontal and vertical duct pairs (traced in green and blue dotted lines, respectively) after removing their medial walls to show their luminal surfaces. The horizontal and vertical duct pairs are separated by a thick wall (stars). (E) SEM image of a lateral view of the medial surface as examined from the inside of the ciliated chambers. The openings of the horizontal ducts (green dotted triangles) and the the vertical ducts (blue dotted triangles) are shown close to the midline partition between the ciliated chambers (dotted line). Between the openings of the PHD and PVD is the opening of the saccular pouch (traced in white dotted lines). The wall separating the horizontal and vertical ducts extend beyond their openings to form the anterior and posterior horizontal maculae (arrows and arrowheads, respectively). (F) A higher magnification of the PHD and PVD openings and posterior horizontal macula (arrowheads).



which was the opening for the vertical macula in the saccular pouch. The partition separating the anterior horizontal and vertical ducts extended between the two openings, projecting as a shelf-like structure in the cavity of the ciliated chambers to form a flat surface for the anterior horizontal macula (Fig. 3E; arrows). Likewise, the partition between the posterior horizontal and posterior vertical duct openings projected in the posterior ciliated chamber, forming the posterior horizontal macula (Fig. 3E,F; arrowheads).

Dorsal view

In this view, the expanded upper surfaces of the two ciliated chambers appeared together as a kidney-shaped surface (Fig. 4A,B). The vertical and horizontal parts of the anterior and posterior lateral ducts appeared on the lateral surface of the ciliated chambers (Fig. 4A; ALD and PLD, respectively; traced in red in B). At the anterior and posterior poles of the ciliated chambers, the lateral ducts dilated to form their respective common ampullae (Fig. 4A; ACA and PCA, respectively) where the cristae were housed. In this view, the horizontal duct cristae, i.e. the vertical arm of the cristae, appeared through the transparent wall of the ampullae (Fig. 4B; traced in black dotted line). From the anterior common ampulla, the anterior horizontal duct proper (Fig. 4A; AHD; traced in green in B) started, running medially and backward following the curves of the posterior ciliated chambers to reach the midline, where it opened close to the midline in the same chamber. Similarly, the posterior horizontal duct (Fig. 4A; PHD; traced in green in B) ran medially and forward to the midline, where it opened in the posterior ciliated chamber. From this view, the anterior and posterior lateral ducts (Fig. 4B; traced in red) and the anterior and posterior horizontal ducts proper (Fig. 4B; traced in green) were small shallow arcs, but together they formed two complete semicircles flanking three sides of their respective ciliated chambers. The anterior and posterior horizontal ducts together almost completely surrounded the circumference of the two ciliated chambers like a belt. In this pattern, the ampullae lay at the peak of the semicircles, rather than at the end of their respective ducts as in other vertebrates. A three-dimensional reconstruction of the dorsal view is shown in Figs 4C, S1 and S2.

In some preparations, we removed the roofs and the lateral walls of the ciliated chambers, including the anterior and posterior common lateral ducts. In these preparations, we also removed the inner wall of the horizontal and vertical ducts proper, separating them from the ciliated chambers, to examine their lumens from the inside. SEM examination of the top view of these preparations demonstrated the tubular nature of the horizontal and vertical ducts (Fig. 4D,E; traced in green and blue, respectively) as they started from their lateral openings to the ampulla until they ended at their medial openings to the ciliated chambers. The horizontal ducts proper started at the common ampulla at the anterior and posterior poles of the ciliated chambers, where their openings to the ampullae were guarded by the vertical cristae (Fig. 4D,E; thin arrows). Similarly, the two vertical ducts started at the common ampullae where their openings to the common ampullae were guarded by the horizontal cristae (Fig. 4D,E; thick arrows). The cavities of the horizontal and vertical ducts proper were separated by a thick partition (Fig. 4D,E; stars), which extended from the cruciate eminence laterally (Fig. 4D,E; arrowheads) to the medial opening of the ducts at the median partition between the ciliated chambers (Fig. 4D,E; dotted vertical white lines).

Ventral view

In the ventral view, the narrow lower ends of the ciliated chambers appeared as heart-shaped structures separated by a median groove. The anterior and posterior lateral ducts (Fig. 5A; ALD and PLD, respectively; traced in red dotted lines in B) ran on the lateral surface of the ciliated chambers to reach their respective ampullae on the anterior and posterior poles of the ciliated chambers, respectively. From the anterior common ampulla, the anterior vertical duct proper (Fig. 5A; AVD; traced in blue dotted line in B) ran a diagonal course, first downward and medially, and then turning sharply up and backward to reach the middle of the posterior border of the anterior ciliated chamber where it ended. The vertical duct crista, i.e. the horizontal arm of the crista (Fig. 5A; arrow; traced in black dotted line in B), was apparent through the transparent inferior surface of the anterior common ampulla. Likewise, the posterior vertical duct proper started at the posterior common ampulla and ran a diagonal course, passing downward and medially, then turned sharply upward and anteriorly to reach the middle of the anterior border of the posterior chambers where it ended. In this view, the anterior and posterior lateral ducts (Fig. 5A; ALD and PLD, respectively; traced in red dotted line in B), and anterior and posterior vertical ducts proper (Fig. 5A; AVC and PVC, respectively; traced in blue dotted lines in B) were small arcs. Nevertheless, the anterior lateral duct and the anterior vertical duct proper together formed a complete semicircle, the anterior vertical duct, as did the posterior lateral duct and the posterior vertical duct proper. In both vertical ducts, the ampullae lay on the peak of the semicircle rather than at the end. In the ventral view, the oblique diagonal course of the vertical ducts permitted the lateral margins on the horizontal ducts proper to appear (Fig. 5B; traced in green). A three-dimensional reconstruction of the ventral view is illustrated in Figs 5C, S1 and S2.

Discussion

Morphological findings

In the present study, the labyrinth of the *Petromyzon marinus* was examined using stereoscopic dissection and SEM methods to reveal the complex morphology of the labyrinth in the lamprey. The main finding of this study is that the labyrinth of the lamprey has a horizontal duct that differs in its organization and topology from jawed vertebrates. In contrast to all jawed vertebrates, which have a single horizontal canal in each labyrinth, our study demonstrates that the lamprey has two horizontal ducts in each labyrinth (Figs 2–5). Furthermore, these ducts are symmetric with symmetric duct cristae associated both anteriorly and

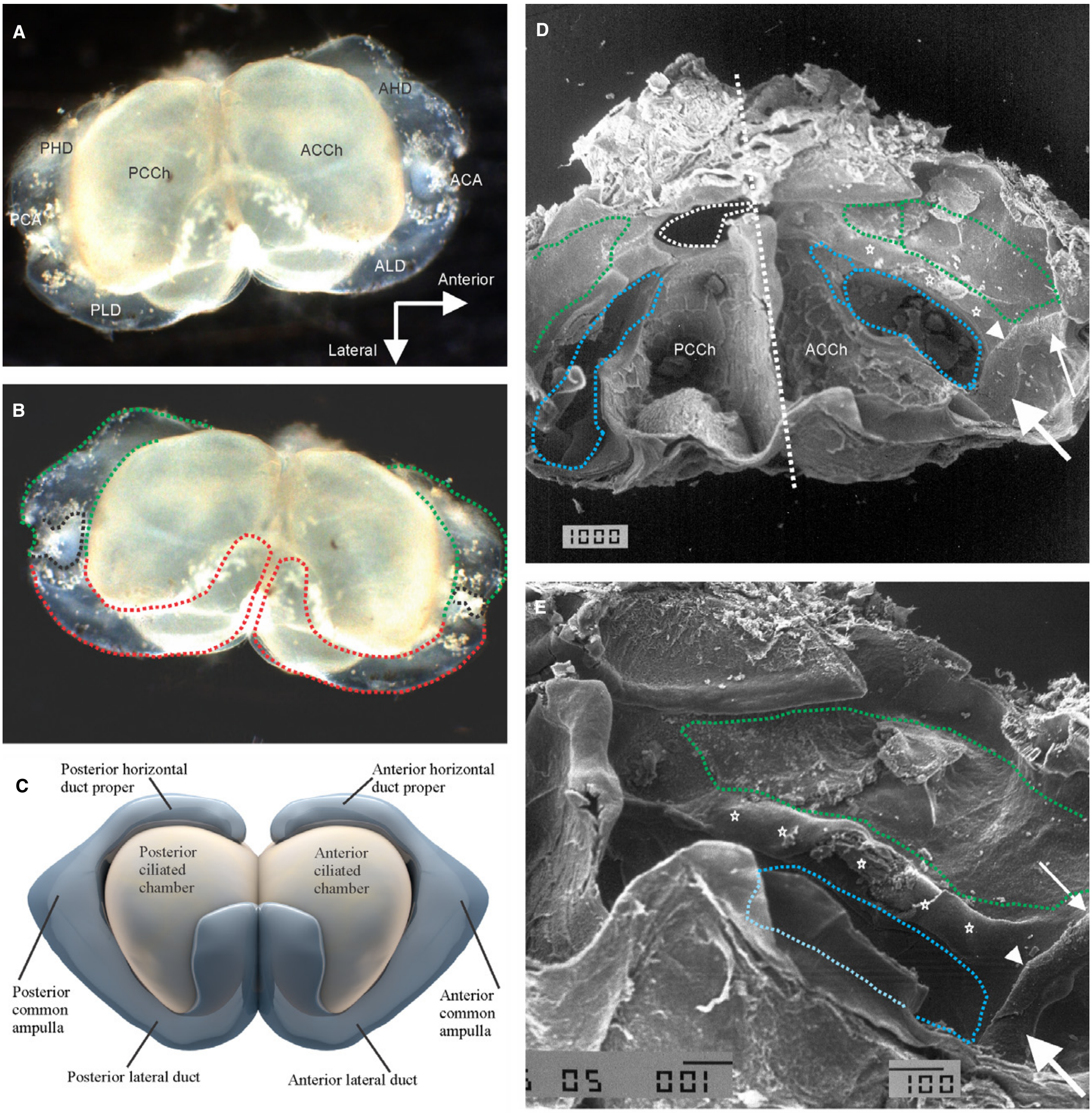


Fig. 4 The anatomical features on the dorsal view of the membranous labyrinth in the lamprey. (A, B) Stereoscopic dissection images of the dorsal view of the membranous labyrinth. The dorsal surfaces of the anterior and posterior ciliated chambers (ACCh and PCCh in A) are shown. The anterior and posterior lateral ducts (ALD and PLD in A; traced in red dotted line in B) wrap around the lateral surfaces of ciliated chambers. The anterior and posterior horizontal ducts proper (AHD and PHD in A; traced in green dotted lines in B) wrap around the medial surfaces of their respective ciliated chambers. Each horizontal duct proper and its respective lateral duct form a complete semicircle flanking their respective ciliated chambers. The anterior and posterior common ampullae (ACA and PCA in A) are at the peak of their respective semicircular ducts. (C) A 3D reconstruction of the anatomical features on the dorsal view of the labyrinth. (D) SEM image of the ventromedial quadrant of the membranous labyrinth in which the lateral walls of the ducts proper have been removed to examine their lumens. The lumen of the horizontal duct is traced in green and that of the vertical duct in blue. At the ampullary end of the horizontal duct proper was the vertical crista (thin arrow) and at the ampullary end of the vertical duct proper is the horizontal crista (thick arrow). The cruciate eminence (arrowhead) is continuous with the thick septum separating the horizontal and vertical ducts (stars). (E) A higher magnification of the anterior half of D.

posteriorly. In contrast to the horizontal canals of gnathostomes, these ducts curve medially around a unique feature of modern lampreys, the ciliated chambers.

Previous studies, using dissection, classical histology and TEM, concluded that the lamprey labyrinth has two vertical canals, the anterior and posterior canals, and that the

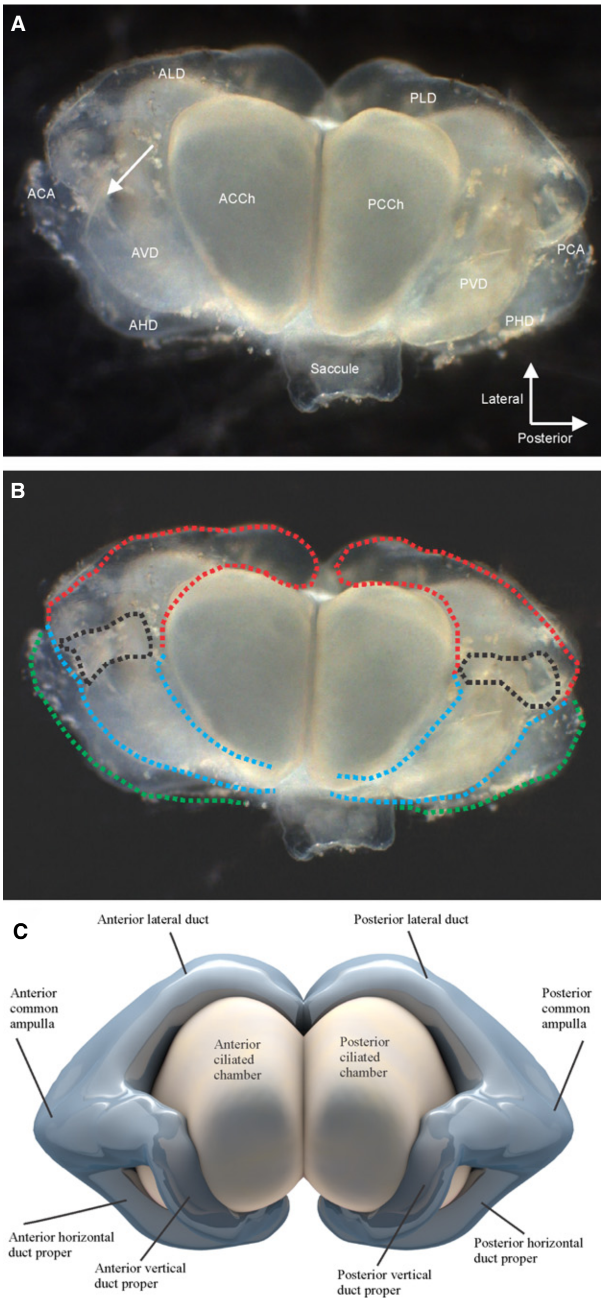


Fig. 5 The anatomical features on the ventral view of the labyrinth of the lamprey. (A, B) Stereoscopic dissection images of the ventral view of the membranous labyrinth. This view shows the narrow ventral surfaces of the anterior and posterior ciliated chambers (ACCh and PCCh in A) and the anterior and posterior lateral ducts (ALD and PLD in A; traced in red dotted line in B). In the ventral view, the entire course of the anterior and posterior vertical ducts proper is shown (AVD and PVD in A; traced in blue dotted line in B). Each lateral duct, with its respective vertical duct proper, forms a complete semicircle with an ampulla on its peak (ACA and PCA in A). The ventral surfaces of the horizontal cristae are shown (arrows in A; traced in black dotted lines in B). The vertical orientation of the vertical ducts allow the lateral outer margins of the horizontal ducts to be seen from the ventral view (traced in green dotted line in B). (C) A 3D reconstruction of the anatomical features as viewed from the ventral aspect of the labyrinth.

horizontal canal is missing (Retzius, 1881; de Burlet & Versteegh, 1930). Later, Lowenstein et al. (1968) and Hagelin (1974) confirmed these observations in their detailed morphological analysis. Based on dissection and interpretation of histological sections of the larval Lampetra, Lowenstein et al. (1968), concluded that the two vertical canals are merely diverticulae from the ciliated chambers, and their ampullae are recesses of the ciliated chambers. In contrast to the observations of Lowenstein et al. (1968), we demonstrated that both ampullae are entirely anatomically separate from the ciliated chambers. We further demonstrated that each ampulla bifurcates into two ducts: a horizontal and vertical duct. Each duct is completely separate from the other, having its own lateral opening in the ampulla and its own medial opening in the medial wall of the ciliated chamber (Fig. 3D–F). The two ducts emerging from each ampulla, which we refer to as the horizontal and vertical ducts proper, are small arcs, but with the common lateral duct added, they form complete semicircles; the horizontal and the vertical semicircular ducts. Each complete semicircle has two openings into their respective ciliated chambers; one lateral opening through the common lateral duct and another through the duct proper. Except at these openings, this semicircular duct system is separate from the ciliated chamber. It is noteworthy that the description of the morphology of the labyrinth in the lamprey in the previous study was based on gross dissection and drawings from these dissections. The labyrinth in lampreys does not lend itself readily for histological sectioning methods due the delicate wall of the duct system. Consequently, it was hard in the previous studies to obtain intact histological sections for reliable reconstruction due to shrinkage and distortion associated with wax embedding. Careful cleaning and dissection combined with improved optics and the use of SEM to study morphological details have enabled us to detect details that were overlooked in previous studies. Our approach has revealed the unique complexity of the lamprey labyrinth. It remains to be determined whether at the biomechanical levels the structures described as ducts in our study and those of earlier investigators are indeed capable of generating endolymph movement upon angular acceleration in different planes.

Physiological interpretations

The vestibular systems in all jawed vertebrates have three semicircular canals arranged in the three orthogonal planes which allow detection of angular accelerations in all directions, and bring about eye movements and postural reflexes. In contrast, the lamprey, a jawless vertebrate, was believed to have only two vertical canals. However, several studies have revealed robust responses to rotations in the yaw plane in the different components of the postural reflex arc: the primary vestibular afferents, the descending vestibulospinal and reticulospinal neurons, and the effector spinal motor neurons (Lowenstein, 1970; Rovainen, 1979; Wannier et al. 1998; Zelenin et al. 2003; Pflieger & Dubuc, 2004; Zelenin, 2005; Karayannidou et al. 2007). These studies made it clear that angular movements in the yaw plane in the lamprey have as robust an effect on the postural neural network as they have for movements in the pitch and roll planes (Deliagina & Pavlova, 2002; Pavlova & Deliagina, 2003). Several publications have addressed the question: What is the source of these responses to horizontal angular stimuli, given the missing horizontal canal? Lowenstein (1970) proposed that the elaborate three-armed shape of the crista in the vertical canals is responsible for responses to horizontal angular stimuli. He stated that the vertical placement of one of these crista arms makes it sensitive to horizontal angular movement. However, this explanation does not satisfactorily account for these robust responses upon angular rotation in the yaw plane. Clearly, an anatomical substrate capable of generating an effective and discrete endolymph flow and cupular deflection upon angular movements in the yaw plane could provide a simpler explanation.

The main finding of our study is that the lamprey, like all other vertebrates, has functional horizontal ducts, albeit it, structurally different. We posit that these new features are the source of responses to horizontal angular stimuli in the previous studies (Lowenstein, 1970; Rovainen, 1979; Pflieger & Dubuc, 2004; Zelenin, 2005; Karayannidou et al. 2007). However, our findings demonstrate differences between the lamprey and other vertebrates. Specifically, the lamprey horizontal ducts are found on the medial surface of the labyrinth and each labyrinth has two horizontal ducts. The medial location should not affect their biomechanical action. The hair cell orientations in the cristae of these horizontal ducts (Lowenstein et al. 1968) make them oppositely tuned for a given horizontal movement. Thus, in lampreys, the anterior horizontal duct will be deactivated by ipsilateral turns, whereas the posterior horizontal duct will be activated by contralateral turns. This push–pull organization, well known between the right and left horizontal canals in jawed vertebrates, may exist unilaterally in lampreys due to the opposing orientations of the anterior and posterior horizontal ducts. In support of our morphological findings, electrophysiological recording from the anterior branch of the vestibular nerve, supplying the anterior horizontal duct crista, revealed activation on contralateral turns and deactivation on ipsilateral turns. In contrast, recording from the posterior branch, supplying the posterior horizontal duct cristae, yielded opposite responses: activation on contralateral turns and deactivation on ipsilateral turns (Lowenstein, 1970). Thus, one labyrinth responds with two antagonistic responses to ipsilateral and contralateral turns comparable to the opposing polarity of hair cells in duct cristae (Maklad et al. 2010).

Furthermore, Karayannidou et al. (2007) demonstrated the presence of two populations of reticulospinal neurons on each side of the lamprey brainstem that respond with opposite tuning to ipsilateral and contralateral turns, supporting the notion of push–pull afferent information, with ipsi- and contralateral turns emanating from one labyrinth. The reticulospinal neurons are the main recipients of vestibular afferent information in lamprey (Northcutt, 1979; Koyama et al. 1989; Fritzsch, 1998), and they convey that information to oculomotor and spinal motor neurons and interneurons (Nieuwenhuys, 1972; Rovainen, 1979; Brodin et al. 1988; Bussières et al. 1999). Thus, our current morphological evidence and previous electrophysiological recordings of others from primary and secondary vestibular neurons, and other targets, such as the reticular nuclei, support the notion of two functional horizontal ducts in each labyrinth in the lamprey. This raises the question of what function these two horizontal ducts perform in lamprey.

Vestibular control of body movement in the lamprey in the yaw plane

Here we propose a model of how the information from these two horizontal ducts is integrated centrally to affect postural control in the yaw plane (Fig. 6). Rovainen (1979), using electrophysiological recordings from vestibulospinal and reticulospinal neurons, suggested that the majority of the secondary vestibular neurons in the octavomotorius area, which receive primary afferents from the anterior branch of the vestibular nerve, project to ipsilateral reticular nuclei. In contrast, the majority of secondary vestibular neurons in the octavomotorius area, which receive primary afferents from the posterior branch of the vestibular nerve, project to contralateral reticulospinal neurons. Rovainen's findings were confirmed by Pflieger & Dubuc (2004). They further added that minor crossed, mostly inhibitory input originates from the octavomotorius domain of the anterior branch to the contralateral reticulospinal neurons, whereas a minor direct, mostly inhibitory input exists from the octavomotorius domain of the posterior branch to the ipsilateral reticulospinal neurons. The targets and functions of these minor and direct inhibitory vestibuloreticular pathways are unknown. However, they might allow disinhibition of the neurons on the activated side or facilitated inhibition of neurons on the deactivated side. These neurons, in turn, could project to excitatory or inhibitory spinal interneurons in a vectorially uniform way.

With this connectivity arrangement, when the lamprey turns its head, for example, to the right side, the endolymph inertial vector will activate hair cells in the crista of right posterior and left anterior horizontal ducts and deactivate hair cells in right anterior and left posterior horizontal ducts. Thus, with any given turn in the yaw plane, there is a mixed output from each labyrinth conveyed to both sides of the brainstem. However, the central connectivity pattern will compartmentalize the facilitatory inputs to the side contralateral to the direction of movement, and all

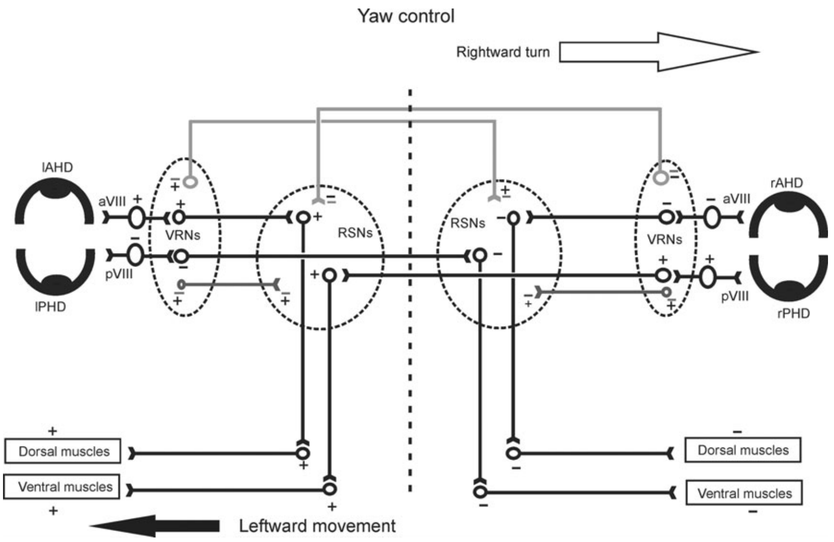


Fig. 6 A model of the role of the horizontal ducts in postural control of movement in the yaw plane. When the animal turns its head to the right, the right posterior and left anterior horizontal ducts are activated (+), whereas the right anterior and left posterior horizontal ducts are deactivated (−). Connectivity patterns of the direct and crossed vestibuloreticular neurons (VRNs) direct the facilitated inputs to reticulospinal neurons (RSNs) projecting to the ventral and dorsal spinal motor neurons on the left side of the body, resulting in a corrective leftward body turn. The minor direct and crossed inhibitory pathways (neurons drawn in gray lines) end as disinhibition on the left side and facilitated inhibition on the right side, further augmenting the effect of the major pathways.

disfacilitatory inputs to the side ipsilateral to movement direction. This will result in activation of motor neurons to dorsal and ventral myotomes of the contralateral side and subsequent movement counteracting the head turn (Fig. 6).

Vestibular control of eye movement in lamprey in yaw plane

In contrast to postural control in the lamprey, which is mediated by reticulospinal neurons, vestibuloocular reflexes are achieved by direct projection of secondary vestibular neurons to oculomotor nuclei. It is also more likely that oculomotor neurons receive direct and crossed vestibular projection from ipsilateral and contralateral sides, respectively. As in other vertebrates, lampreys have six eye muscles; rostral rectus, caudal rectus, dorsal rectus, ventral rectus, rostral oblique and caudal oblique. The rostral and caudal recti pull the eyes anteriorly and posteriorly, respectively, in the horizontal plane, making them the responsive pair for head movement in the yaw plane. The dorsal and ventral recti pull the eyes upward and down, respectively, in the vertical plane, making them the responsive pair for head movement in the roll plane. The rostral oblique rotates the eye upward and backward, whereas the caudal oblique rotates the eye downward and forward, making them the responsive pair for movements in the pitch plane (Rovainen, 1976; Pombal et al. 1996; Fritzsch, 1998). Thus, when the lamprey makes a right turn in the yaw plane, excitatory inputs from the right posterior and left anterior horizontal ducts are conveyed to the right rostral rectus and left caudal rectus, generating rostral horizontal movement in the right eye and caudal horizontal movement in the left eye (Fig. 7). This is the appropriate compensatory movement for this lateral-eyed animal with a rather unusual eye muscle configuration and innervation by unusually distributed eye muscle motor neurons (Fritzsch, 1998).

Evolutionary implications

To date, it was believed that the horizontal canal of the inner ear is absent in the lamprey (Lowenstein et al. 1968) and hagfish, the other extant cyclostome. The horizontal canal is also absent in the ostracoderms (Mazan et al. 2000), extinct jawless armored fish. In contrast, a lateral horizontal canal exists in all extant gnathostomes and placoderms, an extinct gnathostome group of armored bony fish (Janvier, 2007, 2008). It was, therefore, concluded that the presence of a horizontal canal is a gnathostome trait, and its evolution started in their ancestors. Consequently, the lateral horizontal canal is considered a synapomorphic characteristic shared by gnathostomes, and the absence of a horizontal canal in agnathan vertebrates is a primitive characteristic of jawless vertebrates such as lampreys and hagfish. Our morphological findings on the medial horizontal duct system and those of others on the ciliated chambers in lamprey ear (Retzius, 1881; Lowenstein et al. 1968) suggest that these are unique derived features of this cyclostome lineage that have not been found in any other vertebrate or fossil (Hagelin, 1974).

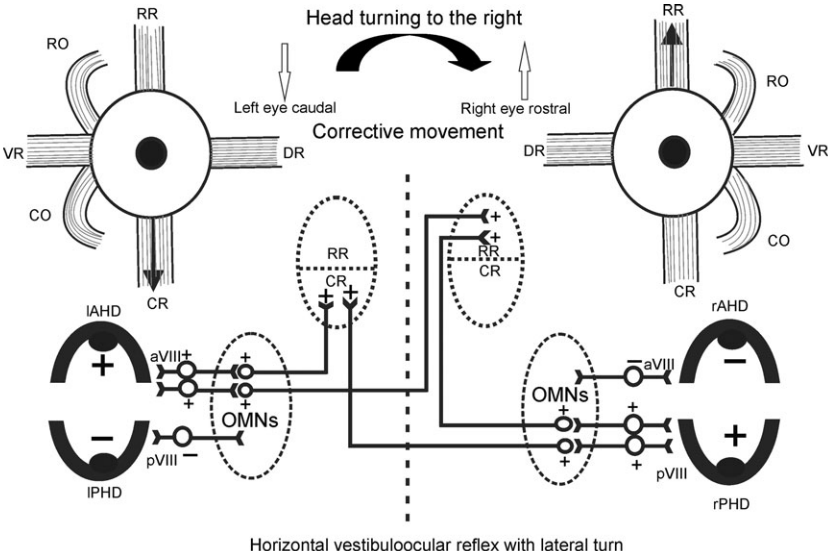


Fig. 7 A model of the role of the horizontal ducts in vestibuloocular reflexes to head rotation in the yaw plane. The secondary vestibular neurons in the octavomotorius (OMNs) project equally bilaterally and directly to motor neurons supplying the extraocular muscles. When the animal turns its head to the right, the right posterior and left anterior horizontal ducts are activated (+), whereas the right anterior and left posterior horizontal ducts are deactivated (−). Afferent information from the activated ducts, carried via the left posterior and right anterior divisions of the vestibular nerve (right pVIII and left aVIII), is projected to the right rostral rectus (RR) and left caudal rectus (C.R.) motor neurons via secondary vestibular neurons in the octavomotorius, generating horizontal rostral and caudal movements in the right and left eyes, respectively. There is no evidence that afferent information from the deactivated ducts has any effect on the antagonistic groups of muscles, i.e. right caudal rectus (C.R.) and left rostral rectus (RR), or any evidence of the presence of inhibitory interneurons between the motor neuron nuclei supplying antagonistic groups of muscle.

The phylogenetic relationship of the lamprey between hagfish and gnathostomes is still controversial (Delarbre et al. 2000, 2002), but most recent molecular evidence support a cyclostome taxon (Smith et al. 2013). Given the presumed monophyly of cyclostomes, future work is needed to determine whether the lack of the medial horizontal duct in either hagfish or ostracoderms was a secondary loss of this feature and reflects the primitive condition of the ear in these two lineages or the medial horizontal duct and ciliated chambers are unique derived features only of lamprey lineage.

At molecular levels, it is widely believed that Otx genes, a homeobox gene family, are responsible for the appearance of several gnathostome characteristics, including the horizontal canal (Morsli et al. 1999; Mazan et al. 2000; Hammond & Whitfield, 2006). The *Otx1* gene null mutant mice sustain complete loss of the horizontal canal, whereas the anterior and posterior vertical canals remain intact, including a horizontal canal crista that resembles in pattern of innervation the dorsal macula of lampreys (Fritzsch et al. 2001), as previously suggested (Hagelin, 1974). In another mutation it was found that the loss of Foxg1 results in formation of a lateral canal without a canal crista (Pauley et al. 2006), implying that evolution of the gnathostomes horizontal canal system may have been a two-step process. First, there was a unique evolution of a separate canal crista that serves in lampreys as the dorsal macula of an unknown function. This was followed by the evolution of the molecular machinery to form a horizontal canal that develops in a molecularly different manner from the two vertical canals in jawed vertebrates (Fritzsch et al. 2007). Our current study suggests the presence of a distinct kind of horizontal duct in the lamprey, not found in any other vertebrates. In lampreys, three Otx gene orthologues were isolated: LjOtxA, LjOtxB and PmOtx (Ueki et al. 1998; Tomsa & Langeland, 1999); a Foxg1 gene is likely present as well. However, none of them is expressed in the developing otocyst. Taken together, it is more likely that a novel expression of Otx genes in the developing otocyst was associated with the acquisition of a novel structure in gnathostomes. Future work is needed to show that the horizontal semicircular ducts of lampreys develop using a distinct set of genes that is different from jawed vertebrates.

Concluding remarks

In summary, we have identified a unique system of horizontal ducts running medial and symmetric along the unique ciliated chamber. We propose that these structures provide the morphological substrate for the unusual mirror-symmetric responses recorded from anterior and posterior duct nerves. In light of the morphological differences and the fact that the ciliated chambers are unknown in any fossil, we suggest that these structures represent a parallel evolution to the gnathostomes' single and lateral horizontal canal. Instead of lacking a horizontal movement sensing system that would put lampreys at a disadvantage to gnathostomes, we suggest that lampreys have evolved a unique system that provides additional input for movement in the yaw plane to enable them to become exceptionally well adjusted ecto-parasites on gnathostomes as evident in the Petromyzon invasion of the Great Lakes.

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Conflict of interests

All authors declare that they have no conflict of interest.

Authors' contributions

All authors had full access to all the data in the study and take responsibilty for the integrity of the data and the accuracy of the data analysis. Study concept and design: A.M., C.R., N.J., B.F. Acquisition of data: A.M., C.R., N.J., B.F. Analysis and interpretation of data: A.M., C.R., N.J., B.F. Drafting of the manuscript: A.M., C.R., N.J., B.F. Interpretation of data: A.M., C.R. Critical revision of the manuscript for important intellectual contents: A.M., C.R., N.J., B.F. Obtained funding: A.M., B.F. Administrative, technical and material support: A.M. Study supervision: A.M., B.F.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. A three-dimensional interactive model in which a complete labyrinth of the lamprey is shown; the two ciliated chambers and the semicircular duct system.

Fig. S2. A three-dimensional interactive model of the labyrinth in lamprey in which only the semicircular duct system is shown after digital subtraction of the ciliated chambers.

Supplementary materials

Figure 1 A three-dimensional interactive model in which a complete labyrinth of the lamprey is shown; the two ciliated chambers and the semicircular duct system. The model is in Quick Time movie format and can be opened by Quick Time player 7, and can be manipulated in 3 dimensions by dragging with the computer mouse. Figure 1, Supplementary material can be accessed through the following URL address:

http://www.umc.edu/Education/Schools/Medicine/Basic_Science/Neurobiology/Adel_Maklad.aspx

Figure 2 A three-dimensional interactive model of the labyrinth in lamprey in which only the semicircular duct system is shown after digital subtraction of the ciliated chambers. The model is in Quick Time movie format and can be opened by Quick Time player 7, and can be manipulated in 3 dimensions by dragging with the computer mouse. Figure 2, Supplementary material can be accessed through the following URL address:

http://www.umc.edu/Education/Schools/Medicine/Basic_Science/Neurobiology/Adel_Maklad.aspx