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RESEARCH ARTICLE

Characterisation of putative oxygen chemoreceptors in bowfin (*Amia calva*)

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ABSTRACT

Serotonin containing neuroepithelial cells (NECs) are putative oxygen sensing cells found in different locations within the gills of fish. In this study we wished to determine the effect of sustained internal (blood) hypoxaemia versus external (aquatic) hypoxia on the size and density of NECs in the first gill arch of bowfin (*Amia calva*), a facultative air breather. We identified five different populations of serotonergic NECs in this species (Types I–V) based on location, presence of synaptic vesicles (SV) that stain for the antibody SV2, innervation and labelling with the neural crest marker HNK-1. Cell Types I–III were innervated, and these cells, which participate in central O_2 chemoreflexes, were studied further. Although there was no change in the density of any cell type in bowfin after exposure to sustained hypoxia (6.0 kPa for 7 days) without access to air, all three of these cell types increased in size. In contrast, only Type II and III cells increased in size in bowfin exposed to sustained hypoxia with access to air. These data support the suggestion that NECs are putative oxygen-sensing cells, that they occur in several locations, and that Type I cells monitor only hypoxaemia, whereas both other cell types monitor hypoxia and hypoxaemia.

KEY WORDS: Chronic hypoxia, Time domains, Neuroepithelial cells, Air breathing fish

INTRODUCTION

In mammals, stimulation of peripheral arterial O_2 chemoreceptors (glomus cells) in the carotid body produces robust cardiovascular and ventilatory responses. Unlike most cells that decrease energy demands during exposure to hypoxia, oxygen chemoreceptors become more metabolically active (Kumar et al., 2009) and increase in size during exposure to sustained hypoxia (Wang et al., 2008). Although the exact mechanism behind this hypertrophy is unknown it is probably related to the increased turnover and synthesis of neurotransmitters associated with the increased activity of the chemoreceptors. In the gills of all fish species studied to date (for review, see Porteus et al., 2012), serotonin-containing neuroepithelial cells (NECs), which are putative peripheral arterial O_2 chemoreceptors, have been found, and evidence suggests that the NECs found in zebrafish (*Danio rerio*) and mangrove rivulus (*Kryptolebias marmoratus*) gills also increase in size during exposure to sustained hypoxia (Jonz et al., 2004; Regan et al., 2011).

NECs are thought to be located in an ideal position on the gills where they can sense changes in oxygen in both water (external) and blood (internal). However, the orientation of the receptors involved in reflex changes in each of the different components of the cardiorespiratory response (breathing frequency, breath amplitude, heart rate, systemic vascular resistance) to hypoxia are highly variable between species of water and air breathing fish (Milsom, 2012). Bowfin, *Amia calva* Linnaeus 1766, are facultative air breathing fish that use their gills to obtain oxygen from the water, but that can use their gas bladder to supplement oxygen uptake by coming to the water surface to breath air. Bowfin respond to acute hypoxia by increasing air breathing frequency and gill ventilation and reducing heart rate (bradycardia) (Porteus et al., 2014a). Branchial denervation and pseudobranch ablation eliminate the air breathing response and the bradycardia and diminish the gill ventilatory response (McKenzie et al., 1991b). These observations are consistent with the finding that bowfin do not possess central oxygen chemoreceptors (Hedrick et al., 1991) and indicate that the gills are the main location for oxygen sensing in bowfin. Furthermore, studies indicate that the reflex hypoxic bradycardia exhibited by this species is mediated exclusively by externally oriented receptors whereas changes in gill ventilation amplitude and frequency are mediated by both internally and externally oriented receptors (McKenzie et al., 1991a; McKenzie et al., 1991b). Internal injections of NaCN had no effect on air breathing in the bowfin (McKenzie et al., 1991b), whereas external NaCN stimulated air breathing (McKenzie et al., 1991b). These studies indicate that although externally oriented receptors are involved in all responses in this species, the internally oriented receptors have a more restricted role (gill ventilation only).

NECs containing serotonin have been described in both the filaments and lamellae of bowfin gills using immunohistochemistry and electron microscopy (Goniakowska-Witalińska et al., 1995). These NECs, however, were not found to be in direct contact with the water in either location. This description of NEC location is inconsistent with the observed reflex responses to hypoxia versus hypoxaemia. The first aim of this study, therefore, was to re-examine the distribution of putative oxygen chemoreceptors in bowfin gills. We hypothesized that there would also be NECs in close contact with the water flowing over the gills. We also hypothesized that there would be NECs with both orientations that were innervated and would contain synaptic vesicles, just like the NECs found in other fish species.

A central reflex arc is made up of an afferent neuron transmitting sensory information to either the brain or the spinal cord, integrative interneurons, and efferent neurons sending information from the central nervous system to an effector (muscles involved in ventilation). For NECs to be oxygen chemoreceptors involved in a central reflex response to hypoxia they must be innervated in order for the information to be relayed to the central nervous system for processing, and they must release their neurotransmitters from vesicles into a synapse onto these nerves. A synaptic vesicle marker SV2 and a neuronal marker zn-12 have been previously used to

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List of symbols and abbreviations

5-HT	5-hydroxytryptamine or serotonin
ACh	acetylcholine
aFA bas	efferent filament artery near basal lamina
aFA epi	efferent filament artery in the top layer of the epithelium
CVS	central venous sinus
DAPI	4′,6-diamidino-2-phenylindole (stains cell nuclei)
eFA bas	efferent filament artery near basal lamina
eFA epi	efferent filament artery in the top layer of the epithelium
HNK-1	human natural killer-1 (marker for a subset of cells derived from the neural crest)
NEBs	neuroepithelial bodies
NEC	neuroepithelial cell
P_{crit}	critical oxygen tension
SH	sustained hypoxia
SV2	synaptic vesicle antibody (synaptic vesicle marker)
VAChT	vesicular acetylcholine transporter (marker for transporter of acetylcholine into storage or synaptic vesicles)
zn-12	zebrafish antibody (neuronal marker)

identify NECs in fish and their nearby innervation (Coolidge et al., 2008; Jonz et al., 2004; Jonz and Nurse, 2003; Regan et al., 2011; Tzaneva and Perry, 2010). Additionally, glomus cells from the carotid bodies of birds and mammals are derived from the neural crest (Pearse et al., 1973). Thus, if fish NECs are phylogenetic precursors of glomus cells we predicted that NECs would also be derived from the neural crest. A mammalian human natural killer (HNK-1) antibody labels a subset of proliferative neural crest cells in fish (Porteus et al., 2013) and the same antibody was used to determine whether the NECs in bowfin gills were proliferative and of neural crest origin.

The second aim of this study was to see whether sustained changes in internal versus external chemoreceptor stimulation would produce changes in the abundance, size and shape of NECs in different locations and enable us to identify putative internal versus external O_2 chemoreceptors. To do this we used three groups of bowfin: bowfin exposed to either normoxia, sustained hypoxia with access to air, or sustained hypoxia without access to air. The level of hypoxia chosen was 6.0 kPa, just below the critical oxygen tension (P_{crit}) of this species (Porteus et al., 2014a). Based on oxygen equilibrium curves from these particular bowfin, we calculated a 70% saturation of haemoglobin at this level of oxygen in bowfin without access to air (Porteus et al., 2014a). Additionally, in trout (*Oncorhynchus mykiss* Walbaum), this level of hypoxia has been shown to maximally stimulate gill oxygen chemoreceptors (Burleson and Milsom, 1993). Bowfin exposed to normoxia rarely air breathe (~1 breath h^{-1}) and utilize their well-developed gills for extracting oxygen from the water (Porteus et al., 2014b). These fish were assumed to be neither hypoxic nor hypoxaemic and their oxygen chemoreceptors to be minimally stimulated under these conditions (Hedrick et al., 1994). Bowfin exposed to aquatic hypoxia without access to air would be both hypoxic and hypoxaemic and both internal and external chemoreceptors would be stimulated. Bowfin exposed to this level of hypoxia with access to air increase air breathing frequency to ~7–10 breaths h^{-1} (Porteus et al., 2014b). As a result, these bowfin have been shown to experience hypoxia but no, or only intermittent hypoxaemia (Hedrick et al., 1994). Therefore, external chemoreceptors would be stimulated far more than internal chemoreceptors in this group.

RESULTS

Identification of putative oxygen chemoreceptors using immunohistochemistry

In longitudinal sections, five types of cells were identified by labelling for serotonin and were classified according to morphology, innervation and location in the first gill arch (Fig. 1). Type I cells were oval, did not have cell projections and were found near the filament arteries. Type II cells were large bipolar neurons found in the middle of the filament, in the lining of the central venous sinus (CVS). Type III cells were round to oval, had no cell projections and were found in the vicinity of the bipolar neurons in the CVS. Type IV cells also did not have any projections and were found in the lamellae. Type V cells had a similar morphology to Type IV cells but were found in the interfilamental support bars.

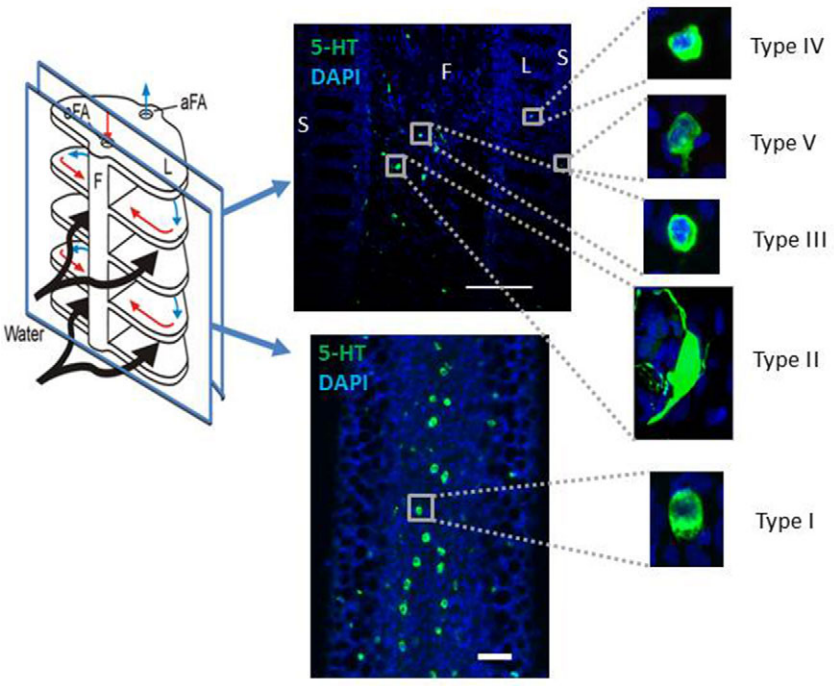

Fig. 1. Schematic diagram of a gill and *Z*-stack compressions showing the location of serotonergic cells in the gills of bowfin. Double labelling of serotonin (5-HT; green) and cell nuclei (DAPI, blue) was used to identify serotonergic cells. Type I cells are located near the filament arteries. Type II cells are bipolar neurons found in the central venous sinus of the filament. Type III cells are round cells without any projections in the central venous sinus of the filament. Type IV cells are round and found in the lamellae, and Type V cells are found in the interfilamental support bars. F, filament; L, lamella; S, interfilamental support bar; eFa, efferent lamellar artery; aFA, afferent lamellar artery. Direction and oxygenation of blood flow through the gill filament are indicated by red (oxygenated) and blue (deoxygenated) arrows. The gill schematic diagram is reprinted from Jonz et al. with permission (Jonz et al., 2004). Scale bars: 200 µm (top panel); 100 µm (bottom panel).

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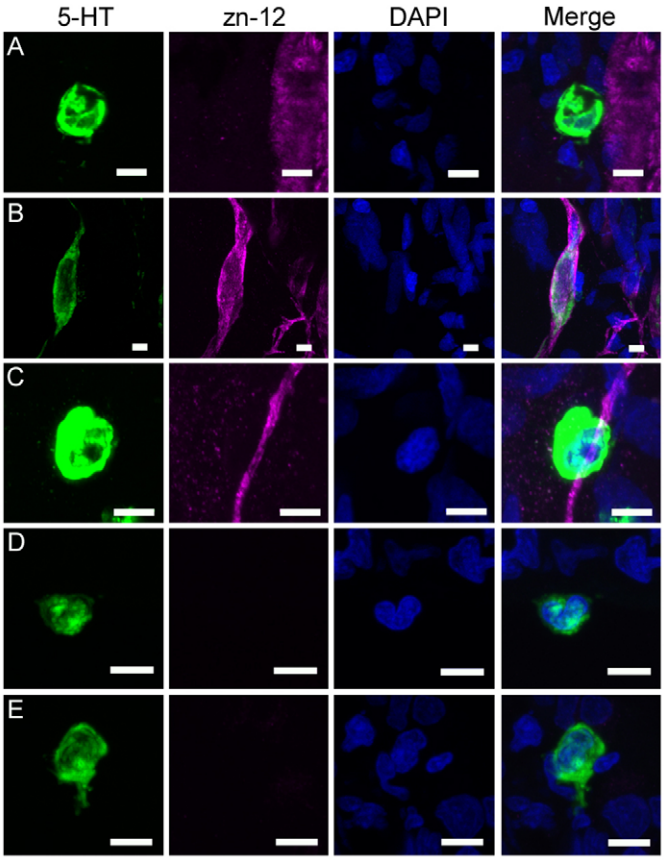

Fig. 2. *Z*-stack compressions showing triple labelling of different cell types in the gills of bowfin with serotonin antibody (5-HT, green) and the neuronal marker antibody (zn-12, magenta), and the cell nuclei with DAPI (blue). (A) Type I cells are found in close proximity to nerve fibres. (B) Type II cells contain synaptic vesicles confirming that they are bipolar neurons. (C) Type III cells were found in close proximity to nerve fibres. (D,E) Type IV (D) and V (E) cells were never located near nerve fibres, therefore were not innervated. Scale bars: 10 µm.

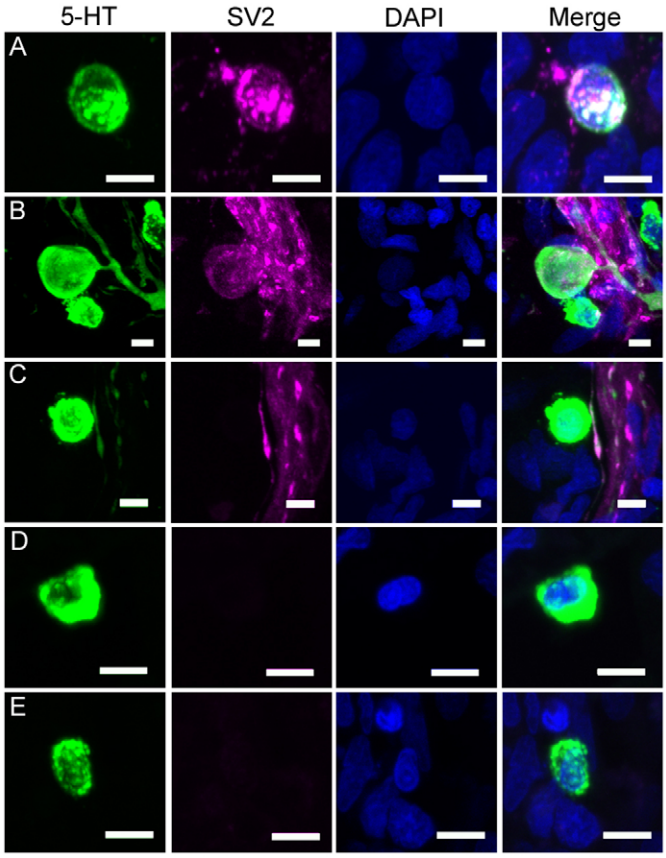

Fig. 3. *Z*-stack compressions showing triple labelling of different cell types in the gills of bowfin with antibodies for serotonin (5-HT, green) and a synaptic vesicle marker (SV2, magenta), and the cell nuclei were labelled with DAPI (blue). (A,B) Type I (A) and II (B) cells labelled for synaptic vesicles. (C) Type III cells did not contain synaptic vesicles but were found in close proximity to nerve fibres that did. (D,E) Type IV (D) and V (E) cells did not contain synaptic vesicles. Coincidence between magenta and green labelling appears white. Scale bars: 10 µm.

Triple labelling with 4′,6-diamidino-2-phenylindole (DAPI), a marker for serotonin (anti-5-HT antibody; hereafter referred to as 5-HT) and a neuronal marker (zn-12), revealed that Type I cells were in close proximity to large nerve bundles (Fig. 2A). Type II cells labelled with zn-12, confirming they were neurons (Fig. 2B). Type III cells did not label with the neuronal marker but were also found in close proximity to nerve fibres (Fig. 2C). Type IV and V cells also did not label with the neuronal marker and were not found near nerve fibres (Fig. 2D,E). Triple labelling with DAPI, a marker for serotonin and a synaptic vesicle marker, SV2 antibody (hereafter referred to as SV2), revealed that Type I and II cells contained synaptic vesicles (Fig. 3A,B). Type III cells did not themselves contain synaptic vesicles, but nearby nerve fibres innervating them did (Fig. 3C). Type IV and V cells did not contain synaptic vesicles (Fig. 3D,E). Labelling with DAPI, a marker for serotonin and the HNK-1 antibody revealed that Type I cells did not label with this antibody (Fig. 4A). Type II cells did label with the HNK-1 antibody (Fig. 4B), and Type III cells labelled with HNK-1 but more weakly than Type II cells or other neurons (Fig. 4C). Type IV and V cells did not label with the HNK-1 antibody (Fig. 4D,E).

Labelling for the vesicular acetylcholine transporter (VAChT), serotonin and with DAPI showed VAChT labelling in the vicinity of Type I cells (Fig. 5A). Rotating the three-dimensional (3-D) projections of these images revealed that VAChT was labelling the innervation of these cells and not the cells themselves (data not shown). Type II cells contained VAChT in both the cell body and its projections (Fig. 5B). The other cell types did not contain VAChT (data not shown).

Cross-sections revealed that Type I cells were found in four locations: near the basal lamina of the epithelium on the efferent filament artery (eFA), within the first cell layer of the epithelium near the eFA and similarly on the afferent filament artery (aFA; Fig. 6A). The cells near the aFA contained synaptic vesicles and were found in the top region of the epithelial layer, often at the base of mucous cells, and most were not in direct contact with the water (Fig. 6A,B). Cells near the basal lamina on the aFA were innervated and contained synaptic vesicles but were rare. The cells near the basal filament artery were less round and often had short projections that contacted the large nerve bundle nearby (Fig. 6A,C). These cells also contained synaptic vesicles (Fig. 6C).

The effect of sustained hypoxia on chemoreceptor morphology and density

Longitudinal sections

Type I cells in bowfin exposed to sustained hypoxia without access to air had a 20% larger projection area than Type I cells in bowfin

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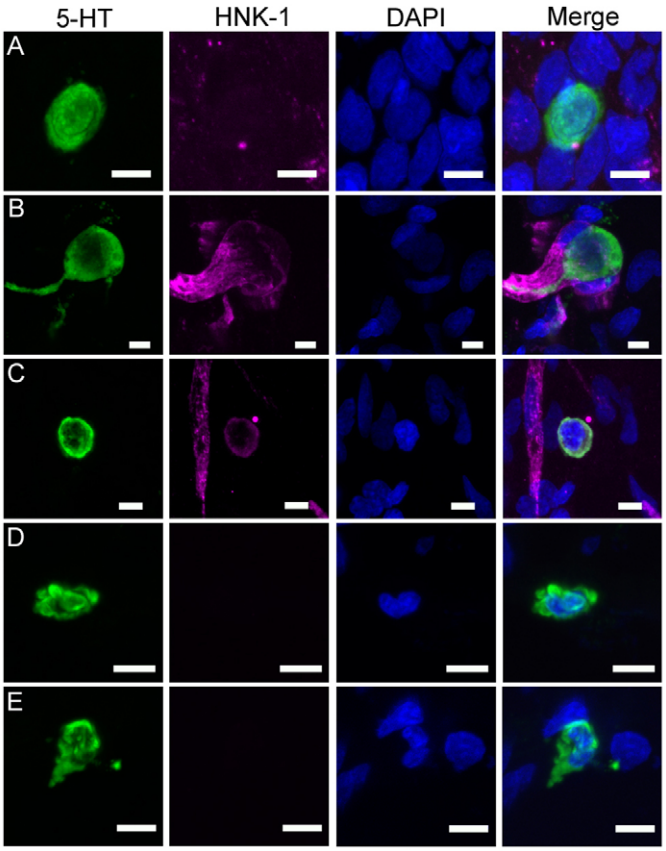

Fig. 4. *Z*-stack compressions showing triple labelling of different cell types in the gills of bowfin with antibodies for serotonin (5-HT, green) and HNK-1 (magenta), and the cell nuclei were labelled with DAPI (blue). (A) Type I cells did not label with the HNK-1 antibody. (B) Type II cells strongly labelled with the HNK-1 antibody. (C) Type III cells weakly labelled with the HNK-1 antibody. (D,E) Type IV (D) and V (E) cells did not label with the HNK-1 antibody. Scale bars: 10 µm.

either exposed to sustained hypoxia with access to air or normoxia ($H_{2,15}$=8.98, P=0.01; Fig. 7A). Type II cells in bowfin exposed to sustained hypoxia with and without access to air had a 25% larger projection area than bowfin exposed to normoxia ($F_{2,15}$=4.31, P=0.03; Fig. 7A). Type III cells in bowfin exposed to sustained hypoxia with and without access to air had a 20% larger projection areas than bowfin exposed to normoxia ($F_{2,15}$=7.35, P=0.006; Fig. 7A).

A shape factor of 1.0 indicates a projected area of a perfect circle. Type I cells were generally round, with a shape factor of 0.74 and there were no differences between treatment groups ($F_{2,15}$=0.30, P=0.743; Fig. 7B). Type II cells had cell projections and an average shape factor of 0.49 in bowfin exposed to normoxia or sustained hypoxia with access to air. There was a trend for a decrease in shape factor (more irregular shape or more cell projections) in bowfin exposed to sustained hypoxia without access to air ($F_{2,15}$=2.65, P=0.10; Fig. 7B). Type III cells were also generally round, with a shape factor of 0.67 and there were no differences in shape factor between treatment groups ($F_{2,15}$=1.66, P=0.22; Fig. 7B).

Cross-sections

Cross-sections revealed that Type I cells were found in four locations: near the basal lamina of the eFA (eFA bas), in the outer epithelial layer of the eFA (eFA epi), in the outer epithelial layer of the aFA (aFA epi) and near the basal lamina of the aFA (aFA bas). There were about 198±54 cells mm^{-1} filament in the outer epithelial layer of the eFA, 424±47 cells mm^{-1} filament near the basal lamina of the eFA, 21±4 cells mm^{-1} filament near the basal lamina of the aFA and 609±43 cells mm^{-1} filament in the outer epithelial layer of the aFA (Fig. 8A). There were no differences in cell density between treatment groups (P>0.05). The number of cells in the outer epithelial layer of the eFA was 183±8 µm^3, 261±11 µm^3 near the basal lamina of the eFA and 208±10 µm^3 in the outer epithelial layer of the aFA (Fig. 8B). There were no differences in cell density between treatment groups (P>0.05). The length of cells in the outer epithelial layer of the eFA was 12±0.6 µm, 14.6±0.9 µm near the basal lamina of the eFA and 14.9±10 µm in the outer epithelial layer of the aFA (Fig. 8C). Cells near the basal lamina of the eFA were larger in bowfin exposed to hypoxia without access to air than in bowfin exposed to normoxia (P<0.05), but not larger than bowfin exposed to hypoxia with access to air (P>0.05). Cells near the basal lamina of the aFA were too few to be able to obtain any reliable measurements.

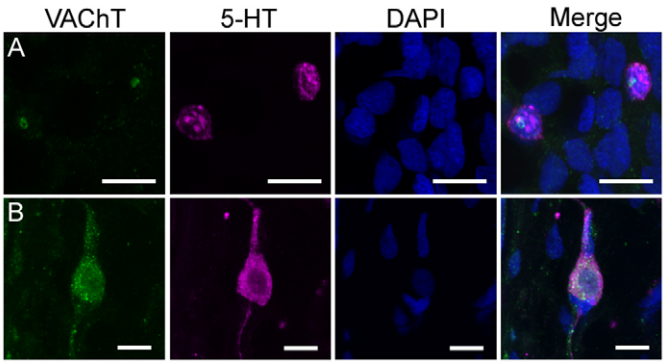

Fig. 5. *Z*-stack compressions showing triple labelling of different cell types in the gills of bowfin with antibodies for vesicular acetylcholine transporter (VAChT, green), serotonin (5-HT, magenta), and the cell nuclei were labelled with DAPI (blue). (A) VAChT labelling was found in the vicinity of some Type I cells probably in synapses of nearby innervations. (B) Type II cells contained VAChT. Scale bars, 10 µm.

DISCUSSION

Our study reveals a diversity of putative oxygen chemoreceptors in the first gill arch of the facultative air breathing bowfin (*A. calva*; Table 1). Contrary to predictions, however, we found that not all NEC cell types were innervated and contained synaptic vesicles. We did identify three populations of cells at different locations in the first gill arch of bowfin that could serve as chemoreceptors involved in chemoreflexes, on the basis of innervation (indicating they could be involved in a central cardio-ventilatory reflex response to hypoxia). Exposure to moderate sustained hypoxia led to changes in cell morphology in all three cell types (Type I–III) supporting this conclusion. Additionally, the Type I cells near the basal lamina of the eFA were identified as specific putative internal oxygen chemoreceptors in bowfin. This is consistent with studies showing that although externally oriented receptors are involved in all cardioventilatory responses in this species, there are internally oriented receptors involved in regulating gill ventilation only (McKenzie et al., 1991b).

Surprisingly, no cell type was identified that could potentially act exclusively as an external chemoreceptor. This was surprising because previous studies have shown that the reflex hypoxic bradycardia exhibited by this species is mediated exclusively by

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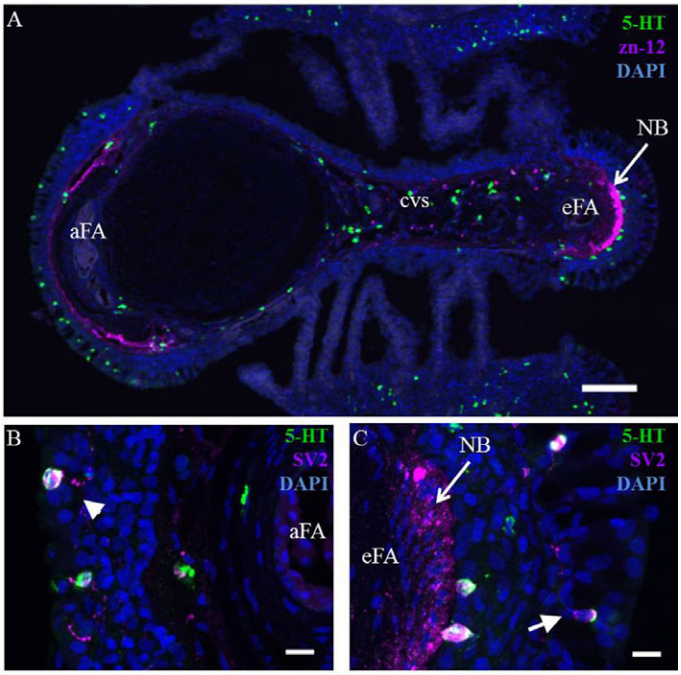

Fig. 6. Cross-section of a gill filament showing Type I cells in bowfin. (A) *Z*-stack compression showing triple labelling with antibodies for serotonin (5-HT, green) and innervation (zn-12, magenta), and the cell nuclei were labelled with DAPI (blue). (B,C) Higher magnification of the area around the efferent filament artery (B) and the afferent filament artery (C) using triple labelling for serotonin (5-HT, green), SV2 (a synaptic vesicle marker; magenta) and cell nuclei (DAPI, blue). Arrowheads indicate nerve fibres innervating nearby cells. Coincidence between magenta and green labelling appears white. Scale bars: 100 µm (A) and 10 µm (B,C). aFA, afferent filament artery; eFA, efferent filament artery; NB, nerve bundle; cvs, central venous sinus.

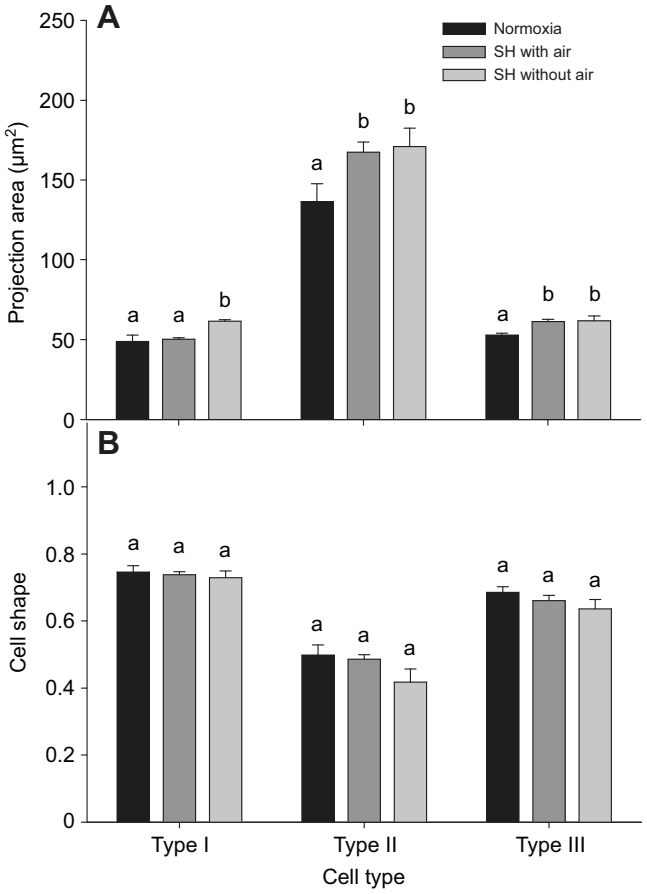

Fig. 7. The effect of exposure to sustained hypoxia (SH; 6.0 kPa for 7 days) on NEC morphology. (A,B) Projection area (cell size; µm^2; A) and cell shape (B) in Type I, II and III cells in bowfin exposed to normoxia or to sustained hypoxia with or without access to air. Type I cells were large in bowfin exposed to hypoxia without access to air. Type II cells were larger in both groups exposed to hypoxia than in normoxia. Type III cells were larger in both groups exposed to hypoxia than in normoxia. For cell shape a value of 1.0 indicates a perfect circle. There were no significant differences in cell shape between the groups for all three cell types. Different letters above the bars indicate significantly different values (P<0.05; mean ± s.e.m.).

externally oriented receptors (McKenzie et al., 1991a; McKenzie et al., 1991b). It should be noted, however, that our study only examined the first gill arch and that elimination of the hypoxic bradycardia required denervation of all four gill arches, and NECs have been found in all four gill arches of all species studied to date (Coolidge et al., 2008; Jonz and Nurse, 2003; Zhang et al., 2011). Thus, it is possible that chemoreceptors exclusively oriented to sample water could be located on other gill arches of bowfin. However, although other gill arches contain NECs, these NECs are generally found in the same locations and have similar distributions to those found in the first gill arch (Coolidge et al., 2008; Jonz and Nurse, 2003; Zhang et al., 2011). This conundrum remains to be resolved.

Type I cells

Type I cells found near the eFA in the first gill arch contained synaptic vesicles (Fig. 2; Fig. 6B) and were innervated by a nerve bundle running in close proximity to them (Fig. 3; Fig. 6A,C). These cells were similar in morphology and location to those previously described in the filaments of all other species of fish studied (Coolidge et al., 2008; Jonz et al., 2004; Regan et al., 2011; Saltys et al., 2006; Tzaneva and Perry, 2010). Additionally, Type I cells, just as in NECs in this same location in goldfish (*Carassius auratus*) and trout (*O. mykiss*), did not label with the HNK-1 antibody, suggesting they are either not derived from the neural crest, or more probably, not proliferative (Porteus et al., 2013), as some neural crest cells stop expressing HNK-1 once they differentiate (Metcalfe et al., 1990). VAChT was found to be present in synaptic vesicles in the proximity of Type I cells suggesting that acetylcholine (Ach) is also released at this synapse but most probably by the post-synaptic neurons. ACh is found in clear synapses in the nerves synapsing with NECs in catfish (*Ictalurus melas*) (Dunel-Erb et al., 1982). Such ACh-positive nerve fibres could have cell bodies either intrinsic or extrinsic to the gill, as both intrinsic and extrinsic neurons containing VAChT have been identified in trout and goldfish (Porteus et al., 2013). Intrinsic nerve fibres, so called because their cell bodies are found within the gill arch, could be those of the type II cells (bipolar neurons). Although a direct connection was not found in our study between the ACh-containing fibres and the cell bodies of Type II cells, in zebrafish, bipolar neurons have been shown to innervate 5-HT-positive NECs in the filaments (equivalent to Type I cells in this study) (Jonz and Nurse, 2005).

In zebrafish (*D. rerio*) and catfish (*Ictalurus punctatus*), dissociated NECs from gill filaments have been shown to depolarize in response to hypoxia (Burleson et al., 2006; Jonz et al., 2004; Qin et al., 2010). These NECs were also larger (but not more numerous)

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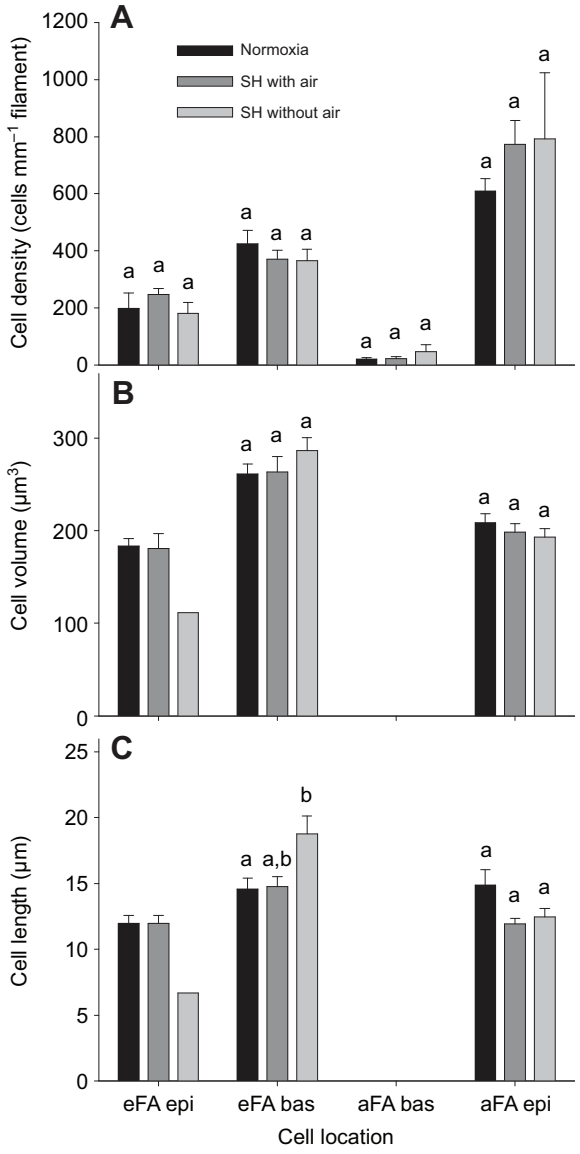

Fig. 8. The effect of exposure to sustained hypoxia (SH, 6.0 kPa for 7 days) on Type I cell morphology. (A) Cell density (cells mm^{-1} filament), (B) cell volume (μm^3) and (C) cell length (μm) in Type I cells in various locations in the gill filament of bowfin exposed to normoxia or to sustained hypoxia with or without access to air. There were no significant differences in cell density and cell volume at the different locations in bowfin exposed to normoxia or sustained hypoxia. Different letters above the bars indicate significantly different values within a certain location (P<0.05). eFA epi, efferent filament artery in the top layer of the epithelium; eFA bas, efferent filament artery near basal lamina; aFA epi, efferent filament artery in the top layer of the epithelium; aFA bas, efferent filament artery near basal lamina. Values are means ± s.e.m.

in zebrafish exposed to sustained hypoxia (4.7 kPa for 60 days) than in control fish (Jonz et al., 2004) as were the Type I cells in bowfin. Taken together, these observations suggest that Type I cells are oxygen chemoreceptors innervated by nerve fibres that contain ACh.

Longitudinal measurements of the projection area of Type I cells revealed that they were larger in bowfin exposed to hypoxia without access to air than in bowfin with access to air or those exposed to normoxia (Fig. 7A). Cross-sections further revealed that this was primarily due to an increase in length of the Type I cells near the basal lamina of the eFA (the most abundant of the Type I cells seen in the longitudinal sections; Fig. 8C). Additionally, Type I cells near the basal lamina of the eFA were longer in bowfin exposed to hypoxia without access to air, whereas Type I cells at other locations tended to be smaller (Fig. 8B). This selective response suggests that Type I cells near the basal lamina of the eFA are internal chemoreceptors [responding to internal hypoxia (hypoxaemia) only], and not external chemoreceptors.

The increase in cell length also indicated that Type I cells were changing shape, either by elongation of the cell itself or by growth of cell projections. Although the cell shape factor was not different between treatments in Type I cells (Fig. 7A), this might simply reflect the mixed population of cells measured longitudinally. Previously, it has been shown that zebrafish exposed to sustained hypoxia (35 mmHg for 60 days) had larger NECs and more NECs with cell processes contacting the nerve bundle than fish exposed to normoxia, suggesting NECs increased neurotransmitter release (Jonz et al., 2004).

Type I cells found near the outer layer of the epithelium on the aFA side of the filament were similar in morphology and had similar immunolabelling to cells near the outer layer of the epithelium of the eFA side of the epithelium, and we suggest that they are the same cell type. Both eFA and aFA Type I cells near the epithelium (epi) contained synaptic vesicles and were innervated by small nerve fibres (Fig. 6), indicating a possible chemosensory role. Type I cells of this description and in this location (eFA and aFA) have not been described in any other fish species to date. Their location close to the water suggests that these cells could be external oxygen chemoreceptors, sensing changes in the partial pressure of oxygen in the water, with afferent nerves completing the chemosensory pathway. However, there was a trend for eFA and aFA Type I cells near the epithelium (epi) to be smaller in bowfin exposed to sustained hypoxia without access to air. While it is possible that these are Merkel-like cells associated with gustation, as previously described in the orobranchial cavity of zebrafish (Zachar and Jonz, 2012), the zebrafish Merkel-like cells also had small projections of 2–3 μm, which were not observed in the present study. It is also possible that they decreased in size due to excessive transmitter release.

Type I cells near the basal lamina of the aFA were scarce and too few to categorize.

Type II cells

As in previous studies on zebrafish, trout (*O. mykiss*) and goldfish, intrinsic bipolar neurons (Type II cells) labelling for serotonin were found in bowfin gills (Jonz and Nurse, 2003; Porteus et al., 2013). Just as in trout and goldfish (Porteus et al., 2013), these intrinsic neurons double labelled for serotonin and VAChT. Type II cells were larger in bowfin exposed to hypoxia with and without access to air (Fig. 7A), indicating that these cells may be involved in the response to both internal and external hypoxia. Owing to their location deep in the filament, not in close proximity to the external water, these cells are unlikely to be external chemoreceptors and are more likely to be involved in transduction in the oxygen-sensing pathway. The overall lower shape factor score in these cells reflects their irregular shape. In other species, intrinsic bipolar neurons innervate the eFA sphincter, thus they are likely to be involved in regulating blood flow through the gill (Jonz and Nurse, 2003). Gill blood flow is modulated during hypoxia by the contraction of the eFA sphincter, which causes an increase in branchial blood pressure, which in turn causes an increase in lamellar recruitment increasing the functional surface area for gas exchange (reviewed by Sundin and Nilsson, 2002).

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Table 1. Summary of immunoreactivity of five types of putative chemoreceptor cells with tested antisera and their location in the bowfin gill

	Antibody					
Structure	5-HT	SV2	zn-12	HNK-1	VAChT	Location
Type I	+	+	+	-	*	eFA
Type II	+	+	+	+	+	CVS
Type III	+	–	+	+	–	CVS
Type IV	–	–	–	–	–	Lamellae
Type V	–	–	–	–	–	S

+, positive immunoreaction; –, no immunoreaction.
5-HT, serotonin; HNK-1, human natural killer; eFA, efferent filament artery; CVS, central venous sinus; S, interfilamental support bars; SV2, synaptic vesicle marker; VAChT, vesicular acetylcholine transporter; zn-12, zebrafish neuronal marker.
*Type I cells did not contain VAChT, but the adjacent neural clefts of some cells did contain VAChT.

Bipolar neurons (Type II cells) also innervate filamental NECs (Type I cells) in zebrafish, suggesting they may be the second step in the chemoreceptor reflex pathway. Additionally, there was an apparent decrease in the cell shape factor (the cells become more irregular in shape) in Type II cells in bowfin exposed to hypoxia without access to air, which was not seen in the other two groups (Fig. 7B), indicating that these cells had more processes. An increase in cell projections in bipolar neurons indicates an increase in interconnectedness with nearby neurons and/or NECs and possibly enhanced sensitivity (neurotransmission), a suggestion that deserves to be further investigated.

Type III cells

To our knowledge, Type III cells have not been described in any fish species studied to date. These cells were located in the filament in the area of the CVS, were innervated, contained serotonin, but did not label with the synaptic vesicle marker SV2 (Fig. 2C; Fig. 3C). The projection area of Type III cells was significantly larger in bowfin exposed to hypoxia both with and without access to air than in bowfin exposed to normoxia (Fig. 7A), indicating that they responded to hypoxia. It is possible that these cells are involved in the hypoxic ventilatory response acting as effector cells, releasing serotonin on nearby vasculature in response to hypoxia, using non-synaptic vesicles. Serotonin caused a dilation of the CVS in Atlantic cod (*Gadus morhua*) (Sundin, 1995), increasing blood flow to the mitochondrion-rich cells. It has also been suggested that dilation of the CVS could divert plasma from the arterio-arterial pathway increasing the haematocrit in the lamellae and improving oxygen uptake (Sundin and Nilsson, 2002), but no anastomoses between these two circulations have been found in bowfin (Olson, 1981). It is also possible that these cells contained synaptic vesicles not labelled by the SV2 antibody. Alternatively, Type III cells could be precursor NECs that differentiate into mature NECs and this explanation is consistent with the weak labelling of these cells with the HNK-1 antibody, which labels a subset of proliferative neural crest cells. In this scenario, Type III cells may have initiated serotonin synthesis and are transforming into NECs but have not yet packaged serotonin into synaptic vesicles. The density of these cells was not determined because of the difficulty in obtaining full longitudinal sections of the gills, but future studies using bromodeoxyuridine (BrdU) would reveal if these cells are indeed proliferating.

Type IV cells

Type IV cells found in the lamellae have been described in most species of fish studied to date (Coolidge et al., 2008; Jonz et al., 2004; Saltys et al., 2006; Vulesevic et al., 2006), but not in trout or mangrove rivulus (Coolidge et al., 2008; Regan et al., 2011; Saltys et al., 2006). In other fish species, Type IV cells typically contain synaptic vesicles and are innervated. In contrast, the lamellar NECs of bowfin did not share these characteristics (Fig. 2D; Fig. 3D). It is unclear why this difference exists. The Type IV cells in bowfin could still be oxygen chemoreceptors releasing serotonin in a paracrine fashion through non-synaptic vesicles on nearby pillar cells. Serotonin causes pillar cells of the lamellae to contract, which serves to redistribute blood flow through the lamellae and thus improve oxygen uptake (Stensløkken et al., 2006). Alternatively, the serotonin released from these cells could be acting on other NECs or nerve endings located elsewhere in the gill arch, therefore, indirectly participating in a reflex response to hypoxia.

In goldfish, NECs in the lamellae are innervated. Goldfish remodel their gills by increasing surface area in warm water or hypoxia, and reducing surface area in cold water or normoxia to reduce osmoregulatory costs (Sollid et al., 2003). Lamellar NECs move during this gill remodelling, remaining in close proximity to the water (Tzaneva and Perry, 2010). These changes are consistent with lamellar NECs being involved in hypoxic cardiorespiratory reflexes. However, because of their small size, no electrophysiological recordings have been made from lamellar NECs in any fish species, to date, and it is not known whether they depolarize in response to hypoxia.

Type V cells

Type V cells were similar in morphology and immunolabelling to Type IV (lamellar) cells (Fig. 2E; Fig. 3E; Fig. 4E) and are probably from the same cell population despite their different location. The interfilamental support bar where Type V cells were found is a tissue joining both adjacent lamellae of the same filament as well as lamellae from the neighbouring filaments (Olson, 1981) (Fig. 1). This structure is, to our knowledge, unique to bowfin and its function remains unknown, but it has been proposed to prevent gill collapse and support the gill curtain during air breathing (Olson, 1981). This tissue is supplied by the outer vascular margins of the lamellae, which are embedded in it (the outer one to three lamellar channels), reducing oxygen uptake capacity in this area of the lamellae (Olson, 1981). Additionally, unlike other fish, bowfin do not have connections (anastomoses) between their nutritive and respiratory circulation (Olson, 1981). Therefore, all of the cardiac output must perfuse the lamellae, which, during air breathing in hypoxia, could lead to a loss of oxygen from the blood to the water. Serotonin released from these cells could act to modify blood flow through the respiratory lamellae or act in a paracrine fashion on NECs or nerve endings in other locations in the gill arch.

Conclusions

A greater diversity of NECs exists in the gills of bowfin than in other species in which NECs have been described. This might be related to the early divergence of this species. Type I cells found near the basal lamina of the eFA may be exclusively internal oxygen chemosensors in bowfin. These cells responded to sustained hypoxaemia by changing size and shape. This study also shows that bipolar neurons (Type II cells) might be involved in the hypoxic response; responding to both hypoxia and hypoxaemia. Additionally, we provide evidence for a novel NEC in bowfin, the Type III cell, that does not contain synaptic vesicles staining for the marker SV2, but that is innervated and also responds to both internal and external hypoxia by increasing in size. NECs found in the lamellae and the interfilamental support bars (Type IV and V cells) showed no SV2 labelling and were not innervated, indicating that these cells may have a paracrine role if involved in oxygen chemoreception. At this point it is not clear to what extent the results described here are unique to bowfin or are common to facultative air breathers but they do provide anatomical evidence of differential distribution of internal versus external O_2 chemosensors.

MATERIALS AND METHODS

Animals

All experiments were run in accordance with the Canadian Council on Animal Care guidelines and were approved by animal care committees from the University of British Columbia and the University of Guelph. Bowfin (340–2500 g), *A. calva*, were caught by a local fisherman from the Bay of Quinte, Ontario, and were transported to the University of Guelph in aerated tanks in a pickup truck. They were kept in recirculated water tanks at the Hagen Aqualab, University of Guelph. Animals were first kept at 8°C for a month, after which the temperature was increased by 1°C per day to 22°C, and the animals were acclimated at that temperature for at least 3 weeks before the start of any experiments. The bowfin were kept on a 12 h:12 h light:dark photoperiod and fed goldfish once a week.

Sustained hypoxia

Bowfin were randomly assigned to either of three groups: one group exposed to normoxia with access to air, one group exposed to hypoxia with access to air and one exposed group to hypoxia without access to air. They were put into 2 m diameter tanks (up to four animals per tank at once) and separated using perforated polyvinyl chloride (PVC) dividers. Each tank was also fitted with a perforated PVC cover either 2 cm below the surface of the water (in the group without access to air) or 10 cm above the surface of the water (in the group with access to air). Sustained hypoxia was achieved by bubbling nitrogen through the water using a Parker Balston nitrogen generator (Model N2-04, Parker Hannifin Corp., Haverhill, MA, USA) into a large header tank (~100 l) as well as into the experimental tank and by controlling the water flow from the header tank into the experimental tank. This design allowed the oxygen level in the experimental tank to be kept within a narrow O_2 range (6.0±0.9 kPa). The animals were exposed to sustained hypoxia or normoxia for 7 days.

Immunohistochemistry

At the end of the experiment bowfin were killed by an overdose of benzocaine followed by a sharp blow to the head. Bowfin were perfused with heparinized (100 i.u. ml^{-1}), ice-cold phosphate-buffered solution (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). Bowfin were perfused through the bulbous arteriosus of the fish using a blunt 25 gauge needle and a 200 ml syringe until the gill filaments appeared clear. The first gill arch was removed and fixed in 4% paraformaldehyde in PBS overnight. Tissues were then rinsed in PBS, cryoprotected in a 30% sucrose solution and frozen in Tissue-Tek® (Sakura Finetek, Fisher Scientific, Ottawa, ON, Canada) at −80°C. Blocks were sectioned using a cryostat (Leica CM3050 S, Leica Microsystems, Wetzlar, Germany) and mounted on Superfrost® plus slides (VWR International, Edmonton, AB, Canada) for immunohistochemistry. Sections were made either longitudinal to the gill filament at 10–12 µm increments or as cross-sections 12 µm thick every 700–1000 µm along the length of the filament.

Slides containing frozen tissue were washed in PBS and blocked in 10% normal goat or donkey serum (Jackson Laboratories, distributed by Cedarlane Laboratories, Hornby, ON, Canada) for 1 h. The slides were incubated overnight at room temperature with the primary antibodies (Table 2), which were diluted in a permeabilizing solution (PBS; 0.1% Triton X-100, 3% normal goat or donkey serum). Then the slides were again washed in PBS. The slides were then incubated with a fluorescently labelled secondary antibody (Table 1) diluted in PBS containing 0.1% Triton X-100 and 3% normal goat or donkey serum at room temperature for 2 h in darkness. Following a final washing with PBS, slides were mounted with coverslips and Vectashield, with DAPI (Vector Laboratories, Burlington, ON, Canada) to prevent photobleaching and to label cell nuclei. Slide boxes were stored at 4°C until viewed under a fluorescence light microscope. Control experiments were performed in which the primary antibodies were excluded to control for effects of the secondary antibody. The results of these control experiments showed only negligible staining with no specific immunolabelling of the gill structures present (data not shown).

Microscopy and cell size quantification using longitudinal sections

Some images were captured using a confocal scanning system (Pascal 2, Axioskop X, Zeiss, Jena, Germany) equipped with argon and helium-neon lasers emitting at 488 nm and using a BP 505-530 filter to detect Alexa Fluor 488. *Z*-stacks of 11–35 optical sections taken 0.22–0.39 µm apart were captured using EC Plan-Neofluar 40×/1.30 oil DIC M27. Additional images

Table 2. Primary and secondary antibodies used for immunohistochemistry

Antibody	Antigen	Manufacturer	Host	Dilution	Cat. no.	Secondary antisera[1]
Primary						
5-HT	Serotonin	Sigma-Aldrich	Rabbit	1:500	S5545	Alexa Fluor 488[b]
5-HT	Serotonin	ImmunoStar	Goat	1:1000	20079	Alexa Fluor 594[d]
SV2	Synaptic vesicles, neuronal and endocrine	DSHB	Mouse	1:200	n/a	Alexa Fluor 594[c]
zn-12	Neuron, surface	DSHB	Mouse	1:50	n/a	Alexa Fluor 594[c]
HNK-1	CD-57	BD Pharmingen	Mouse	1:1000	559048	Alexa Fluor 594[c]
VAChT	Vesicular acetylcholine transporter	Sigma-Aldrich	Rabbit	1:250	V5387	Alexa Fluor 488[e]
Secondary[a]						
Alexa Fluor 488	Rabbit IgG (H+L)[b]	Molecular Probes, Invitrogen	Goat	1:500	A11008	–
Alexa Fluor 594	Mouse IgG (H+L)[c]	Molecular Probes, Invitrogen	Goat	1:300	A11005	–
Alexa Fluor 488	Rabbit IgG (H+L)[e]	Molecular Probes, Invitrogen	Donkey	1:500	A21206	–
Alexa Fluor 594	Goat IgG (H+L)[d]	Molecular Probes, Invitrogen	Donkey	1:300	A11058	–

[a]Secondary antisera were conjugated with a fluorescent marker.
[b–e]Secondary antisera antigen corresponds to primary antibody host.
5-HT, serotonin; HNK-1, human natural killer; eFA, efferent filament artery; SV2, synaptic vesicle marker; VAChT, vesicular acetylcholine transporter; zn-12, zebrafish neuronal marker.

were captured using a confocal microscope (Olympus Fluoview FV10i, Tokyo, Japan) equipped with a solid-state laser emitting at 405, 473 and 559 nm. Z-stacks of 5–12 optical sections taken 1.0 µm apart were captured using the 60× objective of this microscope. Compressed *Z*-stacks of the green channel (serotonin) were used to measure projection area (surface area of a 2D projection of a cell) using the measurement function in Volocity imaging software Version 5.5.1 (Perkin Elmer Inc., Waltham, MA, USA). For each animal 15–20 images, containing approximately 200 cells, were used to measure size of three different cell types: serotonin-containing neuroepithelial cells near the filament arteries, serotonin-containing bipolar neurons and serotonin-containing neuroepithelial cells near the central venous sinus.

Density and cell size quantification using cross-sections

Images were captured using a confocal microscope (Olympus Fluoview FV10i, Tokyo, Japan) equipped with solid state lasers emitting at 473 nm. *Z*-stacks of 5–12 optical sections taken 1.0 µm apart were captured using the 60× objective of this microscope. 3-D projections of the acquired stacks were used to measure cell volume using the measurement function in Volocity imaging software Version 6.1.2 (Perkin Elmer Inc., Waltham, MA, USA). Skeletal length was the maximal length of the 3D projection as measured with the Volocity software and it represents a measure of the cell length. Cell were counted only in cross-sections because it was impossible to obtain perfect longitudinal sections of the entire filament in the same plane (near the efferent filament artery for example) and this would have made comparable cell density counts impossible. Cell counts were used to calculate cell densities (cell mm^{-1} filament) using the lengths of the filaments. Type I cells were counted at four different locations: near the basal lamina of the eFA (eFA bas), in the outer epithelial layer of the eFA (eFA epi), in the outer epithelial layer of the aFA (aFA epi) and near the basal lamina of the aFA (aFA bas). For each animal, 40 images, containing ~20–30 cells, were used to measure Type I cells at three different locations: eFA bas, eFA epi, aFA epi. There were too few cells near the aFA bas to be able to obtain reliable measurements; therefore, the cells in this location were not measured.

Statistical analysis

Data are expressed as means ± s.e.m. For Type I cell projection areas a Kruskal–Wallis one-way ANOVA on ranks was performed because the treatment groups had unequal variances. For Types II and III cells a one-way ANOVA using treatment group as a factor followed by a Tukey's test was used to test for differences between projection area and cell shape. A one-way ANOVA using treatment group as a factor was used to test for differences between Type I densities, cell volume and skeletal (cell) length at the different locations. A significance level of $P<0.05$ was used throughout.

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: C.S.P., W.K.M. and P.A.W.; acquisition of data: C.S.P.; analysis and interpretation of data C.S.P., W.K.M. and P.A.W.; drafting of the manuscript: C.S.P.; critical revision of the manuscript for important intellectual content: C.S.P., W.K.M. and P.A.W.; statistical analysis: C.S.P.; obtaining funding: W.K.M. and P.A.W.; administrative, technical and material support: W.K.M. and P.A.W.; study supervision: W.K.M. and P.A.W.

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