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A comparative analysis of putative oxygen-sensing cells in the fish gill

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SUMMARY

We investigated the distribution of serotonin (5-HT)-containing neuroepithelial cells (NECs), the putative O_2 sensing cells, in the gills of four species of fish: trout (*Oncorhynchus mykiss*), goldfish (*Carassius auratus*), trairão (*Hoplias lacerdae*) and traira (*Hoplias malabaricus*) using immunohistochemical markers for 5-HT, synaptic vesicles and neural innervation. We found that all fish had a cluster of innervated, serotonergic NECs at the filament tips, but there were species-specific distributions of serotonin-containing NECs within the primary gill filaments. Trout gill filaments had a greater number of serotonin-containing NECs than both trairão and traira, whereas goldfish primary filaments had none. Serotonin-containing NECs in the secondary lamellae were most numerous in goldfish, present in trairão and traira, but absent in trout. Those found in the primary filament were generally associated with the efferent filamental artery. Innervated, serotonin-containing cells (NECs or Merkel-like cells) were also found in the gill rakers of trout and goldfish although vesicular serotonin was only found in the gill rakers of goldfish. These differences in serotonergic NEC distribution appear to reflect paracrine *versus* chemoreceptive roles related to hypoxia tolerance in the different fish species.

Key words: fish chemoreceptors, neuroepithelial cells, serotonin, gill raker.

INTRODUCTION

When fish are exposed to acute hypoxia they reduce their heart rate, elevate gill vascular resistance, hyperventilate and increase ventilatory amplitude (Randall, 1982). These acute hypoxic responses are reflexly initiated by oxygen chemoreceptors, but the sites of these receptors have not been definitively established. Previous researchers have proposed that oxygen chemoreceptors in fish could be located in the brain (Smatresk et al., 1986), the arterial/venous vasculature (Randall, 1982), the orobranchial cavity (Milsom et al., 2002), the pseudobranch (Laurent and Dunel, 1980) or the gills (Burleson and Milsom, 1993; Reid and Perry, 2003; Sundin et al., 2000). It has also been proposed that the receptors in the gills are situated in locations that sense either external oxygen tension (P_{O_2}) in the incident flow of water (Pw_{O_2}), internal P_{O_2} within the gill vasculature itself (Pa_{O_2} or Pv_{O_2}), or both (Randall, 1982).

Of note is that the arterial blood supply to the first gill arch of fish is homologous to the carotid artery where the carotid body, the primary peripheral chemoreceptive organ in mammals, is found. Furthermore, putative O_2 chemosensing cells in the first gill arch and carotid body have similar ultrastructure (Bailly et al., 1992; Jonz and Nurse, 2003), patterns and levels of afferent nerve discharge (Milsom and Brill, 1986; Burleson and Milsom, 1993), embryonic origin (Milsom, 1998), innervation by the glossopharnygeal (CN IX) nerve (Sundin and Nilsson, 2002; Gonzalez et al., 1994) and chemoreceptive mechanisms (Jonz et al., 2004).

The putative oxygen chemosensing cells in fish gills are neuroepithelial cells (NECs). Neuroepithelial cells have been characterized in the respiratory epithelia of all classes of vertebrates. First described in the airway epithelia of mammals (Feyrter, 1938) as neuroepithelial bodies (NEBs), this general group of amine-containing cells is characterized by granulated vesicles, proximity to efferent and afferent nerve fibres, and fluorescence when treated with formaldehyde vapour (Pearse, 1969). The first report of NECs in fish gills used formaldehyde-induced fluorescence to reveal both isolated and clustered populations of cells in the primary epithelium of the fish gill, characterized by the presence of dense-cored vesicles (DCV, 80–100 nm in diameter) containing monoamines (serotonin) in proximity to neurons (Dunel-Erb et al., 1982). Since this first report, NECs have been detected in every fish studied to date with the majority of studies focusing exclusively on the first gill arch.

Using antibodies for serotonin (5-hydroxytryptamine; 5-HT), a transmembrane synaptic vesicle marker (SV2) and a general neuronal marker raised in zebrafish (zn12), Jonz and Nurse (Jonz and Nurse, 2003) showed a strong association between NECs and both intrinsic and extrinsic nerve fibres in zebrafish gills. NECs of zebrafish are located along the central axis of the gill filament as well as the edges of the lamellae, locations that would be ideal for sensing both Pa_{O_2} and Pw_{O_2}, respectively. NECs have been previously shown to degranulate during acute hypoxia (Dunel-Erb et al., 1982), suggesting that neurotransmitter release occurs, and subsequent studies found that this was probably mediated by inhibition of background K^+ channels leading to membrane depolarization (Jonz et al., 2004). Chronic hypoxia, however, resulted in NEC hypertrophy and extension of cell processes (Jonz et al., 2004) revealing time–domain effects of hypoxia on NECs and further supporting their role as chemoreceptors.

Rainbow trout *Oncorhynchus mykiss* respond more vigorously to arterial than aquatic hypoxia and have relatively poor hypoxia tolerance (Tetens and Lykkeboe, 1981; Burleson and Milsom, 1990; Burleson and Milsom, 1993). Carp, on the other hand, respond only to aquatic hypoxia and are extremely hypoxia tolerant (Hughes et al., 1983) (A. E. O'Neil, A. L. Lumsden and W.K.M., manuscript in preparation). Given the data presented above, we hypothesized that such differences between physiological responses to hypoxia

in these two species would be associated with different distributions of putative O_2-sensing cells in the gills that reflect each species' hypoxia tolerance. The objective of the present study was to further test the above hypotheses in several species with varying hypoxia tolerances, and to comprehensively analyze all putative O_2-sensing regions within the gills. Thus, as well as examining NEC distributions in trout and carp, species which have been the subject of previous investigations, we collected data from two previously unstudied species of closely related Amazonian fish that differ in their hypoxia tolerance (traira *Hoplias malabaricus* and trairão *Hoplias lacerdae*). Based on our hypothesis, we predicted that the hypoxia-intolerant fish (trout and trairão) would have NEC distributions that favour sensing arterial oxygen levels, whereas hypoxia tolerant species (goldfish *Carassius auratus* and traira) would have distributions that favour sensing environmental oxygen levels.

MATERIALS AND METHODS

Animals

Adult rainbow trout *Oncorhynchus mykiss* Walbaum (N=6, 520–1190 g) and goldfish *Carassius auratus* L. (N=6, 20–100 g) were kept in outdoor, flow-through, and aerated tanks in the Zoology Aquatic Facility at the University of British Columbia. Trairão *Hoplias lacerdae* (Ribeiro) (N=6, 90–200 g) and traira *Hoplias malabaricus* (Bloch) (N=6, 140–260 g) were maintained in 250 l outdoor tanks at 25±1°C at the Federal University of São Carlos (São Paulo, Brazil) and supplied with aerated water. Fish used for tissue analysis were randomly selected and killed by an overdose of MS-222 (3-aminobenzoic acid ethyl ester) followed by a sharp blow to the head.

Tissue preparation

The first and second gill arches were excised from each fish and washed in ice-cold phosphate-buffered saline (PBS) containing (in mmol l^{-1}): NaCl, 137; Na_2HPO_4, 15.2; KCl, 2.7; KH_2PO_4, 1.5; buffered to pH 7.8 with 1 mol l^{-1} NaOH (Jonz and Nurse, 2003). Individual gill arches were syringe-perfused with heparinized PBS (1000 i.u. ml^{-1}) until filaments appeared clear, and fixed by immersion in 4% paraformaldehyde in PBS at 4°C overnight. Tissues were then rinsed in PBS and cryoprotected in a 30% sucrose solution. Segments of the first and second gill arches were then frozen in Tissue-Tek® (Sakura Finetek, Fisher Scientific, Ottawa, ON, Canada) at –80°C until the blocks were either sectioned longitudinal to the gill filament at 10–30 μm or transverse to the gill filament at 10–14 μm using a cryostat (Leica CM3050 S, Leica Microsystems, Nussloch, Germany) and mounted on Superfrost® plus slides (VWR International, West Chester, PA, USA) for immunohistochemistry.

Immunohistochemistry

Slides were washed in PBS and blocked in 10% normal goat serum (Jackson Laboratories, distributed by Cedarlane Laboratories, Hornby, ON, Canada) for 1 h. Primary antibodies (used individually or in combination) were diluted in a permeabilizing solution (PBS, 0.2% Triton X-100, 0.1% sodium azide) according to the optimal dilutions, determined in preliminary experiments, detailed in Table 1, and set on the slides to incubate overnight at room temperature. Following incubation of the primary antibodies, the slides were again washed in PBS. The slides were then treated with fluorescently labelled secondary antibodies diluted in PBS (Table 1) and left to incubate at room temperature for 2 h in darkness. Following a final wash with PBS, coverslips were mounted with Vectashield (Vector Laboratories, Burlington, ON, Canada) to prevent photobleaching, and the edges of the coverslips were sealed with nail polish. Slides were stored at 4°C until viewed under an epifluorescence light microscope (Axioplan 2, Zeiss, Jena, Germany). Control experiments were performed in which the primary antibodies were excluded to control for non-specific binding of the secondary antibody, and normal serum (IgG) from primary antibody host species was used (at an equivalent protein concentration) to test for non-specific reaction of the antibodies' primary hosts. The results of these control experiments showed only negligible staining with no specific immunolabelling of the gill structures (data not shown). Immunolabelled slides were observed under a light microscope (Axioplan 2, Zeiss, Jena, Germany) and digitally captured using a Q-Imaging (Burnaby, BC, Canada) CCD camera and analyzed using image analysis software (Northern Eclipse, Empix Imaging, ON, Canada). A few representative sections cut at 16–30 μm were further examined using a confocal scanning system (Pascal 2, Axioskop X, Zeiss, Jena, Germany) equipped with argon (Ar) and helium-neon (He-Ne) lasers with peak outputs of 488 nm and 543 nm, respectively. Images obtained from the epifluorescence light microscope were subsequently used for quantification of staining intensity and cell counts.

Quantification

Distribution patterns of 5-HT immunoreactive (5-HT-IR) cells were obtained through analysis of average staining intensity from the tips of the lamellae to the midline of the filament after subtracting background (threshold) intensity. This distance was expressed relative to lamellar length, where 0 represents the lamellar tip and 100 represents the filament midline, to standardize the distance

Table 1. Details of primary and secondary antibodies used for immunohistochemistry

Antiserum	Dilution	Antigen	Host	Source	Secondary antiserum*
Primary					
5-HT	1:400	Serotonin	Rabbit	Sigma-Aldrich	Alexa Fluor® 488
SV2†	1:200	Synaptic vesicles, neuronal and endocrine	Mouse	DSHB‡	Alexa Fluor® 594
zn12†,§	1:25	Neuron, surface	Mouse	DSHB‡	Alexa Fluor® 594
Secondary*					
Alexa Fluor® 488	1:500	Rabbit IgG (H+L)	Goat	Molecular Probes, Invitrogen	–
Alexa Fluor® 594	1:300	Mouse IgG (H+L)	Goat	Molecular Probes, Invitrogen	–

*Secondary antisera were conjugated with a fluorescent marker.
†Monoclonal antibody.
‡Developmental Studies Hybridoma Bank, University of Iowa.
§Zebrafish-specific antibody.

measured in each individual fish for varying gill sizes and across all species examined. Intensity thresholds were created for each individual fish, and line intensity measurements were made along the relative lamellar length of 140 individual lamellae from six individual fish per species. This method of quantification displays the pattern of immunoreactivity analyzed pixel by pixel from numerous microscope images. Threshold and intensity analysis were conducted using SigmaScan Pro 5.0 (SPSS, Chicago, IL, USA). Additionally, the numbers of 5-HT-IR cells were counted per mm of filament (McCormick et al., 2003) from six representative images per individual fish.

Transmission electron microscopy

Gill arches were fixed in 2.5% glutaraldehyde in a 0.1 mol l^{-1} sodium cacodylate buffer (pH 7.35), rinsed with 0.1 mol l^{-1} sodium cacodylate and postfixed in 2% osmium tetroxide in 0.1 mol l^{-1} sodium cacodylate. After washing in distilled water, the preparations were dehydrated and embedded in Spurr's resin. Sections (60 nm thick) were cut using a Leica Ultracut T (Vienna, Austria) using a 35° Diatome diamond knife, and mounted on 150 mesh copper grids. Specimens were viewed with a Hitachi H7600 (Hitachi Ltd, Tokyo, Japan) transmission electron microscope and pictures were captured using a 1K AMT (Advanced Microscopy Techniques Corp., Danvers, MA, USA) side mounted digital camera.

Statistics

Data are presented as mean average intensity with 95% confidence limits, and as mean ± s.e.m. for 5-HT-IR cell number. Differences between cell numbers in filaments and lamellae within each species were compared using a Student's paired *t*-test. One-way analysis of variance (ANOVA) followed by a Tukey–Kramer *post-hoc* test was used to test differences in filament and lamellar cell number among the four species. $P<0.05$ was used to reject the null hypothesis. Statistical analyses were performed using the software package JMP 5.1.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

There were no observable differences in morphology or immunoreactive staining between the first and second gill arches; therefore, the results from analysis of both first and second gill arches were pooled together. There were distinct differences in the distribution of serotonergic cells along the filament among the four species examined in this study. In trout, 5-HT-IR cells were found along the primary filament, frequently near filamental blood vessels (Fig. 1A) as well as at the filament tip. This contrasted sharply with what was seen in the goldfish, where serotonergic cells were located in the lamellae (Fig. 1B) as well as at the filament tip. In trairão (Fig. 1C) and traira (Fig. 1D), 5-HT-IR cells were found along the filament, in the lamellae and at the filament tip. In the lamellae of goldfish, trairão and traira the 5-HT-IR cells were primarily located at tips. Quantification of staining intensity for 5-HT from lamellar tips to the central axis of the filament (the median line between the efferent and afferent sides of the filament) confirmed this difference in distribution (Fig. 2). Trout showed no staining along the lamellae, but had a strong peak of intense staining beginning at the filament epithelium (Fig. 2A). The average intensity profile of the goldfish revealed the opposite pattern, with staining intensity highest near the lamellar tip (Fig. 2D). The two species of *Hoplias*, trairão and traira, had similar staining profiles, with stronger staining intensity towards the tip of the lamellae and gradually less intense staining towards the filament (Fig. 2B,C).

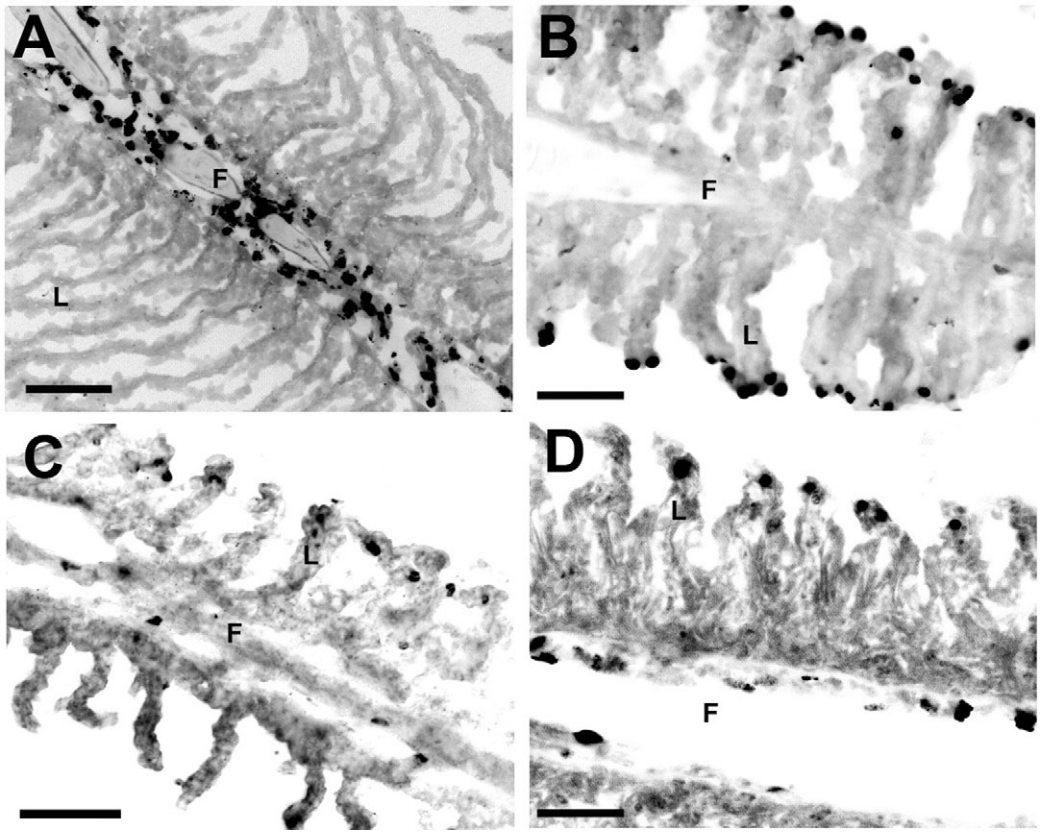

Fig. 1. Distribution of serotonergic immunoreactive cells (darkly stained cells) along the filament (F) and lamellae (L) in (A) *O. mykiss*, (B) *C. auratus*, (C) *H. lacerdae*, and (D) *H. malabaricus*. Scale bars, (A) 200 μm, (B–D) 100 μm. Images were colour-inverted to emphasize bright immunofluorescent structures.

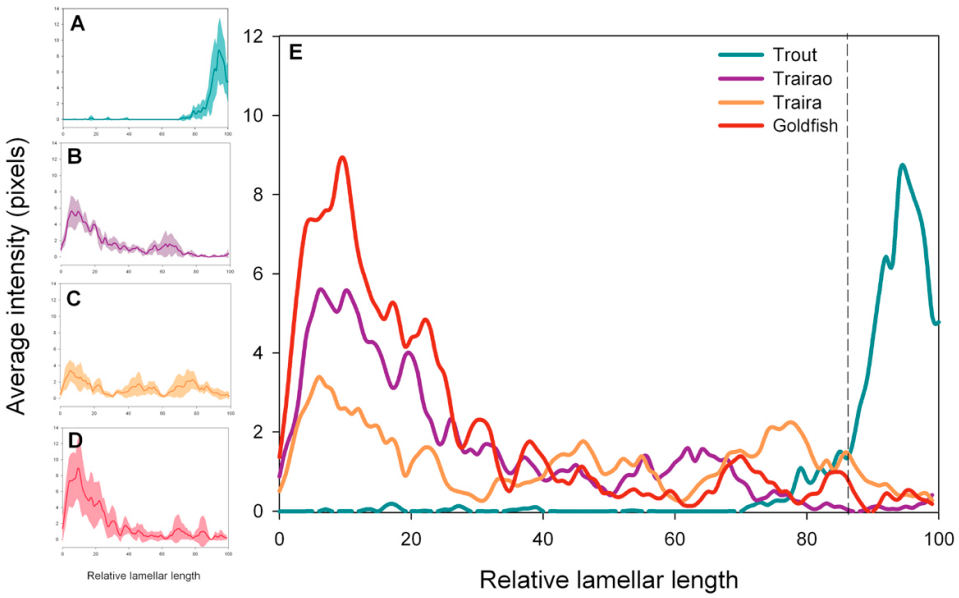

Fig. 2. Distribution of 5-HT-IR cells expressed as the average staining intensity (pixels) from lamellar tips (0) to the central axis of the filament (100) in (A) *O. mykiss*, (B) *H. lacerdae*, (C) *H. malabaricus* and (D) *C. auratus*. Shaded area represents the 95% confidence interval associated with the mean distribution (solid line) for each fish species. (E) Comparison of the mean distribution of 5-HT-IR cells among the four species. Broken line represents the division between lamellae and filament.

Counts of 5-HT-IR cells produced results similar to the average intensity profiles. The number of cells on the filaments compared with the lamellae of each species were significantly different in all fish (P<0.001) (Fig. 3). As well, significant differences were found between species (Fig. 3). Rainbow trout had a higher number of immunoreactive cells on the filaments than the three more hypoxia-tolerant species. Additionally, there was a significant difference in the numbers of 5-HT-IR cells in the lamellae between the species of *Hoplias*.

Double immunolabelling of the gills with antibodies for a synaptic vesicle membrane protein (SV2) and serotonin, produced a strong colocalization in all four species (Fig. 4). Synaptic-vesicle-containing cells were located along the central filament and on the lamellae in all four species. The majority of cells immunoreactive for SV2 were also 5-HT positive; however, not all SV2-IR cells contained 5-HT (arrowheads, Fig. 4B,C). Serotonergic cells of the lamellae in goldfish, trairão and traira often colocalized with SV2, which was especially apparent in the goldfish (Fig. 4B), but whereas the SV2 marker did appear in the lamellae of trout, 5-HT did not (Fig. 4A). Furthermore, serotonin in cells was not always found in synaptic vesicles (Fig. 4D, arrow).

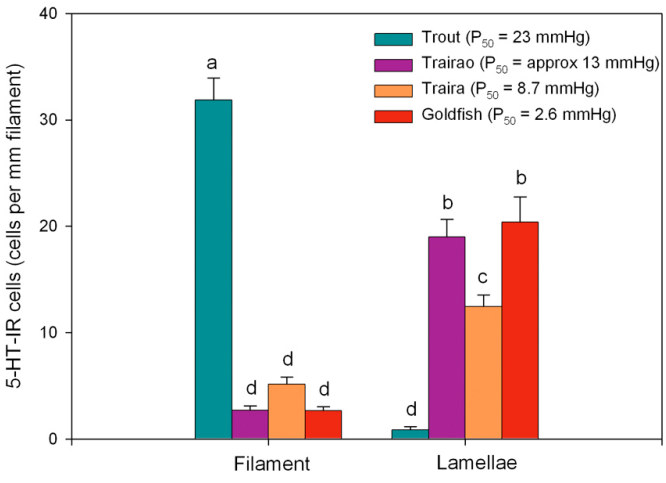

Fig. 3. Number of 5-HT-IR cells on the filament and lamellae in rainbow trout (*O. mykiss*), trairão (*H. lacerdae*), traira (*H. malabaricus)* and goldfish (*C. auratus*). Values are expressed as means ± s.e.m. At least 50 cells per individual and six individuals per species were measured. Letters (a–d) indicate cell counts significantly different from columns labelled with different letters. P_{50} values are given in the key as good indicators of relative hypoxia tolerance.

Confocal images of gill sections doubly-labelled with anti-5-HT and a general neuronal marker (anti-zn12) traced nerve fibres solely down the filament in trout, traira and trairão (Fig. 5A,C,D), whereas in goldfish nerve fibres extended down the filament (Fig. 5B) and into the secondary lamellae (inset). There did not appear to be any nerve fibres extending out of the primary epithelium into the lamellae of trout, traira and trairão. Thus, although the 5-HT-IR cells along the lamellae of goldfish were innervated, in traira and trairão they did not appear to be innervated.

A closer examination of NECs in the filaments of rainbow trout (Fig. 6A) and the lamellae of goldfish (Fig. 6B) using transmission electron microscopy revealed NECs characterized by dense-cored vesicles (insets) that were near to red blood cells (RBC) and pillar cells (pc). NECs in the filaments of rainbow trout were associated with nerves (N), near vasculature (RBC), and were located within the filament epithelium, as denoted by the basal lamina (BL; Fig. 6A). NECs of the goldfish lamellae were in close proximity to the ambient environment (H_2O), red blood cells (RBC) and pillar cells (pc; Fig. 6B).

To further define the location of the serotonergic cells located within the central filament, cross sections of individual gill filaments were immunolabelled and examined. Trout filament cross sections showed 5-HT-SV2-IR cells primarily located around the efferent filament artery (eFA), with a few cells located near the afferent filament artery (aFA) and central venous sinous (cvs; Fig. 7A). Cross sections of goldfish filaments supported the observation of serotonergic distribution at the lamellae tips, with no cells located in the central filament (Fig. 7B). Results from both trairão and traira also supported the findings from longitudinal sections (Fig. 4), showing 5-HT-SV2-IR cells in the lamellae and less frequently in the filament (Fig. 7C,D). Cross-sectional nerve tracings (of zn12)

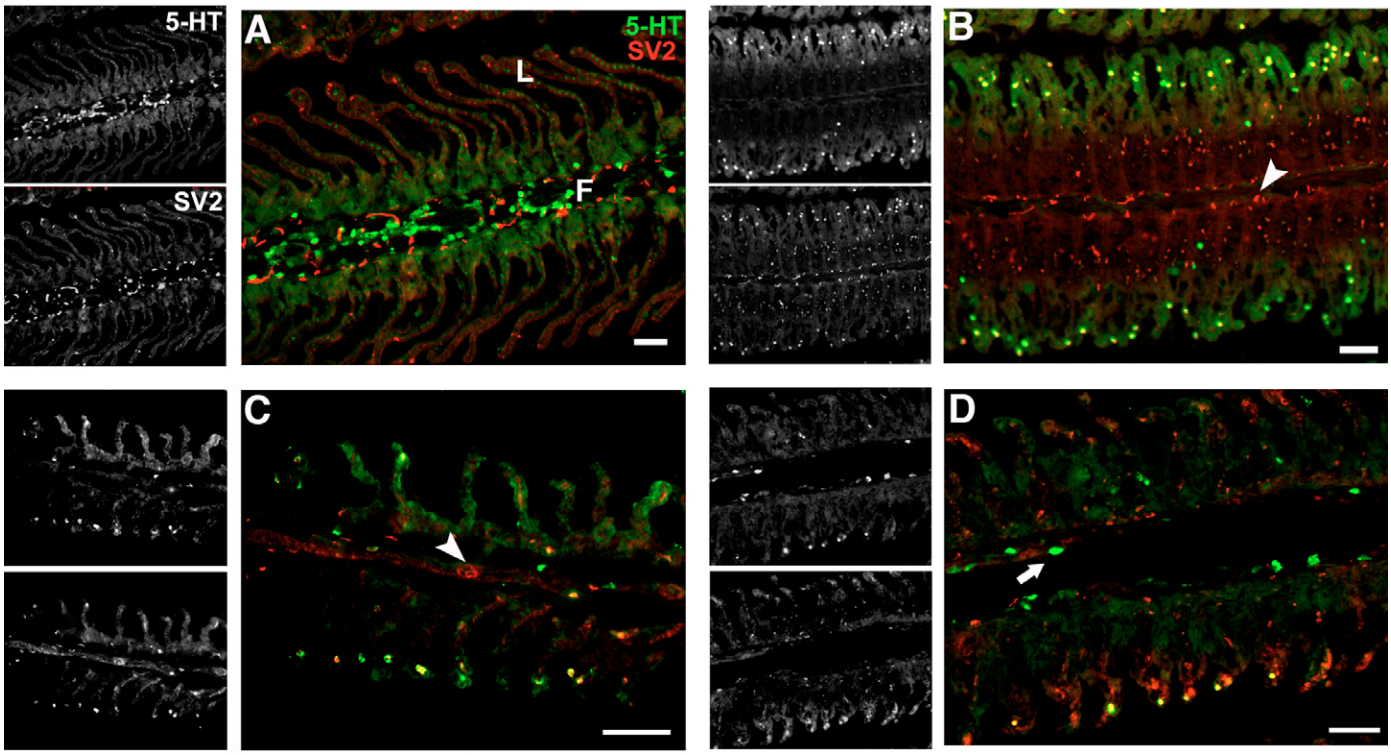

Fig. 4. Double immunolabelling of serotonin (5-HT, green) and synaptic vesicles (SV2, red) along a single gill filament with multiple lamellae in (A) *O. mykiss*, (B) *C. auratus*, (C) *H. lacerdae* and (D) *H. malabaricus*. Scale bars, 100 μm. Colocalization appears yellow. Arrowheads indicate non-5-HT-IR but SV2-IR cells and arrows indicate non-SV2-IR but 5-HT-IR cells.

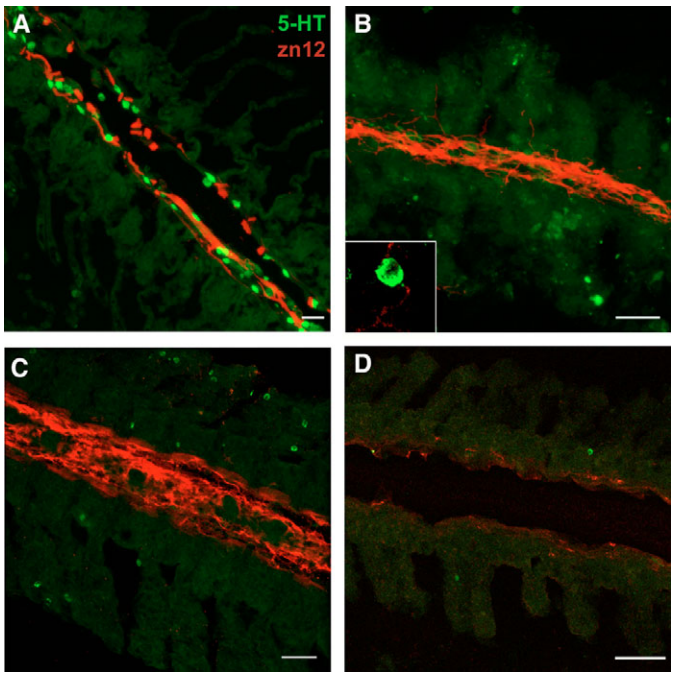

Fig. 5. Double immunolabelling of serotonin (5-HT; green) and nerves (using a neuronal marker zn12; red) in longitudinal sections (30 μm thick) of the gill filament and lamellae of (A) *O. mykiss*, (B) *C. auratus*, (C) *H. lacerdae* and (D) *H. malabaricus*. Images were captured using a confocal scanning system. Inset in B shows close association of nerves (red) and 5-HT-IR cell at the tip of the lamellae of *C. auratus*.

further corroborated our failure to find evidence of innervation of the lamellae in both trairão and traira, while supporting the finding of nerves extending into the lamellae of goldfish. 5-HT-positive cells within the central filament again showed a strong association with nerves, especially around the eFA.

Although there were distinct differences in the distribution of 5-HT-IR cells along the lamellae and filament, the distribution of 5-HT-IR cells at the tips of the gill filaments was consistent among the four species examined (Fig. 8). In all four species, clusters of 5-HT-SV2-positive cells were found at the tip of every filament, where the lamellae gradually decreased in size and the filament was directly exposed to the ambient environment (Fig. 8). A general neuronal marker (zn12) traced nerve profiles into the tip of the filament, and higher magnification images using confocal microscopy showed a close association between the 5-HT-IR cells and nerves (Fig. 9). This finding was characteristic of all fully intact filaments in all four species.

5-HT-IR cells were also found in the epithelium surrounding of both trout and goldfish. Double immunolabelling of the gills with antibodies for a synaptic vesicle membrane protein (SV2) and serotonin, produced a strong colocalization in goldfish but not in trout (Fig. 10). In the gill rakers of goldfish, as in the gill filament and lamellae, most SV2-IR cells were also 5-HT-IR; however, not all SV2-IR cells contained 5-HT (Fig. 10, arrowheads). In both trout and goldfish, confocal images of gill rakers doubly-labelled with anti-5-HT and a general neuronal marker (anti-zn12) traced nerve fibres to 5-HT-IR cells (Fig. 11). Thus, 5-HT-IR cells in the gill rakers of trout and goldfish were associated with nerve fibres. Gill raker tissue from traira and trairão was not available for immunostaining.

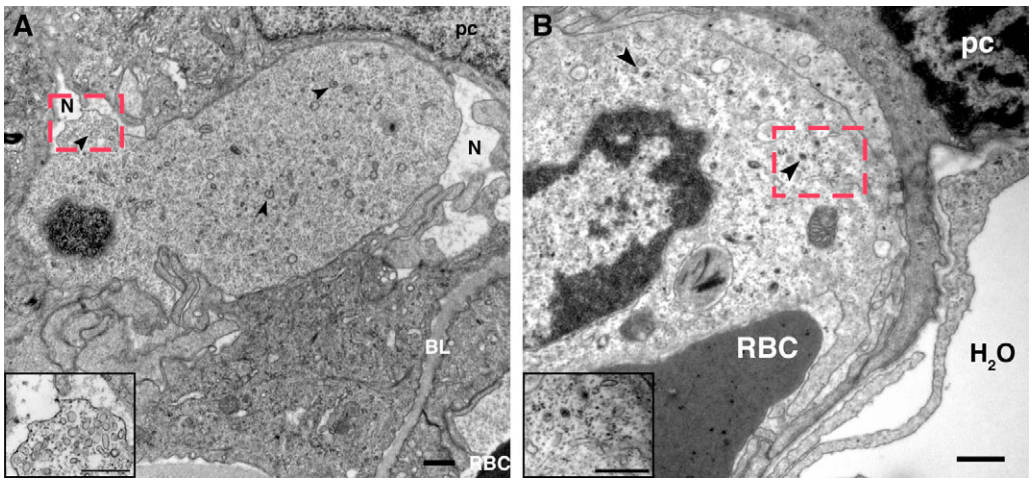

Fig. 6. TEM images of neuroepithelial cells (NECs) in (A) the filament of *O. mykiss* and (B) the lamella of *C. auratus*. Lower left-hand insets (areas indicated by broken red boxes) show the presence of dense-cored vesicles (arrowheads). Note the proximity of NECs in both A and B to a red blood cell (RBC), as well as a nerve (N) and the basal lamina (BL) in A. The NEC of the goldfish lamella (B) is also juxtaposed to a pillar cell (pc) and near the ambient environment (H_2O). Scale bars, 500 nm.

DISCUSSION

Denervation of the glossopharyngeal and vagus nerves innervating the gills has provided indirect evidence for a role of the gills in oxygen sensing (Burleson and Smatresk, 1990; McKenzie et al., 1991; Sundin et al., 2000; Florindo et al., 2006). Additionally, electrophysiological recording of chemoreceptive nerve fibres in the first gill arch (Milsom and Brill, 1986; Burleson and Milsom, 1993) and patch-clamp recording of isolated neuroepithelial cells (NECs) (Jonz et al., 2004) provided direct evidence of the chemoreceptive responses of the gill and NECs to hypoxia. Since their first identification (Dunel-Erb et al., 1982), gill NECs have been documented in every fish species studied to date, and their putative role in oxygen sensing and chemoreception has been supported by histological and electrophysiological examination. Characterized by the presence of serotonin stored in vesicles, NECs degranulate and depolarize in acute hypoxia (Dunel-Erb et al., 1982; Jonz et al., 2004), presumably releasing vesicular serotonin either on target cells, vasculature, or afferent nerves.

Within the fish gill, there are numerous potential sites for oxygen sensing. Sites capable of monitoring arterial or venous oxygen levels include the efferent filament artery (eFA), the central venous sinus (cvs) or the afferent filament artery (aFA); aquatic hypoxia ($P_{W_{O_2}}$) may be detected where the lamellar, filament, or gill raker epithelium is exposed to the ambient water. Oxygen-sensing cells at these different sites could also be either chemoreceptive or paracrine. A respiratory chemoreceptive cell releases a neurotransmitter directly onto an afferent nerve thereby transmitting information to the central nervous system to produce hypoxic reflexes, as in the case of the glomus cells of the carotid body (Krammer, 1978; Gonzalez et al., 1994; Nurse, 2005). Alternatively, non-innervated cells releasing chemical signals play a paracrine role (Nurse, 2005), releasing neuroendocrine transmitters directly onto a nearby target cell, such as vascular smooth muscle (Zaccone et al., 1992). In our comparative analysis of potential O_2-sensing sites, we observed NECs that could serve both chemoreceptive and paracrine roles based on their location and innervation. Furthermore, our results indicated a striking difference in the distribution of putative O_2-sensing cells (chemoreceptive and paracrine), as identified by colocalization of serotonin (5-HT) and synaptic vesicles (SV2), among the four species examined.

Chemoreceptive NECs

In all species examined in this study, we observed a cluster of innervated cells containing serotonin in vesicles at the filament tips (Figs 8, 9). NECs at the filament tips have also been described in cod (Sundin et al., 1998a), the Antarctic borch *Pagothenia borchgrevinki* (Sundin et al., 1998b) and zebrafish (Jonz and Nurse, 2003), and thus this appears to be a consistent location for chemoreceptive neuroepithelial cells across species. NECs at the filament tip are in an ideal location to sense changes in $P_{W_{O_2}}$, releasing serotonin directly onto an afferent nerve, transmitting the signal to the central nervous system (CNS) and producing a reflex hypoxic response. The presence of such a mechanism is supported by physiological data, indicating that all fish included in this study reflexly respond (increase ventilation) to aquatic hypoxia (Burleson and Milsom, 1993; Sundin et al., 1999) (A. E. O'Neil, A. L. Lumsden and W.K.M., manuscript in preparation). More specifically, denervation of the glossopharyngeal and vagus nerves in rainbow trout (Reid and Perry, 2003) and traira (Sundin et al., 1999) abolished the hyperventilation induced by application of exogenous (aquatic) NaCN, thereby localizing the external chemoreceptors to the gills. Subsequent studies supported this finding in other fish species (Milsom et al., 2002; Florindo et al., 2006), indicating that external NaCN stimulates hyperventilation by chemoreceptors located in the gills monitoring the aquatic environment.

Although innervated NECs were consistently present at the filament tip, we found species differences in the distribution of innervated NECs along the centre of the filament. Innervated cells containing serotonergic vesicles were present in high number in the filament core of rainbow trout, less so in trairão and traira, but not at all in goldfish (Figs 4, 5). The filament is flanked by an efferent filament artery (eFA) and afferent filament artery (aFA), with a central venous sinus (cvs) running down the middle of the filament, making it an ideal location to sense internal oxygen levels ($P_{a_{O_2}}$ or $P_{v_{O_2}}$). Examination of cross sections of the filament in trout, traira, and trairão (Fig. 7) revealed that most NECs along the central filament were associated with the eFA, a location that would sense the oxygenated blood returning to the systemic circulation. NECs in the centre of the filament may play a chemoreceptive role, sensing internal ($P_{a_{O_2}}$) hypoxia and producing a reflex response.

Examination of cross sections of goldfish filaments failed to identify any NECs in the filament (Fig. 7). This is in contrast to the finding of a previous study that identified innervated 5-HT-IR cells in the primary filament of goldfish (Saltys et al., 2006). Although the discrepancy between our findings and those of Saltys et al. (Saltys et al., 2006) may be a reflection of differences in the preparations used in the two studies, this seems highly unlikely. Saltys et al. (Saltys et al., 2006) used whole-mount preparations

and we used transverse slices. Although slices are thinner and there is the possibility that NECs are confined to a specific plane that we failed to examine this seems unlikely. Furthermore, our findings are supported by our inability to produce a response in carp to internal injections of NaCN (A. E. O'Neil, A. L. Lumsden and W.K.M., manuscript in preparation). Others (Eclancher and Dejours, 1975), however, have found a response to internal injections of NaCN. Thus, at the moment we cannot explain the discrepancy.

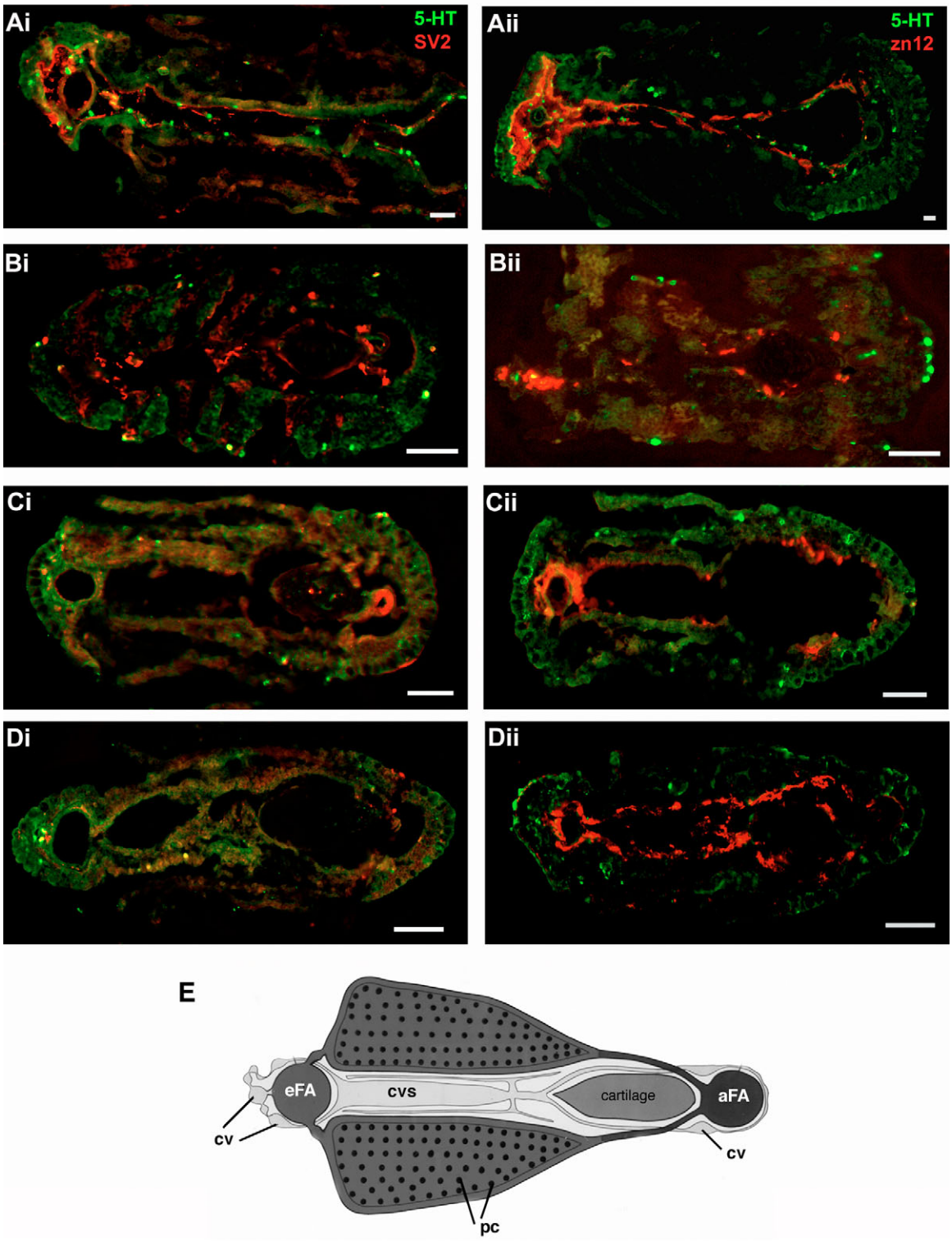

Fig. 7. Cross sections of gill filaments from (A) *O. mykiss*, (B) *C. auratus*, (C) *H. lacerdae* and (D) *H. malabaricus* showing double immunolabelling of 5-HT (green) and SV2 (red; Ai–Di) and 5-HT (green) and zn12 (red; Aii–Dii). (E) Schematic of a filament cross section showing the location of the efferent filament artery (eFA), central venous sinus (cvs), afferent filament artery (aFA), lamellar pillar cells (pc) and companion vessels (cv) [adapted from Farrell (Farrell, 1979)]. Note that lamellae are rarely sectioned in a straight plane. Scale bars, 100 μm.

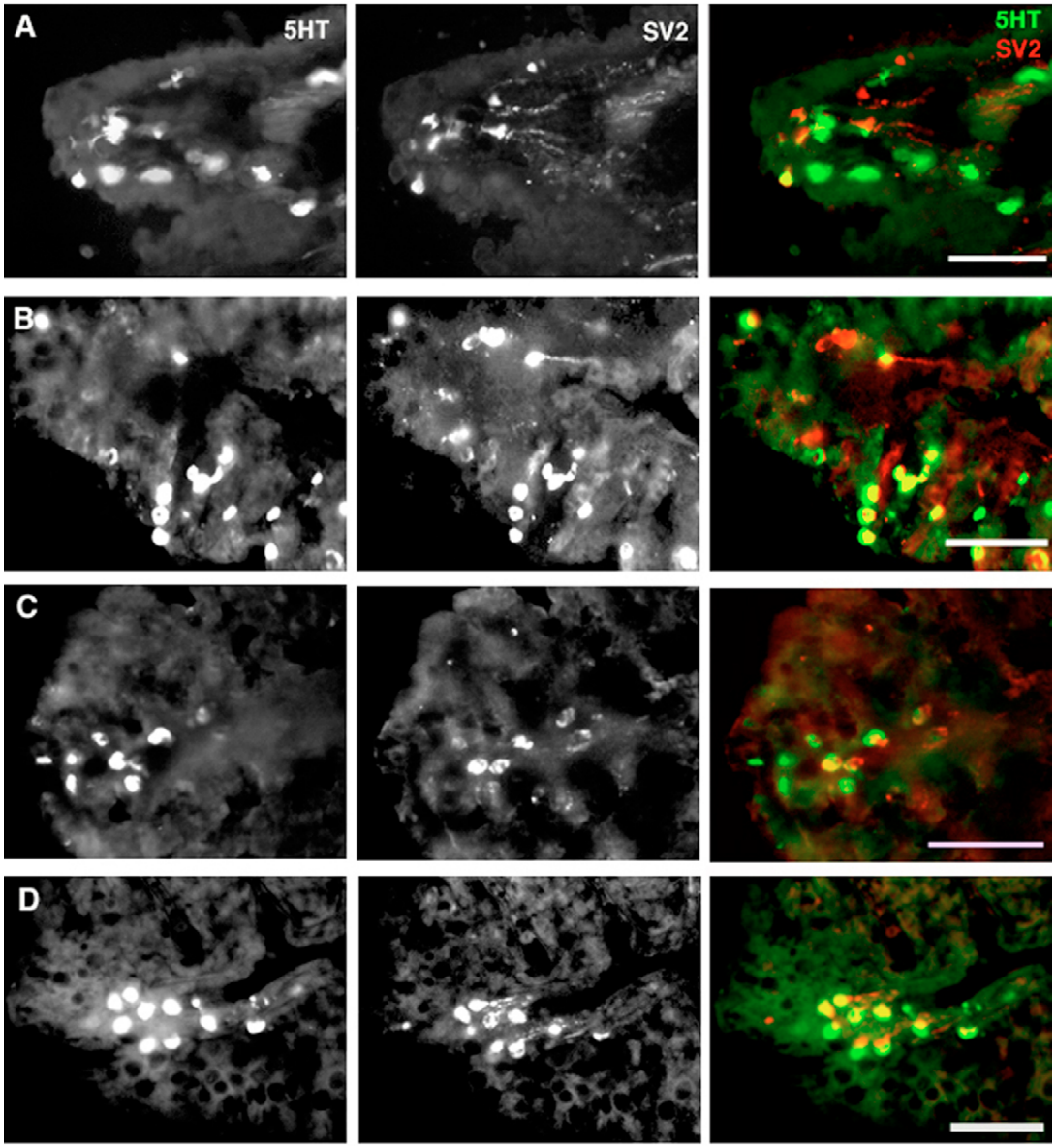

Fig. 8. Comparison of the tip of the filaments in all four species using antibody immunolabelling of serotonin (5-HT) and synaptic vesicle marker (SV2): (A) *O. mykiss*, (B) *C. auratus,* (C) *H. lacerdae* and (D) *H. malabaricus.* Colocalization appears yellow. Scale bars, 100 μm.

In goldfish, the NECs in the secondary lamellae were innervated, which is consistent with previous findings (Saltys et al., 2006). Furthermore, aquatic hypoxia caused lamellae to protrude in the crucian carp *Carassius carassius*, increasing the respiratory surface area as an adaptive and reversible morphological change (Sollid et al., 2003). A similar gross morphological alteration was seen in both crucian carp and goldfish as a response to temperature (Sollid et al., 2005), and it is likely that this morphological change due to environmental factors may be characteristic of *Carassius*. Therefore, it would be especially advantageous for the O_2-sensing cells of carp to be located at the tips of the lamellae as opposed to the middle of the lamellae (Fig. 4), where they are continually exposed to the ambient water regardless of increases in intra-lamellar cell mass associated with hypoxia- and temperature-induced changes.

The finding of NECs in the filament and lamellae that are innervated suggests that they release neurotransmitter (serotonin) across a synapse, transmitting a signal to the CNS. Thus, these cells may serve a chemoreceptive role, and physiological data supports the presence of hypoxic reflexes stimulated by internal hypoxia in these species. Altering internal O_2 levels by hypoxemia, injection of hypoxic blood, or reduced blood flow to the gills stimulates ventilation in the rainbow trout (Holeton, 1971; Smith and Jones, 1982). In traira, ventilatory amplitude increased with both internal hypoxia (Pa_{O_2}) and bolus injections of NaCN (Sundin et al., 1999). As well, single fibre nerve recordings of the trout gills displayed chemoreceptive afferent transmission in response to both internal and external hypoxia (Burleson and Milsom, 1993). However, carp *Cyprinus carpio* did not respond to internal injection of NaCN in some studies (A. E. O'Neil, A. L. Lumsden and W.K.M., manuscript in preparation), which is consistent with our failure to find innervated filamental NECs in a location able to sense blood oxygen levels.

Additionally, we found innervated NECs in the gill rakers of goldfish and trout, an ideal location for sensing external hypoxia. However, double immunolabelling of these cells for a synaptical vesicle protein and serotonin identified a strong colocalization in goldfish (Fig. 10B), but not in trout (Fig. 10A). Moreover, when these cells were observed under a higher magnification, staining patterns in goldfish were consistent with those found in lamellar

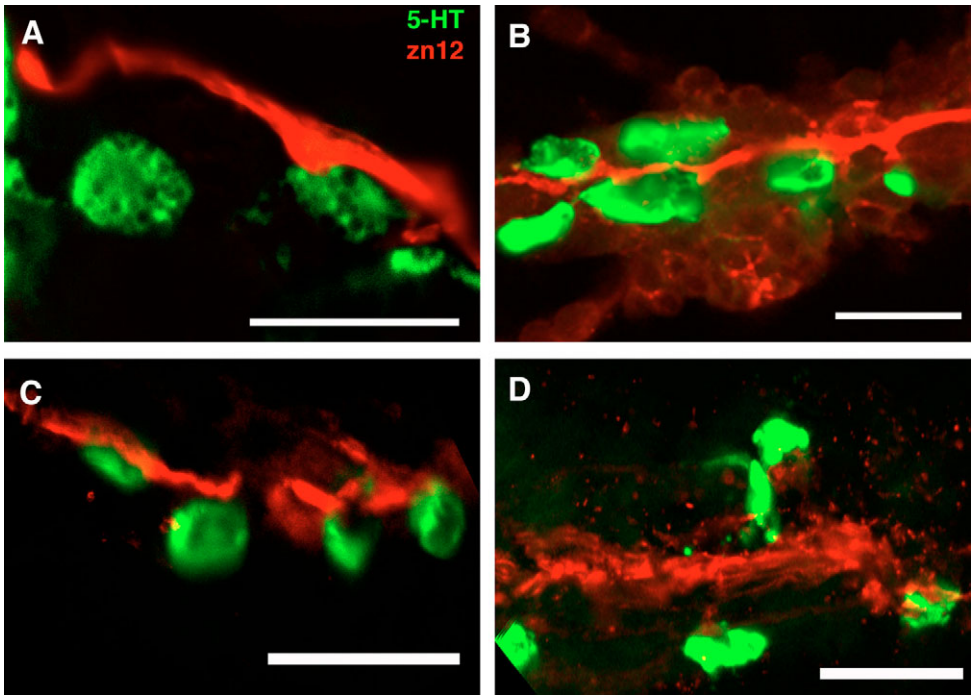

Fig. 9. Innervated serotonergic cells located at the filament tips in (A) *O. mykiss*, (B) *C. auratus,* (C) *H. lacerdae* and (D) *H. malabaricus* immunolabelled for serotonin (5-HT, green) and a neuronal marker (zn12, red). Scale bars, 25 μm.

NECs (compare Fig. 4B with Fig. 10B inset), whereas in trout they were different from NECs found in the filament core (compare Fig. 4A with Fig. 11A, inset). These findings imply that 5-HT-IR cells in the gill rakers of goldfish release 5-HT as a neurotransmitter, but those in trout do not. In goldfish, NECs in the gill rakers can be an additional location for sensing changes in Pw_{O_2}, consistent with the ability to respond to external hypoxia (A. E. O'Neil, A. L. Lumsden and W.K.M., manuscript in preparation). In trout, these 5-HT-IR cells might be Merkel-like cells associated with taste buds (reviewed by Zacconne et al., 1994). Merkel-like cells are serotonergic and innervated, similar to the cells seen in trout gill rakers. However, Merkel cells in teleosts are located at the base of the taste bud, whereas the 5-HT-IR cells found in trout gills are located exclusively in the top layer of the gill raker epidermis. 5-HT-IR cells in the gills were often neighbouring cells containing dense cored vesicles but not serotonin, thus these cells might be taste bud cells.

Paracrine NECs

The cells with serotonin-containing vesicles in the lamellae of traira and trairão cannot play a direct role in afferent signalling to the CNS, as there were no nerve fibres extending out of the primary epithelium and into the lamellae (Figs 5, 7). Saltys et al. (Saltys et al., 2006), using the same neuronal marker (zn12), also did not see nerves extending into the lamellae of juvenile trout using whole-mount fixed samples examined with confocal microscopy. Jonz and Nurse, however, did see innverated lamellae in the zebrafish (Jonz and Nurse, 2003). However, re-examination of our data with confocal microscopy (Fig. 5) still did not reveal innervated lamellae in trout, traira and trairão. Furthermore, detailed reviews of gill morphology and branchial innervation do not describe any innervation extending deep into the lamellae (Laurent and Dunel, 1980; Wilson and Laurent, 2002; Sundin and Nilsson, 2002).

Thus, our data suggest that in addition to the chemoreceptors monitoring the Pw_{O_2} at the filament tip, and Pa_{O_2} in the efferent

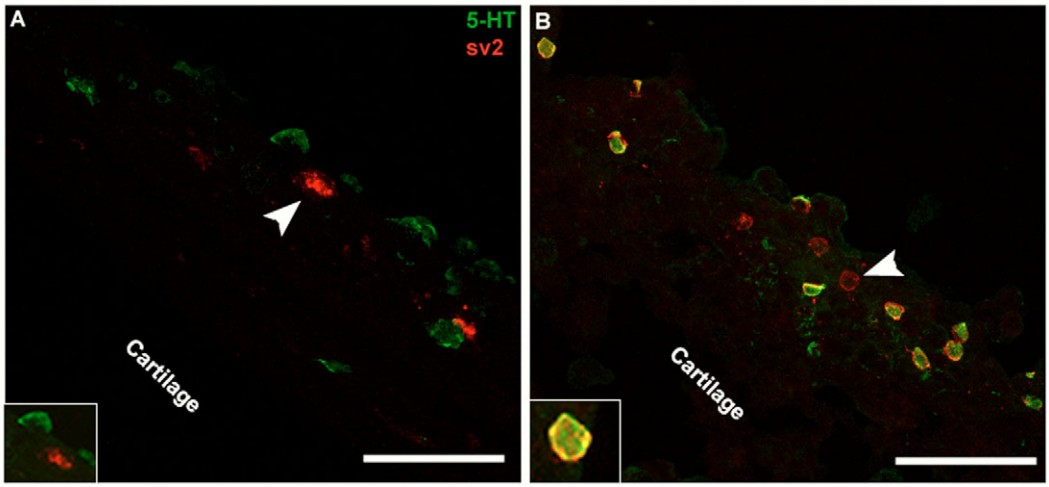

Fig. 10. Innervated serotonergic cells located in the gill rakers in (A) *O. mykiss* and (B) *C. auratus* immunolabelled for serotonin (5-HT, green) and a synaptic vesicle marker (sv2, red). Insets show magnification of individual cells. Colocalization appears yellow. Arrowheads indicate non-5-HT-IR but SV2-IR cells. Scale bars, 50 μm.

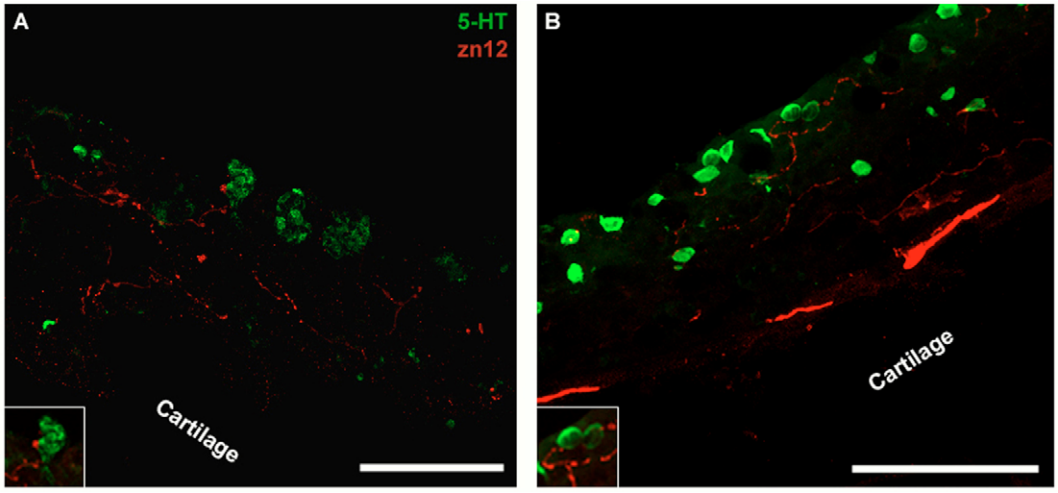

Fig. 11. Innervated serotonergic cells located in the gill rakers in (A) *O. mykiss* and (B) *C. auratus* immunolabelled for serotonin (5-HT, green) and a neuronal marker (zn12, red). Insets show magnification of innervated 5-HT-IR cells in both trout and goldfish. Scale bars, 50 μm.

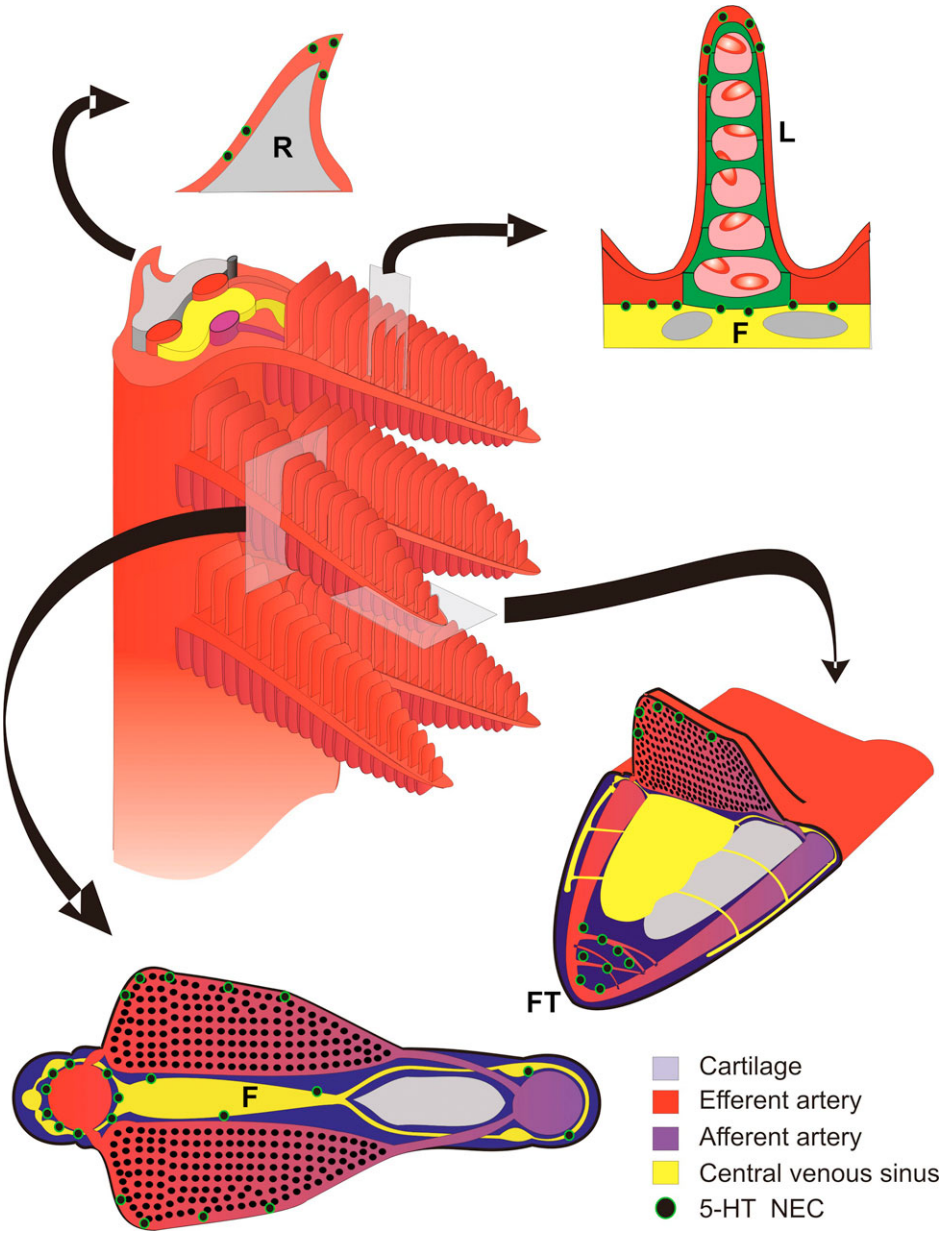

Fig. 12. Schematic of proposed locations of putative O_2 receptor cells in fish gills: gill rakers (R), lamellae (L), tips of filaments (FT) and centre of filament (F) (efferent filament artery). Note that all fish species contain putative O_2 receptor cells in at least two of these proposed locations and variation between species seems to be related to hypoxia tolerance.

filament artery (trout, traira, and trairão), traira, and trairão also have paracrine cells potentially monitoring $P_{W_{O_2}}$ in the lamellae (Fig. 4). Acting in a paracrine fashion, these cells may release serotonin, a potent vasoconstrictor, directly onto the contractile pillar cells of the lamellae, thereby optimizing respiratory surface area. Under resting conditions, fish decrease the loss of ions across the gill by perfusing only two-thirds or less of the lamellae (Booth, 1978; Farrell et al., 1979); however, hypoxic conditions require microcirculatory alterations to increase the respiratory surface area to its maximum. Pillar cell contraction increases lamellar blood spaces and drives blood across the full lamellar sheet, which has a significant effect on matching the respiratory surface area with the oxygen demands of the fish (Smith and Johnson, 1977; Stenslokken et al., 2006). In this way, these lamellar 5-HT-IR cells found in the traira and trairão potentially monitor the hypoxic waters they frequently inhabit, functioning to increase the respiratory surface area in response to lowered $P_{W_{O_2}}$. Lamellar NECs were not present in the trout, which indicates that this fish species may not require additional monitoring of the external environment, as rainbow trout rarely encounter hypoxic waters. Additionally, *in vivo* studies of the microcirculation of the rainbow trout gill did not show direct vasodilation in the lamellae *via* pillar cell contraction (Sundin and Nilsson, 1997), which is consistent with our failure to find NECs in the trout lamellae (Figs 1, 4). In addition to chemoreceptive responses, the NECs of the filament may act in a paracrine manner. *In vivo* examination of rainbow trout gill vasculature exposed to hypoxia demonstrated constriction of the eFA resulting in overflow to the cvs and a continuous flow through the lamellae (Sundin and Nilsson, 1997).

NECs in relation to hypoxia tolerance

Hypoxia-tolerant fish are well known to have lower metabolic rates (less oxygen demand) and higher haemoglobin–oxygen (Hb–O_2) affinities (greater ability to load oxygen into the blood) than hypoxia-intolerant fish. Thus P_{50}, the partial pressure at which Hb is 50% saturated, is a good indicator of relative hypoxia tolerance. This is true of the species in the present study, whose P_{50} values are 23 mmHg for trout (Tetens and Lykkeboe, 1981), 2.6 mmHg for goldfish (Burggren, 1982), and 8.7 mmHg for traira (Perry et al., 2004). There is no available data on the P_{50} value for trairão, but an approximation of 13 mmHg can be made based on the critical oxygen tensions ($P_{C_{O_2}}$) of traira and trairão (Rantin et al., 1992) and P_{50} values of other Erythrinids (Perry et al., 2004). In general, there was a positive correlation between the number of 5-HT-IR cells in the filament and the P_{50}, and a negative correlation with the number of 5-HT-IR cells in the lamellae (Fig. 3).

This trend, however, is not supported by other lines of evidence. Single fibre nerve recording from tuna gills revealed more fibres responsive to alterations in internal hypoxia than external hypoxia, supporting the suggestion that active, hypoxia-intolerant fish are more sensitive to a drop in $P_{a_{O_2}}$ than $P_{W_{O_2}}$ (Milsom and Brill, 1986). However, the rainbow trout gill exhibited an equal number of fibres responsive to internal or external hypoxia (Burleson and Milsom, 1993), making this generalization speculative at best. Furthermore, although there was a large difference in the neuroepithelial cell distribution in the rainbow trout compared to the goldfish, traira and trairão, we did not observe the hypothesized difference in cell distribution related to hypoxia tolerance between the two *Hoplias* species. Although the traira is well adapted to life in shallow, stagnant, hypoxic lakes, the trairão inhabits well-aerated waters of central and southern Brazil (Rantin et al., 1993). Consequently, the traira has a higher hypoxia tolerance ($P_{C_{O_2}}$=20 mmHg), higher Hb-O_2 affinity (P_{50}=8.7 mmHg), larger respiratory surface area, lower metabolic rate and higher anaerobic capacity compared with the trairão ($P_{C_{O_2}}$=35 mmHg) (Fernandes et al., 1994; Rantin et al., 1993; Perry et al., 2004). Filament and lamellar cell counts were different in the two *Hoplias* species, but the specific cell numbers did not produce the expected correlation to hypoxia tolerance (Fig. 3). We would have expected the more hypoxia-tolerant traira to have relatively more putative NECs in the lamellae and fewer in the filament compared with trairão, which was not the case. However, the difference in hypoxia tolerances of these two *Hoplias* species is slight compared to the larger difference in P_{50} between trout and goldfish. Based on the data from the two *Hoplias* species, however, for now we cannot distinguish whether the striking difference we see in NEC distribution between trout and goldfish is due to differences in hypoxia tolerance or differences in phylogeny. Attributing the difference in distribution to differences in physiological responses to internal *versus* external hypoxia, and to different actions of the putative O_2-sensing cells must remain speculative.

In conclusion, we describe innervated NECs at the filament tips that were present in all species in a prime location to sense $P_{W_{O_2}}$, in agreement with physiological data, indicating that all fish studied to date respond to aquatic hypoxia (Fig. 12). It also appeared that there were putative chemoreceptors monitoring $P_{a_{O_2}}$ surrounding the eFA, but the presence of these internal chemoreceptors were species specific and correlated with the ability to respond to internal (arterial) hypoxia. Additionally, we describe putative O_2 chemoreceptors in the gill rakers of goldfish. As well, we propose a paracrine role for the non-innervated NECs we found in the lamellae, acting directly on the pillar cells to enhance respiratory surface area when exposed to aquatic hypoxia. Finally, we speculate that differences in NEC distribution are not due to phylogeny, but instead appear to be adaptations related to hypoxia tolerance.

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
5-HT-IR	5-HT immunoreactive
AFA	afferent filament artery
BL	basal lamina
cvs	central venous sinous
DCV	dense-cored vesicle
EFA	efferent filament artery
N	nerve
NEB	neuroepithelial body
NEC	neuroepithelial cell
$P_{a_{O_2}}$	arterial P_{O_2}
PBS	phosphate-buffered saline
pc	pillar cell
P_{O_2}	external oxygen tension
$P_{V_{O_2}}$	venous P_{O_2}
$P_{W_{O_2}}$	oxygen tension in water
RBC	red blood cell
SV2	synaptic vesicle marker

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