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Vascularization of the lateral line organ in the Atlantic cod: involvement of the secondary vascular system

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Abstract

Sections of the lateral line organ, primary and secondary blood vessels and skin from the Atlantic cod *Gadus morhua*, Linnaeus 1758, were examined by light and transmission electron microscopy (TEM). The lateral line organ showed a structural analogy to the semicircular canals of the mammalian inner ear. A pericanalicular sinus (PCS), a canal of very loose connective tissue, surrounded the lateral line canal (LLC), separated by a multilayered epithelial wall. Located dorsal and ventral to the lateral line organ secondary vessels of capillary dimensions were found in association with the PCS. TEM of the wall of these dorso-ventral vessels showed single tight junction contacts between the endothelial cells, allowing paracellular fluid exchange between the secondary vascular system and the PCS, an indication supported by horseradish peroxidase (HRP) tracer experiments, which showed reaction products in the PCS within 2 h after injecting HRP into the systemic circulation. The multilayered epithelial wall of the LLC showed multiple tight junctions between cells, making this boundary permeable only through transcellular transport.

Introduction

The mechanosensory lateral line organ is present in all teleost fishes and aquatic amphibians (Dijkgraaf, 1963; Blaxter, 1987) detecting pressure gradients in the surrounding water (Kalmijn, 1989). Neuromasts enable mechanosensory reception via the shearing of numerous stereovilli and a single kinocilium on the apical surface of the neuromast. The villi are protruding into a gelatinous cupula stretching across the width of the lateral line canal (LLC) (Dijkgraaf, 1963; Blaxter, 1987). The lateral line organ bears a structural resemblance to the semicircular canals of the mammalian inner ear; the neuromast found in the LLC of the teleost fishes is virtually identical to the mammalian receptive unit sensing angular acceleration located in the ampullae of the semicircular canals (Widmaier, Raff & Strang, 2004). In the mammalian inner ear, the apical stereovilli are suspended in the endolymph of the semicircular duct with a high potassium and a low sodium concentration, whereas the basal membrane is in intimate contact with the perilymph characterized by low potassium and high sodium concentration (Germann & Stanfield, 2002). Because the LLC closely resembles the semicircular duct, the canal fluid is believed to have a high K^+ concentration (Kroese & van Netten, 1989).

The unique angio-architecture of the secondary vascular system (SVS) renders the fluid of this vascular system virtually devoid of erythrocytes (Vogel, 1981*b*; Steffensen, Lomholt & Vogel, 1986), making it a likely candidate for supplying the LLC with the plasma-carried K^+-ions.

The SVS was first described in teleost and holost fishes by Vogel (1981*a*,*b*) and Vogel & Claviez (1981). Vascular corrosion casts supplied evidence for secondary vessels arising from the primary vasculature through interarterial anastomoses (Vogel & Claviez, 1981; Skov & Bennett, 2004).

Secondary capillaries have been found to supply the skin, the fins, the peritoneum and the mucosa of the mouth and pharynx (Vogel, 1985), which eventually drain into secondary veins and in turn into the lateral, dorsal and ventral collecting vessels (Vogel, 1985). The lateral collecting vessels (LCV) are located medial and parallel to the lateral line in several teleost species. Fluid flow through the SVS is believed to be considerably slower than for the primary vascular system (PVS); correspondingly, the pressure within the secondary blood vessels is considerably lower. The volume of the SVS in *Gadus morhua* constitutes 50% of that of the PVS or one-third of the total blood volume (Skov & Steffensen, 2003), while it has been shown to have a volume 1.5 times greater than that of the PVS in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Steffensen & Lomholt, 1992).

Research has focused mainly on the morphology and angio-architecture of the SVS. While there is little experimental evidence for the physiological function of the SVS, ion and water balance (Satchell, 1991), oxygen uptake (Satchell, 1991) and hypercapnic acid–base regulation (Ishimatsu *et al*., 1992) have been suggested as possible functions maintained in part by the SVS.

The aim of the present study was to investigate possible connections between the SVS and the lateral line organ in

the Atlantic cod *G. morhua*, in order to provide an insight into the functional and anatomical organization of these features of the teleost anatomy.

Materials and methods

Atlantic cod *G. morhua* (Linnaeus, 1758) with a size in the range of 430–650 g (L 38–41 cm) were caught by trawl in the northern part of Øresund (55°57′N, 12°38′E), and kept in laboratory holding tanks provided with continuously recirculating seawater (salinity 30‰, 10 °C). Animals were allowed to recover for a minimum of 2 weeks before use.

Lateral line morphology

Perfusion-fixed animals were used for morphological examination of the lateral line. The animals were anaesthetized in benzocaine (0.06 g L^{-1}) and pre-heparinized (0.5 mL 5000 UI) by injecting a blood vessel within the haemal arch. The heparin was allowed to circulate for a few minutes before the animal was euthanized. The heart was exposed by a ventral midline incision posterior to the gills, and the heart was cannulated normograde using PE-50 polyethylene tubing. The cannula was secured at the bulbo-ventricular junction and an incision was made in the atrium to allow drainage. The animal was flushed with phosphate-buffered saline (PBS, pH 7.8) (0.1 M phosphate buffer in 0.9% saline) until the effluent was clear of any red blood cells, followed by 450 mL 2.5% glutaraldehyde in PBS from a pressurized reservoir at a rate of 50 mL min^{-1}. The animal was subsequently immersed in the same fixative and stored at 4 °C until use.

Tissue blocks of suitable size were cut from the lateral line and decalcified for 1 week at room temperature in 20% (w/v) EDTA (Paisley, Scotland) in Karnovsky's fixative to soften the scales before sectioning. Samples for sectioning tangential to the skin surface were pinned flat during post-fixing.

Primary and secondary vessel identification

Immersion fixation of whole animals with intact vasculature allowed identification of secondary vessels due to the *in situ* fixed erythrocytes in the blood vessels. For morphological examination, animals were euthanized by an overdose of benzocaine (0.1 g L^{-1}) and immersion fixed in Karnovsky's fixative (2.5% glutaraldehyde, 1% paraformaldehyde in 0.11 M phosphate buffer) at 4 °C. Tissue blocks of suitable size were cut from lateral muscle tissue around a segmental artery. Blocks were dehydrated in a graded series of ethanol [70% 1 h, 70% overnight, 96% 2 × 1 h, 96% overnight and in absolute alcohol (abs) for 3 × 1 h], infiltrated with xylene 2 × 1 h and finally infiltrated with paraffin at 60 °C for 2 h and an overnight change. Sections were cut using a Leica Histoslide 2000 (Wetzlar, Germany), collected on poly-lysine-coated microscope slides and dried for 20 min at 60 °C. Sections were deparaffinized in 3 × 4 min changes of xylene, hydrated in a graded series of ethanol (absolute 2 × 1 min, 96% 1 min, 70% 1 min) and water for 1 min, stained with haematoxylin for 10 min, rinsed in water (10 min) and then stained with eosin (2 min) and briefly rinsed in water, dehydrated in a graded series of ethanol (70%, 96%, 3 × abs) and dried at 37 °C for 20 min, coverslipped using Depex (Poole, UK) and photographed using a Zeiss Axioplan2 light microscope (Oberkochen, Germany).

Ultrastructure of SVS capillaries and LLC epithelium

Small (2–3 mm^3) blocks containing the lateral line organ were cut from perfusion-fixed animals and post-fixed in 2% OsO_4 in 0.1 M phosphate buffer (pH 7.8) for 1 h, rinsed in distilled water for 1 min, dehydrated in a graded series of ethanol (70% 5 min, 96% 5 min, abs 5 min, abs 3 × 20 min), infiltrated with propylene oxide for 2 × 10 min and embedded in Epon following standard procedures. Ultrathin sections were cut on a Reichert-Jung Ultracut E microtome (Wetzlar, Germany) and collected on one-hole copper grids with Formvar supporting membranes, stained with uranyl acetate and lead citrate and examined on a Philips EM 400 transmission electron microscope (Eindhoven, the Netherland) operated at an accelerating voltage of 60 kV.

Transport of horseradish peroxidase (HRP) from the vasculature to the lateral line organ

Two animals of 205 and 270 g body weight, respectively, were anaesthetized in benzocaine (0.06 g L^{-1}) until the animals were unresponsive to tactile stimulation. HRP 0.5 mg g^{-1} body weight (P-8250 SIGMA peroxidase type II, Steinheim, Germany) was injected through the ventral midline posterior to the second dorsal fin into a dorsal vessel within the haemal arch. Animals were allowed to recover in a holding tank, for 2 and 22 h, respectively, and subsequently euthanized and perfusion fixed as described above, immersed in 2.5% glutaraldehyde and stored at 4 °C. Animals that had not been injected with peroxidase, but otherwise treated identically, served as controls.

Tissue samples were cut from the lateral line *c.* 2/3 down the length of the fish. Samples were frozen in isopentane cooled by liquid nitrogen, mounted on section mount using Tissue-Tek® (4583 Sakura O.C.T™ Compound, Zoeterwonde, the Netherlands), sections (8 μm) (Zeiss Microm HM560, Oberkochen, Germany) were collected on poly-lysine-coated microscope slides and air dried for 20 min, incubated for 10 min in 1 μL mL^{-1} 30% hydrogen peroxide in 3,3-diaminobenzidintetrahydrochloride dihydrate 0.5 mg mL^{-1} (Kem-En-Tec, Copenhagen, Denmark), stained with toluidine blue for 2 min, rinsed in water and dehydrated shortly in a graded series of ethanol (70%, 96%, 2 × abs). Sections were coverslipped and photographed using a Zeiss Axioplan2 light microscope.

Results

Lateral line morphology

The schematic drawing in Fig. 1 provides a guideline to the position and orientation of the light micrographs. The lateral line organ in the Atlantic cod *G. morhua* was located lateral to

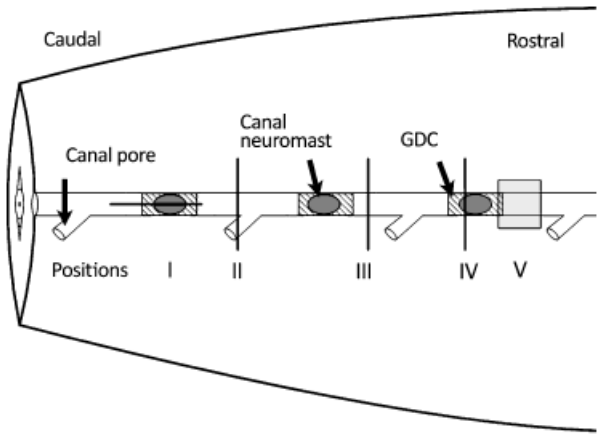

Figure 1 Schematic drawing of the lateral line organ in the Atlantic cod, caudal towards the left in the picture. Positions I–V relate to the position and orientation of sections of the light micrographs in Figs 2, 4 and 6. Pos. I represents the longitudinal section through the lateral line canal and canal neuromast, pos. II–IV transverse section and pos. V section tangential to the skin surface. The extent of gutter of dense connective tissue (GDC) is indicated by the hatched area.

the scales of the skin (Fig. 2a). Histological examination showed that the lateral line organ was in essence a tube (the pericanalicular sinus, PCS) with a tube of a smaller diameter inside (the LLC), separated by a wall of multilayered epithelium (Fig. 2a). The stereovilli of the neuromast hair cells were embedded in a gelatinous cupula, the remains of which can be observed in Fig. 2b,c. The LLC was suspended in the PCS by very loose reticular tissue composed of stellate cells in a matrix of an amorphous intercellular substance that stained metachromatically with toluidine blue. Around segments of the LLC that contained neuromasts, the PCS was reinforced by a laterally open gutter of very dense connective tissue (Fig. 2b). This gutter was observed only at the level of the neuromast (Fig. 2d). An aperture in the gutter gave passage for the neuromast afferent nerve (Fig. 2d). The LLC was connected to the external environment via canal pores that departed at acute angles, oriented caudally, whereas the PCS was closed.

Primary and secondary vessel identification

The vessel wall of primary and secondary capillaries was structurally identical at the light microscopy level.

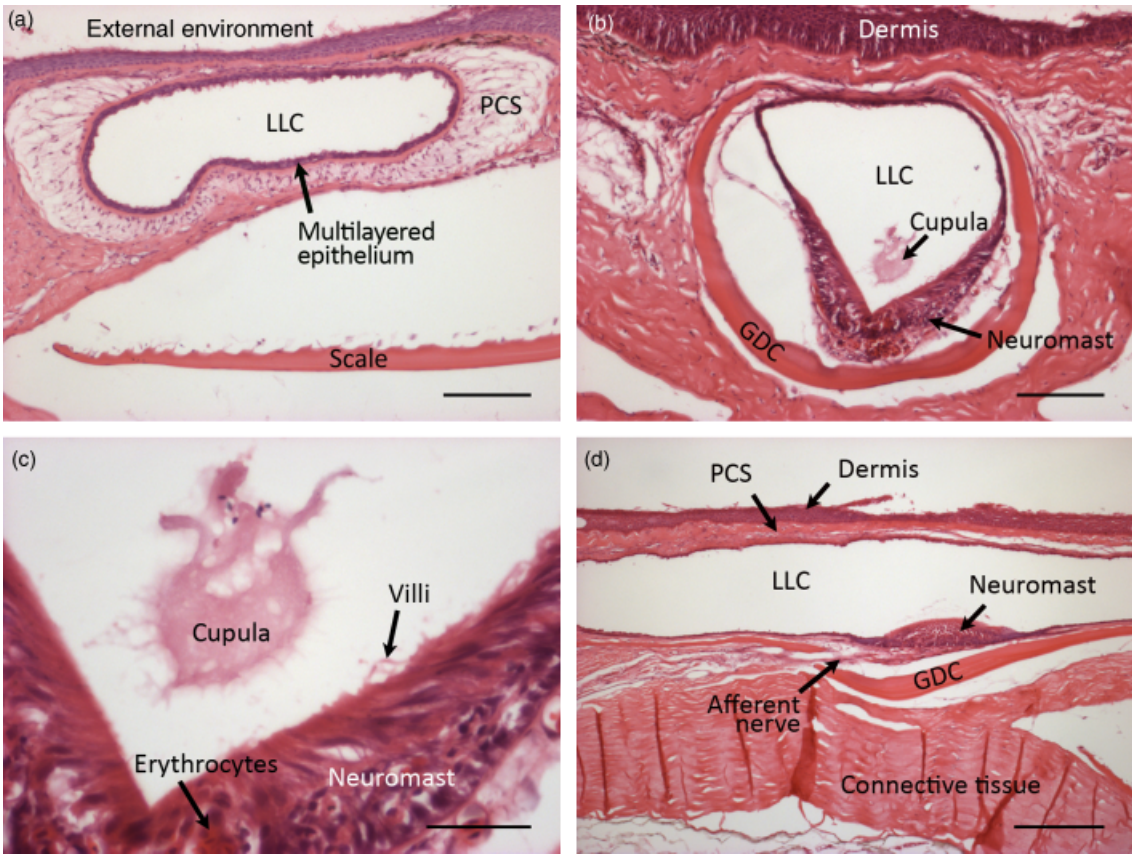

Figure 2 Transverse (a, b and c, pos. III, IV and IV in Fig. 1, respectively) and longitudinal section (d, pos. I in Fig. 1) of the lateral line. (a) shows the lateral line canal (LLC) with the surrounding pericanalicular sinus (PCS) and the multilayered epithelium. The stereovilli of the LLC neuromast protrude into a gelatinous cupula, the remains of which are visible in (b) and (c). The lower magnification of (b) displays the gutter of dense connective tissue (GDC) that supports the LLC at the level of the neuromast. The longitudinal section of the LLC, (d), shows the neuromast and associated afferent nerve fibre. Scale bars (a) and (c) 100 μm; (b) 30 μm; (d) 200 μm. H&E staining. (a–c) 8 μm; (d) 2 μm.

Immersion fixation of the animals, without puncturing the circulatory system, allowed identification of the secondary vessels. The vessels of the primary vasculature were all filled with erythrocytes, while the secondary vessels were virtually devoid of erythrocytes, but still contained fluid (Fig. 3). Secondary arteries can be seen running parallel to the segmental artery in Fig. 3. Had the virtual absence of erythrocytes been an artefact, the vessels would have been empty, not filled with plasma.

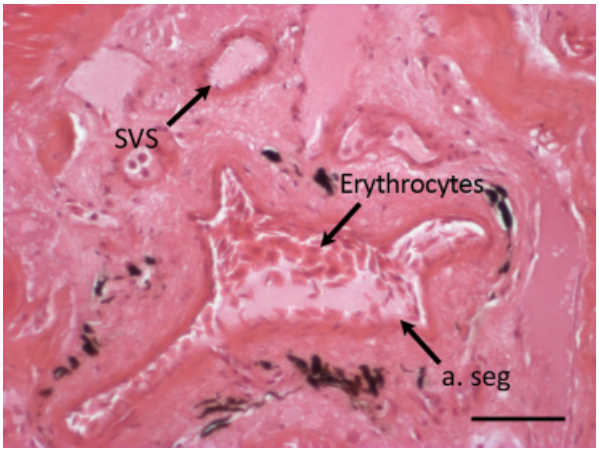

Figure 3 Transverse section of the segmental artery (a. seg) and associated secondary vessel (SVS). The lumen of the primary artery is filled with erythrocytes, whereas the secondary vessel is plasma filled and devoid of erythrocytes. Scale bar 50 μm. H&E staining, 2 μm sections.

Secondary blood vessels supplying the PCS ran along the dorsal and ventral side of the lateral line organ (Fig. 4a) and were often quite numerous (Fig. 4b). Immersion-fixed capillaries adjacent to the PCS were always plasma filled and devoid of erythrocytes, indicating that they were of secondary origin. Efferent and afferent vessels could not be distinguished. The wall of the SVS capillaries adjacent to the PCS had a remarkably thin endothelium (Fig. 4a), suggesting the possibility of fluid exchange. An extensive SVS skin capillary network was observed (Fig. 4c) and so were primary capillaries supplying the scales (Fig. 4d).

Ultrastructure of SVS capillaries and LLC epithelium

Transmission electron microscopy of the endothelium of the capillaries in contact with the reticular tissue of the PCS

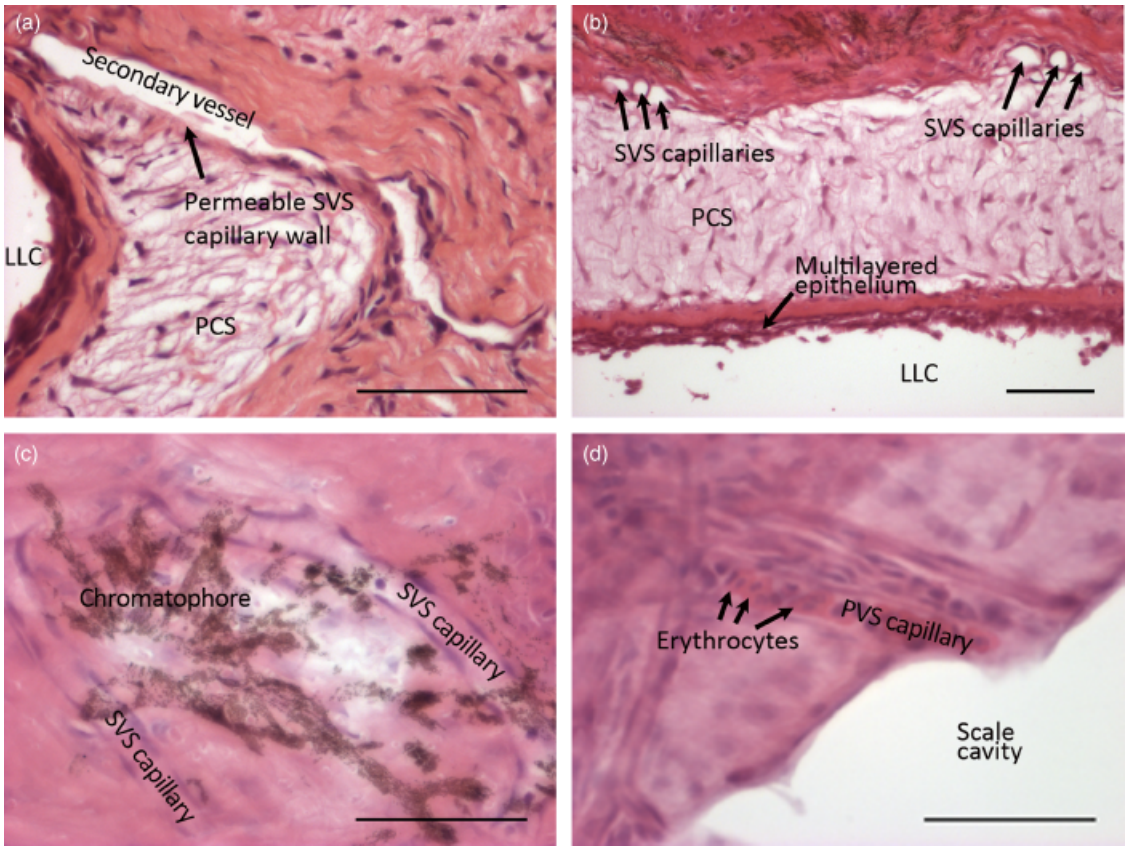

Figure 4 Transverse section (a) and section tangential to the skin (b–d) of the lateral line and associated vessels. (a) and (b) show the dorso-ventral vessels in longitudinal (pos. III in Fig. 1) and transverse (pos. V in Fig. 1) sections, respectively. (c) shows capillaries of the dermis (pos. V in Fig. 1). The primary capillary of the scale containing erythrocytes is shown in (d) (pos. V in Fig. 1). The scale itself dissolved in the decalcification process. Scale bars 50 μm. H&E staining. (a) 2 μm; (b) 4 μm; (c) 4 μm and (d) 1 μm sections.

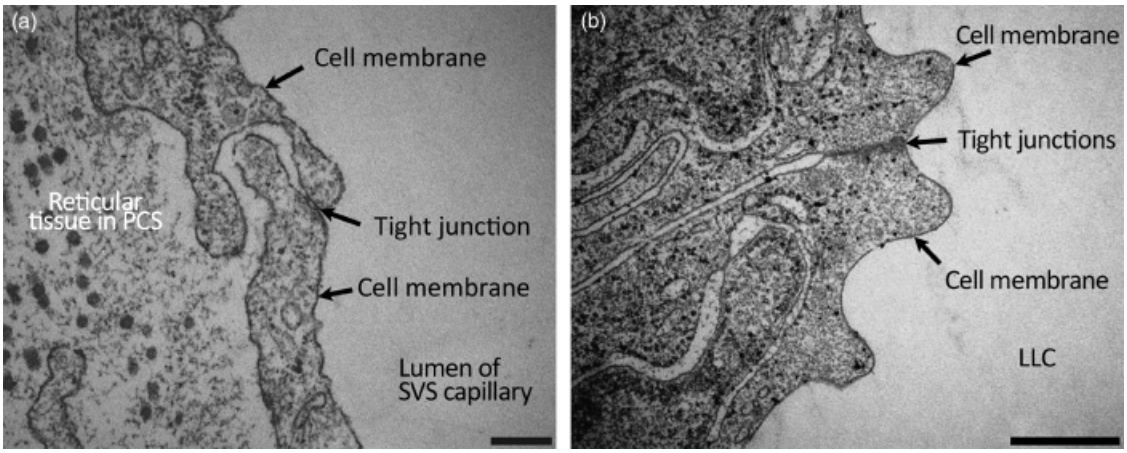

Figure 5 (a) Transmission electron micrographs of a secondary capillary similar to the one shown in Fig. 4a. The endothelium of the secondary vessel is connected by a few tight junctions. (b) Epithelial cells of the wall of the lateral line canal (LLC) are connected by several tight junctions and thus impermeable to fluid. Scale bars (a) 200 nm; (b) 500 nm. Lead citrate staining, 80 nm sections.

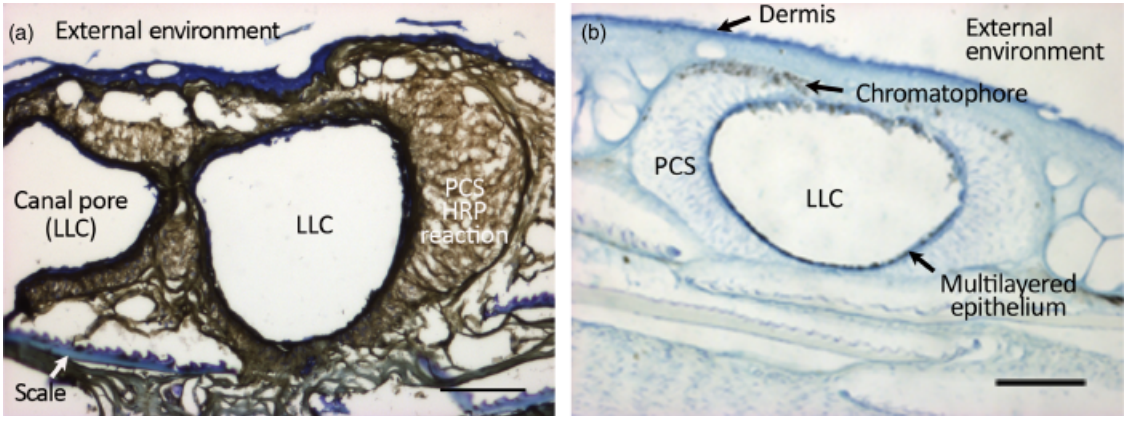

Figure 6 Light micrographs of transverse cryo sections of the lateral line. (a) Sites of HRP reaction (black to brown) following injection of HRP into the primary vascular system after 2 h (pos. II in Fig. 1). (b) Control (pos. III in Fig. 1) showing faint endogenous peroxidase activity in the multilayered epithelium of the lateral line canal (LLC). Pericanalicular sinus (PCS). Scale bars 100 μm. Toluidine blue staining, 8 μm sections.

showed tight junctions between adjacent cell luminal membranes (Fig. 5a). Tight junctions between endothelial cells were mostly singular, whereas cells in the multilayered epithelium of the LLC were connected by several tight junctions, enabling only transepithelial transport between the PCS and the LLC (Fig. 5b).

Transport of HRP tracer from the vasculature to the lateral line organ

HRP-injected animals showed HRP transfer to the PCS within 2 h of injection (Fig. 6a). Judged from the intensity of the HRP reaction, HRP did not build up further during the next 20 h. HRP is a small molecule (molecular mass 40 kDa), allowing HRP to enter the SVS, and is passed readily through the endothelium of the dorso-ventral vessels to the PCS. The epithelial cells of the LLC were strongly coloured by the HRP reaction product, indicating active uptake of HRP from the reticular tissue of the PCS or endogenous peroxidase activity. In control animals, a faint endogenous peroxidase activity was observed in the epithelium lining of the LLC, but not in amounts that could explain the activity observed in the HRP-injected animals (Fig. 6b).

Discussion

Owing to the unique angio-architecture of the SVS, vessels of this system are virtually devoid of erythrocytes. In this study, immersion-fixed animals with an intact vasculature showed two types of vessels indistinguishable on the basis of endothelial morphology: one filled with erythrocytes, and the other virtually devoid of erythrocytes (Fig. 3). Because fluid is fixed in the lumen of both types, it is possible to determine the origin of the vessels by the presence of erythrocytes. Dorso-ventral vessels were observed in association with the PCS and ultrastructural examination

showed that paracellular transport was possible between the vessels and the PCS (Fig. 5a). The absence of erythrocytes in the dorso-ventral vessels of the PCS and the capillary beds of the dermis, in immersion-fixed animals, provide strong evidence that these vessels originate from the SVS. In addition, erythrocytes were never observed in the PCS. The HRP experiments showed that the loose connective tissue in the PCS has a spongeous character, probably allowing fluid flow through the sinus. This is seen by the extent of coloration of the connective tissue in the PCS (Fig. 6a).

The function of the lateral line is well documented, both in terms of stimulus and nervous response (Dijkgraaf, 1963; Blaxter, 1987; Kroese & van Netten, 1989). The type of ions carrying the depolarizing current is presently unknown, but the transduction channel is open to monovalent cations with little specificity (Corey & Hudspeth, 1979). Most likely K^+ is the predominant ion to carry the depolarizing current, considering the generally high K^+ concentration at the apical surface of hair cells (Kroese & van Netten, 1989). Although the LLC is connected with the external environment, the canal pores are occluded by a gelatinous substance. The ionic permeability of the gelatinous substance is not known, but being the only barrier between the LLC and the external environment, it can be considered to be a semi-open state requiring a high rate of ion transfer to the LLC in order to maintain the high K^+ environment necessary for the optimal function of the canal neuromast. The cells of the epithelial wall of the LLC are connected by several tight junctions, and the multilayered epithelium is paracellular impermeable (Fig. 5b). Ions can only enter the LLC from the external environment through the gelatinous sealed pores or through transcellular transport from the PCS. Most likely, the ions are supplied by the fluid in the PCS, which is indicated by the strong content of HRP reaction product in the LLC epithelium (Fig. 6a), a content that cannot be explained by endogenous peroxidase activity (Fig. 6b). A similar function of the SVS could be present in superficial neuromasts because skin capillaries are predominantly secondary. Russell & Sellick (1976) reported an elevated K^+ concentration in neuromast cupula of *Xenopus laevis*. The gelatinous cupula could possibly retard ion outflux, thus maintaining a higher K^+ concentration around the neuromast hair cells.

Studies have been conducted to determine the nature of the fluid in the SVS. Ishimatsu *et al.* (1992) reported significantly fewer erythrocytes in the LCV associated with the secondary vasculature than in the primary system. Of the major ions (Na^+, Ca^{2+}, K^+, Mg^{2+} and Cl^-), only K^+ showed a significantly lower concentration in the SVS. Accurate measurements of ionic composition in the LLC are desirable, but the LLC epithelium is very delicate, making fluid sampling difficult. Rupturing of the epithelium will contaminate the LLC fluid with that of the PCS, which presumably has an 'intermediate' ionic composition.

In summary, the arrangements of secondary vessels, as well as their morphology and ultrastructure, the rapid transfer of HRP and the findings by Ishimatsu *et al.* (1992) provide strong evidence that the SVS is involved in fluid and ion transfer to the PCS and possibly K^+ ion transfer to the LLC of the lateral line organ in *G. morhua*. Because the hair cells of the LLC are both structurally and functionally similar to the hair cells of the semicircular canals and the otholitic organs, it could be speculated that the SVS also supply these structures. A connection has never been shown, but numerous interarterial anastomoses are present on the internal carotid artery in several species of teleost fishes (Skov & Bennett, 2004). Branches of the internal carotid artery supply the brain and surrounding areas, and it is therefore possible that the SVS is responsible for the fluid supply to the semicircular canals and the otholitic organs.

The apparent widespread distribution of SVS capillaries suggests that this vascular system may be involved in other transepithelial exchange processes.

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