The Journal of Experimental Biology 211, 2371-2378
Published by The Company of Biologists 2008
doi:10.1242/jeb.010587

Commentary

New developments on gill innervation: insights from a model vertebrate

Michael G. Jonz[1,*] and Colin A. Nurse[2]

[1]Department of Biology, University of Ottawa, Ottawa, ON, Canada, K1N 6N5 and [2]Department of Biology, McMaster University, Hamilton, ON, Canada, L8S 4K1

*Author for correspondence (e-mail: mjonz@uottawa.ca)

Accepted 28 May 2008

Summary

The fish gill is a highly specialized and complex organ that performs a variety of important physiological functions. In this article, we briefly review the innervation of important structures of the branchial region, such as the gill filaments, respiratory lamellae and pseudobranch, and discuss the physiological significance of this innervation within the context of homeostatic functions of the gill, such as oxygen sensing and ion regulation. Studies in zebrafish utilizing techniques of confocal microscopy and immunolabelling, with specific antibodies against neuronal markers, have recently led to the characterization of innervation patterns in the gills not attained with traditional techniques of histochemistry and electron microscopy. We will discuss the association of putative sensory nerve fibres with O_2-chemoreceptive neuroepithelial cells and the implications of dual sensory pathways for cardiorespiratory and vascular control. In addition, the idea of the neural control of ion regulation in the gill based on the apparent innervation of mitochondria-rich cells, and the role of innervation in the pseudobranch, will be presented.

Key words: innervation, gill, neuroepithelial cell, NEC, mitochondria-rich cell, MRC, pseudobranch, zebrafish, oxygen sensing, ion regulation.

Introduction

The fish gill is a multifunctional organ made up of several cell types. Many processes, such as the motor control of the branchial musculature, and several sensory modalities (e.g. chemoreceptors, baroreceptors, nociceptors), are made possible because of the efferent (motor) or afferent (sensory) innervation of the gills. Of particular interest to the present article are the homeostatic functions that the gills perform, such as respiration and ion regulation. While the role of branchial nerves in respiratory regulation has received much attention, the idea of the neural control of other important processes in gill physiology remains speculative. That such questions have remained unanswered is perhaps due to the limitations of traditional approaches based on serial section histochemistry or electron microscopy to characterize the full extent of innervation to branchial structures. However, recent studies utilizing confocal immunofluorescence techniques and whole-mount gill preparations in the model vertebrate, the zebrafish, have provided novel insights into the structural and potential functional aspects of gill innervation. The zebrafish is amenable for such studies because of its small size and relative transparency of gill tissue, allowing entire populations of nerve fibres and other cell types to be visualized without the need for serial section microscopy. In addition, the commercial availability of zebrafish-specific antibodies against neuronal markers (e.g. zn-12) (see Trevarrow et al., 1990) allows labelling of the entire branchial nervous system.

Several pioneering histological and ultrastructural studies have established the cellular morphology and innervation of the branchial structures in fish, particularly those of Pierre Laurent and colleagues (Laurent and Rouzeau, 1972; Laurent and Dunel-Erb, 1984; Dunel-Erb et al., 1982; Dunel-Erb et al., 1989; Bailly et al., 1989; Bailly et al., 1992). However, as it is not the goal of this Commentary, we will not attempt to review this extensive body of work. The purpose of this Commentary is to briefly introduce important concepts of nervous innervation of the fish gill to the non-specialist reader and to highlight recent contributions in the field that have made an impact on our current understanding of the functional significance of this innervation. The present article will draw from selected studies and reviews, as well as recent investigations by the authors that have used confocal microscopy to map branchial innervation patterns in the zebrafish, and indicate how these may provide insights into the fields of oxygen sensing and ion regulation.

Organization of the gills and branchial nerves

The gills of fish are derived from the embryonic aortic arches observed in all vertebrates (Weichert, 1967) and are differentiated to perform specialized homeostatic functions in an aquatic environment (Fig. 1A). In teleost fish, each of four bilateral pairs of arches gives rise to two parallel rows (hemibranchs) of gill filaments that are each further subdivided into a series of secondary lamellae (Fig. 1A,B). The filaments and lamellae are covered by a thin epithelium composed of several cell types that perform such processes as gas exchange, ion regulation, acid–base balance and excretion (for a review, see Evans et al., 2005). The gill arches connect the ventral aorta to the paired dorsal aortae (Fig. 1A,C). The heart, therefore, pumps deoxygenated blood rostrally and dorsally for gas exchange and other processes across the gill epithelium and for delivery of oxygenated blood to tissues *via* the systemic circulation. Afferent and efferent branchial arteries carry blood through the gill arches, while afferent and efferent filament arteries carry blood to and from the lamellae, respectively (Fig. 1B). Lamellar arterioles allow passage of blood to a vascular sinus of the lamellae and back to the filament arteries (Olson, 2002).

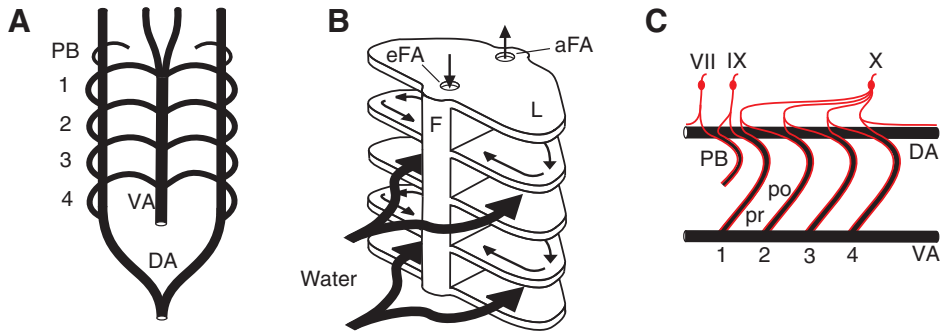

Fig. 1. Schematic representation of the gill arches, pseudobranch and their innervation. (A) Ventral view of the organization of the gill arches (numbers 1 to 4) and pseudobranch (PB) in teleosts. VA, ventral aorta; DA, dorsal aortae. Based on Weichert (Weichert, 1967). (B) Illustration of the organization of gill filaments and lamellae in a typical teleost fish. Large arrows indicate the flow of water through the gills, and small arrows indicate the flow of blood. aFA, afferent filament artery; eFA, efferent filament artery; F, filament; L, lamellae. (Modified from Jonz et al., 2004.) (C) Left lateral view of the nerve supply to the gill arches and pseudobranch from cranial nerves (VII, IX and X). Branchial structures are innervated by postganglionic divisions (red) of pre-trematic (pr) and post-trematic (po) rami.

The organization of gill innervation is discussed only briefly here, but the reader is referred to other reviews (Nilsson, 1984; Sundin and Nilsson, 2002) for a more detailed account. Eleven pairs of cranial nerves are found in fish. These include the terminal (0), olfactory (I), optic (II), oculomotor (III), trochlear (IV), trigeminal (V), abducens (VI), facial (VII), acoustic (VIII), glossopharyngeal (IX) and vagus (X) nerves (Nilsson, 1984; Sundin and Nilsson, 2002). In addition, the cranial nerves may carry somatic sensory, somatic motor, visceral sensory or visceral motor fibres (Nilsson, 1984; Sundin and Nilsson, 2002). However, only the facial, glossopharyngeal and vagus nerves innervate the gill region and are therefore called the 'branchial nerves'. In jawed fishes, such as teleosts, the glossopharyngeal and vagus nerves primarily innervate the gill arches and form large nerve trunks that enter the gill arches dorsally (Fig. 1C). Branches of the branchial nerves are further divided into pre-trematic (anterior) and post-trematic (posterior) rami that straddle the gill slits (Nilsson, 1984; Sundin and Nilsson, 2002). Thus, each gill arch is innervated by a post-trematic and pre-trematic ramus from two different cranial nerve branches (see Fig. 1C). Gill arches 2, 3 and 4 are innervated entirely by branches of the vagus nerve, while the first gill arch is innervated by both the glossopharyngeal and vagus nerves (Fig. 1C). In addition to innervation of the gill arches (i.e. arches 1–4), a vestigial gill-like structure, called the 'pseudobranch', primarily receives innervation from the pre-trematic branch of the glossopharyngeal nerve and may receive additional innervation from the post-trematic branch of the more anterior facial nerve (Laurent and Dunel-Erb, 1984; Nilsson, 1984).

Innervation of the gill filaments and secondary lamellae

The branchial nerves traverse the gill arches and send projections into each gill filament and, in some species (see below), also the secondary lamellae. Innervation to the filaments and lamellae has significant physiological implications and will be discussed here and in the following sections. As shown in the confocal image of a whole-mount preparation in Fig. 2A, the branchial nerve of the gill arch is labelled with the neuronal marker zn-12 and gives rise to a single nerve bundle that extends into each gill filament in zebrafish (Jonz and Nurse, 2003). The nerve bundle of the filament continues distally and runs adjacent to the efferent filament artery

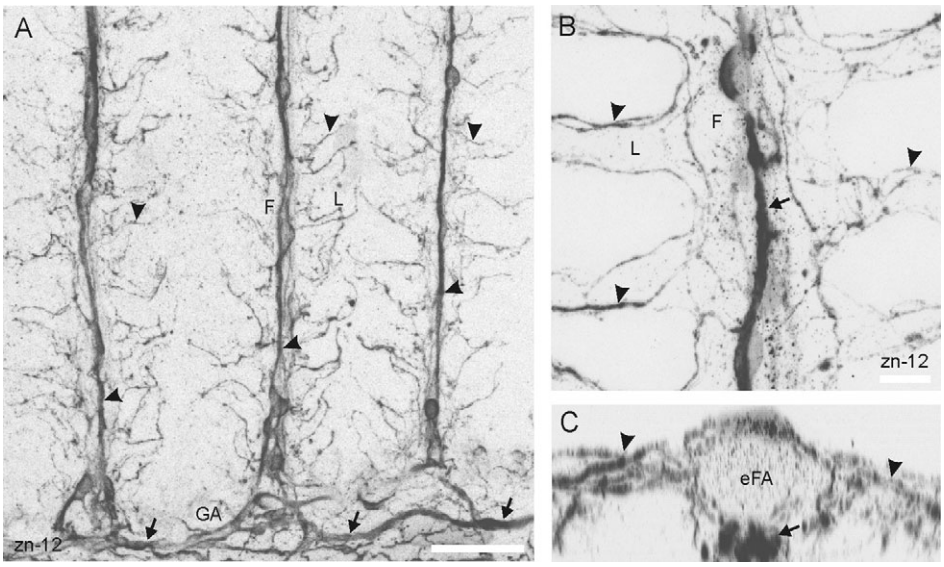

Fig. 2. Innervation of the filament and lamellae of the zebrafish gill, as indicated by zn-12 confocal immunohistochemistry. (A) zn-12-immunoreactive nerve trunks (arrows) of the gill arch (GA) extend fibres (arrowheads) into the filaments (F) and lamellae (L). Scale bar, 50 μm. Modified from (Jonz and Nurse, 2003). (B) Higher magnification confocal image showing a nerve bundle (arrow) of the filament and nerve fibres (arrowheads) of the lamellae in zebrafish gill. Scale bar, 10 μm. (C) Image in B tilted back 90° showing that the nerve bundle of the filament sends fibres that wrap around the efferent filament artery (eFA) to innervate the filament and lamellar epithelium.

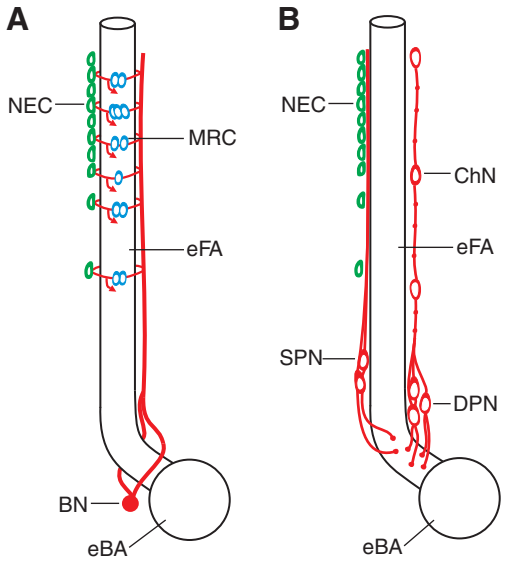

Fig. 3. (A) Summary of extrinsic innervation of the zebrafish gill illustrating formation of a nerve bundle composed of nerve fibres emanating from the BN of the gill arch that gives rise to a nerve plexus surrounding the eFA. Fibres of the nerve plexus associate with NECs (green) and MRCs (blue) and extend out to the respiratory lamellae (indicated by arrows). (B) Intrinsic innervation showing nerve endings of SPNs and DPNs terminating at the base of the eFA, and extension of SPN and DPN nerve fibres towards NECs and ChNs (with varicose processes), respectively. BN, branchial nerve; eBA, efferent branchial artery; eFA, efferent filament artery; ChN, chain neuron; DPN, deep proximal neuron; MRC, mitochondria-rich cell; NEC, neuroepithelial cell, SPN; superficial proximal neuron. (Modified from Jonz and Nurse, 2003.)

(eFA). Furthermore, fibres of this nerve bundle give rise to an extensive nerve plexus that wraps around the eFA, heavily innervating the superficial and interlamellar regions of the filament, and extends branches into the secondary lamellae (Fig. 2B,C). Previous studies have described nerve fibres of the gill filaments, including those identified as adrenergic, cholinergic, nitrergic and peptidergic (Donald, 1984; Donald, 1987; Dunel-Erb et al., 1982; Dunel-Erb et al., 1989; Bailly et al., 1989; Bailly et al., 1992; Sundin et al., 1998a; Zaccone et al., 2006). Only recently was innervation of the secondary lamellae characterized in zebrafish with zn-12 (Jonz and Nurse, 2003), and similar nerve fibres of the lamellae were identified in goldfish and catfish (Saltys et al., 2006; Zaccone et al., 2006). However, only minimal labelling of nerve fibres in the lamellae of trout was observed with zn-12 (Saltys et al., 2006), and in the tropical species traira and trairao no lamellar nerve fibres were observed (Coolidge et al., 2008). This suggests that innervation of the secondary lamellae in fish may be species specific. The implications of the presence or absence of innervation in the secondary lamellae are discussed further in a subsequent section, but these species differences may be related to adaptation to different aquatic environments or conditions, such as temperature, oxygen availability and salinity.

Experiments designed to determine the origin of innervation to the gill filaments and lamellae in zebrafish involved the removal (and consequent denervation) of the gills and maintenance of this tissue in explant culture for 48 h. These studies showed that this denervation resulted in the complete degeneration of the filament nerve bundle, plexus and lamellar fibres, suggesting that the parent cell bodies of these nerve fibres were extrinsic to the gill (Jonz and Nurse, 2003). This class of nerve fibre represents the majority of innervation to the zebrafish gill and is summarized in Fig. 3A. These experiments in the zebrafish gill also indicated the presence of intrinsic innervation, i.e. those nerve fibres whose parent cell bodies are located within the gill filaments, and indicated the presence of three populations of intrinsic neurons. These observations confirmed previous reports of intrinsic neurons in teleosts (Bailly et al., 1989; Sundin et al., 1998a). The intrinsic innervation in the zebrafish gill is summarized in Fig. 3B. Labelling of whole-mount gill preparations in zebrafish with zn-12 revealed the presence of two populations of neurons that were clustered near the proximal regions of the filaments. These neurons are depicted in Fig. 4 and are referred to as ‘deep’ or ‘superficial proximal neurons’ (DPNs or SPNs) in zebrafish based on their location relative to the eFA (Jonz and Nurse, 2003). These proximal neurons in the filaments are multipolar and have also been labelled with antibodies against the synaptic vesicle protein SV2 (Fig. 4A,B). This antibody identifies a highly conserved protein of synaptic vesicle membranes (necessary for neurosecretion) found in neurons and other neurosecretory cells (Buckley and Kelly, 1985). The

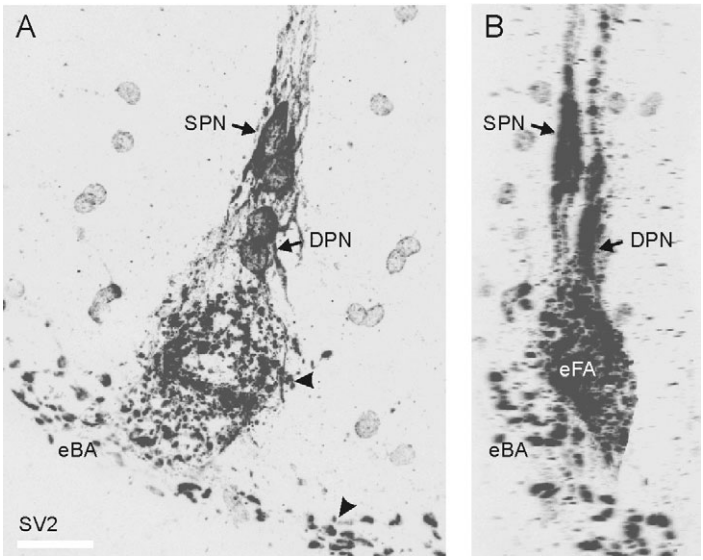

Fig. 4. Intrinsic neurons at the proximal region of the gill filaments in zebrafish innervate the base of the efferent filament artery. Confocal imaging was performed using antibodies against the synaptic vesicle protein SV2. (A) Multipolar superficial proximal neurons (SPNs) and deep proximal neurons (DPNs) extend processes towards the base of the efferent filament artery (eFA), where SV2-immunoreactive nerve endings (arrowheads) are found. Extrinsic nerve terminals surrounding the efferent branchial artery (eBA) are also visible. Scale bar, 20 μm. (B) Image in A rotated 70° depicting SPNs and DPNs as two distinct neuronal groups. Note the shape of the eFA outlined by SV2-positive nerve terminals. (Modified from Jonz and Nurse, 2003.)

significance of proximal neurons in chemoreception, and potentially in the regulation of blood flow within the gill, will be discussed in the following sections. A third group of intrinsic neurons in zebrafish was reported and these cells are referred to as 'chain neurons' because of their apparent linear organization adjacent to the eFA along the entire length of the gill filament (Jonz and Nurse, 2003). A role for these neurons has not been proposed, but the presence of distinct varicosities along chain neuron fibres suggests that these bipolar neurons may form synaptic connections with other structures. For example, in other teleosts, intrinsic neurons innervate afferent lamellar arterioles and may provide a means of vascular or hemodynamic control (Bailly et al., 1989; Dunel-Erb et al., 1989). All three populations of intrinsic neurons in zebrafish have been shown to contain synaptic vesicles and the neurotransmitter serotonin [5-hydroxytryptamine (5-HT)] (Jonz and Nurse, 2003). In addition, serotonergic neurons in the gill filaments have been previously described and appear to be typical in teleost fish (Bailly et al., 1989; Sundin et al., 1998a; Sundin et al., 1998b; Sundin and Nilsson, 2002). Thus, the prevalence of this neurotransmitter in the cytoplasm of intrinsic gill neurons suggests that release of 5-HT *via* neurosecretion from these cells may play an important physiological role in the gill. For example, it has been shown that exogenous application of 5-HT induced vasoconstriction of the eFA and modified blood flow (Sundin et al., 1995).

Having now established the organization and innervation of the fish gill, the following three sections will discuss further the physiological significance of this innervation within the context of O_2 sensing, vascular control during periods of hypoxia, and ion regulation.

Role of innervation in O_2 sensing

As in all vertebrates, fish respond to a decrease in O_2 availability with reflex physiological responses. These include hyperventilation, decreased heart rate and changes in gill vascular resistance (Sundin and Nilsson, 1997; Nilsson and Sundin, 1998; Burleson and Milsom, 2003). A group of neurosecretory paraneurons, called neuroepithelial cells (NECs), are found in the primary epithelium of the gill filaments, where they are exposed to the incident flow of water over the gills during ventilation (see Fig. 1B, Fig. 5), and have long been implicated in O_2 chemoreception and respiratory regulation in fish (Burleson and Milsom, 2003). NECs store 5-HT in cytoplasmic synaptic vesicles near the plasma membrane (Fig. 5) and have a morphology and distribution that is highly conserved among fish (Dunel-Erb et al., 1982; Zaccone et al., 1994; Zaccone et al., 1997; Sundin et al., 1998a; Jonz and Nurse, 2003; Saltys et al., 2006; Coolidge et al., 2008). Experiments have demonstrated that gill NECs isolated from zebrafish respond to hypoxia with K^+ channel inhibition and membrane depolarization, thus confirming their O_2 sensitivity, and a cellular model for O_2 sensing in fish has been proposed where Ca^{2+}-dependent release of neurotransmitter results in activation of postsynaptic sensory nerve fibres (Jonz et al., 2004). Such a process would ultimately lead to a physiological response to hypoxia, such as hyperventilation. Thus, because of these morphological and physiological characteristics, NECs are strongly believed to bear a phylogenetic relationship with mammalian O_2-chemoreceptors (see González et al., 1994; López-Barneo, 2001) and allow for O_2 chemosensory responses in the gill.

The innervation of NECs in the gill filaments represents an important feature of peripheral O_2 chemoreception because it provides a pathway through which the transmission of the cellular response to hypoxia may occur from the periphery to other sites, such as the central nervous system (CNS). Similarly, mammalian O_2 chemoreceptors, such as the type I cells of the carotid body and neuroepithelial bodies (NEBs) of the lung, receive extensive innervation primarily from fibres of the glossopharyngeal and vagus nerves, respectively (González et al., 1994; Cutz and Jackson, 1999). NEC innervation has been verified at the ultrastructural level in teleosts (Dunel-Erb et al., 1982; Bailly et al., 1992) and has more recently been studied using confocal microscopy in whole-mount gill preparations (Jonz and Nurse, 2003; Saltys et al., 2006), facilitating identification of entire populations of nerve fibres and innervation patterns. These studies have shown that NECs of the gill filaments receive innervation from both extrinsic and intrinsic sources (see Fig. 3), indicating a complex pattern of innervation. Since gill NECs retain both synaptic vesicles and the neurotransmitter, 5-HT (Dunel-Erb et al., 1982), they can be identified using antibodies against these antigens. In zebrafish, gill NECs labelled with antibodies against 5-HT or the synaptic vesicle protein SV2 are intimately associated with zn-12-positive fibres of a nerve plexus that branches from a large nerve bundle of the filament (Fig. 5). This indicates a putative sensory pathway through which NECs stimulated by hypoxia may communicate with the nervous system. In addition, NECs are positioned in a linear arrangement along a separate bundle of nerve fibres that lies superficial to the eFA and originates from the superficial proximal neurons (described in the previous section)

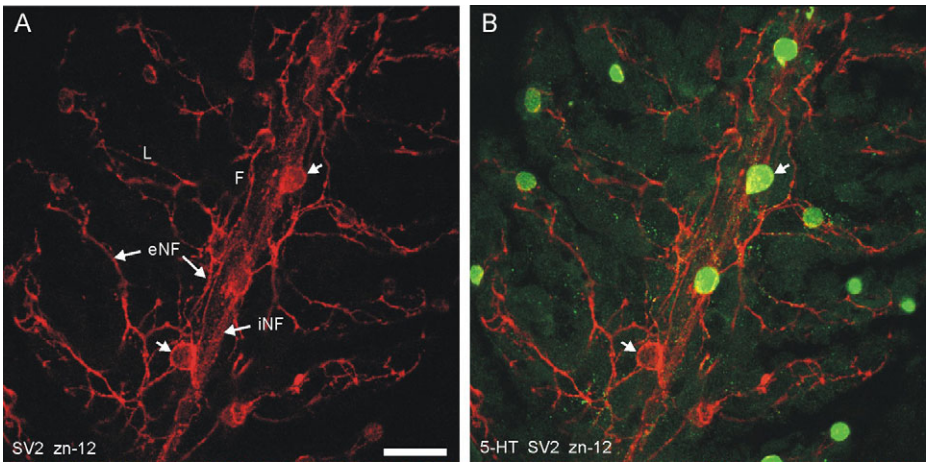

Fig. 5. Confocal imaging of neuroepithelial cells (NECs) and associated innervation of the gill filaments and lamellae in zebrafish. NECs were labelled with antibodies against 5-hydroxytryptamine (5-HT; green) and SV2 (red in A; red or yellow in B), and nerve fibres were identified by zn-12 immunoreactivity (red). (A) SV2-positive NECs (arrows) were found in the filament (F) and received innervation from intrinsic nerve fibres (iNF) and a plexus of extrinsic nerve fibres (eNF). Extrinsic nerve fibres were also found in the lamellae (L). Scale bar, 20 μm. (B) Dual exposure image in A showing, in addition, 5-HT-positive immunofluorescence (green) and that some NECs containing SV2 are also serotonergic.

near the filament base (Fig. 3B). In zebrafish, these fibres also make contact with NECs (Fig. 5A,B) (see Jonz and Nurse, 2003). Morphological evidence suggests that at least part of the innervation to NECs is sensory, as would be required for O_2 sensing, and indeed extracellular nerve recordings have identified sensory fibres of the branchial nerves that increased their discharge during hypoxia (Burleson and Milsom, 1993). Early studies indicated that NECs in the trout gill were degranulated at the ultrastructural level, suggestive of exocytosis, following exposure to hypoxia (Dunel-Erb et al., 1982). In zebrafish, synaptic vesicles were localized to the basal cytoplasm of NECs, adjacent to nerve fibres, and a greater number of NECs devoid of a detectable amount of 5-HT was found following chronic exposure to hypoxia, suggesting hypoxic release of 5-HT (Jonz and Nurse, 2003; Jonz et al., 2004). These morphological features may implicate the neurosecretion of 5-HT induced by hypoxia and a sensory role for nerve fibres that innervate NECs. Furthermore, in developing zebrafish, the correlation between innervation of gill NECs and a significant rise in the hyperventilatory response to hypoxia at 7 days post-fertilization (d.p.f.) (Jonz and Nurse, 2005) suggests that these nerves are indeed sensory. Therefore, in the case of stimulation of gill NECs in fish during periods of hypoxic exposure, the release of neurotransmitters from NECs following membrane depolarization would potentially activate postsynaptic sensory nerve fibres and induce centrally mediated changes in ventilation or heart rate *via* the extrinsic neural pathway (Fig. 3A) or lead to changes in gill vascular resistance *via* the intrinsic neural pathway (Fig. 3B).

While there is a wealth of evidence indicating that NECs of the teleost gill contain 5-HT (see above references), the neurotransmitter underlying hypoxic signalling from NEC to sensory nerve fibre is currently unknown. Several studies have indicated that other neurochemical candidates may include nitric oxide or catecholamines (Zaccone et al., 2006; Burleson et al., 2006; Milsom and Burleson, 2007), and a variety of neuropeptides may also be involved (for reviews, see Zaccone et al., 1994; Zaccone et al., 1997). It is conceivable that the neurochemical basis of O_2 sensing in the gill may involve multiple neurotransmitters or neuropeptides and, perhaps, a diversity of excitatory, inhibitory and modulatory mechanisms, as has been described in the mammalian O_2-sensing organ, the carotid body (González et al., 1994; Nurse, 2005; Prabhakar, 2006; Lahiri et al., 2006). Interestingly, a variety of neurochemicals, such as acetylcholine, 5-HT and dopamine, applied exogenously to the fish gill have been shown to have stimulatory effects on sensory nerve fibres and cardiorespiratory reflexes (Burleson and Milsom, 1995a; Burleson and Milsom, 1995b).

Despite the evidence for afferent innervation, the possible contribution of efferent nerve fibres to NEC innervation must also be considered. Many nerve fibres associated with NECs of the zebrafish gill, especially the intrinsic fibres, contain synaptic vesicles and may potentially release neurotransmitters onto NECs (Jonz and Nurse, 2003). In addition, serotonergic and nitrergic fibres in the gill filaments contact NECs (Jonz and Nurse, 2003; Zaccone et al., 2006). These results may suggest an additional neuroendocrine role for NECs in the gill, whereby NEC stimulation would result in local vascular changes, or a role for efferent nerve fibres in modulating the chemosensory response to hypoxia. Similarly, pulmonary NEBs receive afferent and efferent innervation (Adriaensen and Scheuermann, 1993), and a mechanism of modulation of O_2 sensing *via* efferent glossopharyngeal nerve fibres has been described in the mammal carotid body (Campanucci and Nurse, 2007).

Role of innervation in vascular control during hypoxia

The fish gill possesses an intricately complex vascular network, perhaps due to the diverse range of regulatory functions performed by this organ (Olson, 2002). Control of vascular resistance in the gill is, therefore, of particular interest because homeostatic processes, such as respiration, are dependent on haemodynamic changes (Evans et al., 2005). The autonomic and pharmacological control of vascular changes within the gill of several fish taxa has been extensively reviewed (Nilsson, 1984; Sundin and Nilsson, 2002; Evans et al., 2005; Zaccone et al., 2006), but recent studies in zebrafish have identified potential roles for intrinsic and extrinsic sources of innervation in vascular control and respiratory regulation during hypoxic stress (Jonz and Nurse, 2003). Interestingly, in zebrafish, the intrinsic proximal neurons, which also send fibres distally to innervate O_2-chemoreceptive NECs (Fig. 5A,B), appear to be the same neurons that send fibres rich in secretory vesicles proximally to terminate at a muscular segment at the base of the eFA (Fig. 3B, Fig. 4A,B) (Jonz and Nurse, 2003). Such a relationship between NECs and intrinsic neurons was previously proposed (Bailly et al., 1992). This evidence suggests that SPNs may mediate adjustments in vascular tone through vasomotor control of the eFA during hypoxic stimulation of NECs. This putative sensory pathway would indicate a mechanism of local neural control within the gill and allow for modulation of gill blood flow without involvement of the CNS. Innervation of the contractile segment of the eFA by filament neurons has been previously described in other species and may operate through serotonergic or cholinergic mechanisms (Bailly et al., 1989; Dunel-Erb et al., 1989). Furthermore, vasoconstriction of the proximal region of the eFA appears to occur during hypoxia (Sundin and Nilsson, 1997) and may contribute to the hypoxic increase in gill vascular resistance observed in teleosts (Nilsson and Sundin, 1998; Smith et al., 2001). A subsequent elevation in lamellar perfusion pressure may lead to a process known as ‘lamellar recruitment’ that may underlie perfusion of distal lamellae (which are apparently not normally utilized) during periods of hypoxia to increase respiratory surface area and gas exchange (Booth, 1978; Booth, 1979; Sundin and Nilsson, 1997). It should be noted that since nitrergic and serotonergic nerve fibres in the gills contact NECs (Jonz and Nurse, 2003; Zaccone et al., 2006), a presynaptic role for these fibres cannot be ruled out. Such a role may release stored neurochemicals from NECs, having an effect on surrounding tissue, or modify the cellular response to hypoxia.

The discovery of a rich network of extrinsic nerve fibres in the secondary lamellae in zebrafish (Jonz and Nurse, 2003) (Fig. 5) suggests that other cell types of the lamellae in this and other species may be innervated and under neural control, possibly affecting respiratory or osmoregulatory functions of the gill. Pillar cells of the lamellae, which provide structural support and allow deoxygenated blood to pass through the vascular sinus, contain proteins that allow these cells to contract and increase gill vascular resistance (Smith and Chamley-Campbell, 1981; Stensløkken et al., 1999; Mistry et al., 2004). If extrinsic nerve fibres of the lamellae contact pillar cells, this innervation may allow for pillar cell contraction and provide a mechanism by which blood flow through the respiratory lamellae could be rapidly adjusted by the CNS as needed during changes in ambient or arterial O_2.

Do branchial nerves contribute to ion regulation?

As a consequence of aquatic respiration across such a relatively large surface area, such as that of the gill epithelium, aquatic organisms are susceptible to the diffusion of important ions down

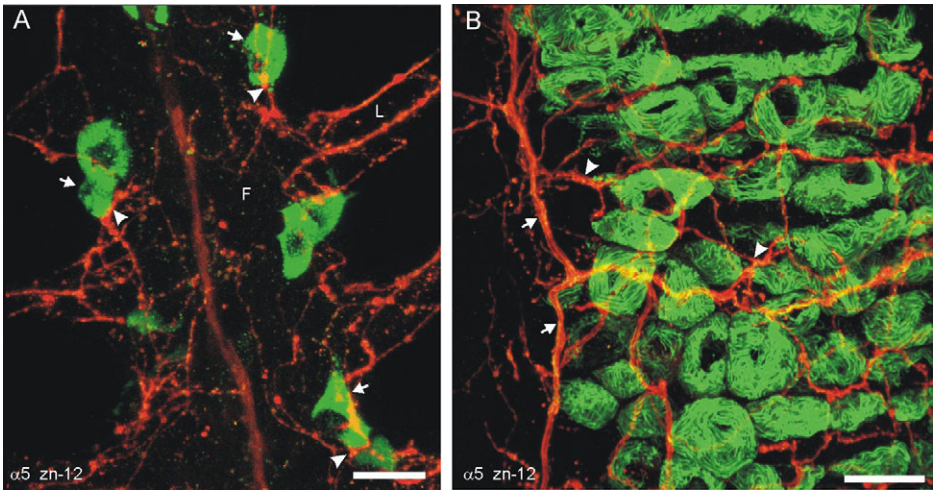

Fig. 6. (A) Mitochondria-rich cells and associated innervation in the gills of zebrafish. Mitochondria-rich cells (MRCs; green) of the interlamellar regions were immunoreactive for antibodies against the α5 subunit of Na^+/K^+-ATPase, and nerve fibres (red) were labelled with the neuronal marker zn-12. The confocal image illustrates the association of MRCs (arrows) of the gill filaments (F) with nerve fibres (arrowheads indicate apparent points of contact). Scale bar, 10 μm. Reprinted with permission (Jonz and Nurse, 2006). (B) Innervation of the pseudobranch. Methods similar to those used in A were used to label α5-positive pseudobranch cells (PBCs; green) and zn-12-positive nerve fibres (red) in zebrafish. Larger nerve bundles (arrows) of the supportive filaments gave rise to a network of nerve fibres (arrowheads) that ramify among the fused lamellae and innervated PBCs. Scale bar, 20 μm.

their concentration gradients (Evans et al., 1999). The gill is a major site of osmotic and ion regulation in fish, and the primary functional unit of ion regulation in the teleost gill is the mitochondria-rich cell (MRC). MRCs are located in the interlamellar region of the gill filament epithelium (Fig. 6A) and are specialized for ion transport. These cells are characterized by a high density of mitochondria, an abundance of the enzyme Na^+/K^+-ATPase and a complement of ion channels, exchangers and pumps (Perry, 1997; Wilson and Laurent, 2002; Evans et al., 2005). In freshwater teleosts, they mediate Na^+, Ca^{2+} and Cl^- uptake across the epithelium into the blood, while in saltwater teleosts MRCs (also called 'chloride cells') mediate ion extrusion (Perry, 1997; Marshall, 2002; Evans et al., 2005).

The control of ionoregulatory mechanisms by the gill is a vibrant field of research, but this process is not completely understood. The movement of ions across gill or opercular epithelia appears to be mediated by circulating hormones and neurotransmitters (McCormick, 2001; Evans, 2002; Marshall, 2003), and evidence has accumulated suggesting that ion transport by MRCs may be under neural control. Nerve stimulation in isolated gill and opercular membrane preparations has been shown to result in alterations in Ca^{2+} and Cl^- flux across epithelia (Donald, 1989; Marshall et al., 1998). In addition, earlier studies demonstrated that cutting the branchial nerves produced a depletion of gill MRCs and altered ion transport and water permeability (Pequignot and Gas, 1971; Mayer-Gostan and Hirano, 1976), suggestive of MRC innervation and neural control. A more recent study used confocal immunofluorescence techniques and provided morphological evidence for such innervation of MRCs in zebrafish (Fig. 6A) (Jonz and Nurse, 2006). MRCs of the efferent filament epithelium appeared to make contact with only extrinsic fibres derived from the nerve plexus of the filament, which would indicate the presence of a mixed nerve supply in the gill since similar fibres also innervate O_2-chemoreceptive NECs. Developmental studies using zebrafish showed that gill filament primordia receive innervation as early as 3 d.p.f., but the site of termination of these fibres was not determined at this stage (Jonz and Nurse, 2005). However, nerve fibres in zebrafish larvae were found to make contact with MRCs as early as 5 d.p.f. (Jonz and Nurse, 2006), suggesting that ion regulatory neural pathways may be established in the gill during larval development before O_2-chemosensory pathways, which occur at 7 d.p.f. These results were consistent with an earlier study that reported that the gills are needed for ion regulation before they are functional as respiratory organs (Rombough, 2002).

While MRC innervation has not been investigated in other species, collectively, the above studies point to the neural control of ion regulation in the gill. Similarly, the neural control of ion homeostasis in the mammalian kidney is well established (DiBona and Kopp, 1997). Nerve fibres in the fish gill are associated with the basolateral regions of MRCs (Fig. 6A), where Na^+/K^+-ATPase activity is localized (Evans et al., 2005), and apical regions of MRCs are positioned toward the external environment. This may suggest that putative presynaptic nerve terminals release neurochemicals to influence the activity of membrane-bound ion channels, or other intracellular processes of postsynaptic MRCs, thereby controlling ion regulation across the gill epithelium. Neurotransmitters and neuropeptides, such as catecholamines, acetylcholine, nitric oxide, vasoactive intestinal polypeptide, endothelin and prostaglandins, have been shown to mediate the movement of ions across gill and opercular epithelia, and stimulation of Cl^- extrusion is mediated by adrenergic receptors (Evans, 2002; Marshall, 2003; Evans et al., 2004).

Although evidence appears to suggest efferent innervation of MRCs, sensory innervation of these cells must also be considered. Evidence suggests the presence of ionoreceptors in fish that are sensitive to changes in plasma ion concentration that may elicit rapid ion transfer during adaptation to salinity change (Evans et al., 2005; Marshall et al., 2005). Moreover, sensory receptors and afferent innervation of transport epithelia in the mammalian kidney have been described, including nerve fibres near the renal tubules and vagal innervation (DiBona and Kopp, 1997).

The pseudobranch

The pseudobranch is a nonrespiratory, reduced gill-like organ that has remained an enigma since its initial discovery [see

Laurent and Dunel-Erb for a historical review (Laurent and Dunel-Erb, 1984)]. While pseudobranch structure is variable between fish species, it resides within the cranial portion of the opercular epithelium, where it is not exposed to the external environment, and its primary cellular component is that of the pseudobranch cell (PBC). These cells are characterized by apical localization of the enzyme Na^+/K^+-ATPase and an extensive cytoplasmic tubular network (Fig. 6B; Laurent and Dunel-Erb, 1984; Quinn et al., 2003; Jonz and Nurse, 2006). Early studies investigating the function of the pseudobranch suggested that it may act as a gland, a sensory organ involved in O_2 chemoreception, or take part in osmoregulation (Laurent and Rouzeau, 1972; Laurent and Dunel-Erb, 1984). It was recently concluded, however, that the hypoxic release of catecholamines from chromaffin cells mediated by O_2 chemoreceptors did not involve the pseudobranch in trout (Reid and Perry, 2003), and only one or two 5-HT-containing cells resembling O_2-sensitive gill NECs were found in each pseudobranch filament in zebrafish (Jonz and Nurse, 2003). Thus, it would seem unlikely that the pseudobranch plays any significant role in O_2 sensing, at least in these species. Other recent evidence has suggested that PBCs supply O_2 to the retina and regulate blood pH (Bridges et al., 1998; Kern et al., 2002) and that a role for these cells in osmoregulation, if any, is limited (Quinn et al., 2003).

Most striking, however, is the extensive innervation found in the pseudobranch and the fact that the physiological significance of this innervation has not yet been determined. Pseudobranch innervation was characterized at the ultrastructural level (Laurent and Dunel-Erb, 1984) and, more recently, in zebrafish with confocal microscopy (Jonz and Nurse, 2006). In zebrafish, PBCs are densely packed within the fused lamellae and are innervated by a rich network of nerve fibres that arise from supportive filaments (Jonz and Nurse, 2006). There is evidence that the teleost pseudobranch receives afferent innervation (Laurent and Rouzeau, 1972; Laurent and Dunel-Erb, 1984), but similar nerve fibres in zebrafish are immunopositive for the synaptic vesicle protein SV2 (Jonz and Nurse, 2003), suggesting a neurosecretory or efferent role of these fibres. Moreover, since innervation of PBCs occurs apically, rather than basolaterally (Laurent and Dunel-Erb, 1984; Jonz and Nurse, 2006), the physiological significance of these cells would appear to be limited to internally oriented functions that may take place between the basolateral membrane and the vasculature, such as hormone secretion or chemical sensing of the blood. While the precise function of the pseudobranch and its component cells is currently speculative, further study of the nature of PBC innervation may reveal significant clues about the role of this potentially important organ in fish.

Conclusions and remaining questions

The rich supply of nerve fibres found in the gills indicates an important role for the branchial nervous system in gill function. This paper has highlighted selected studies that have provided morphological evidence of the innervation of branchial structures, with a particular emphasis on recent work with the zebrafish model, and has presented the potential physiological significance of this innervation in homeostatic processes, such as respiratory and ion regulation. Several important questions surrounding the specific roles of gill innervation remain unanswered. Evidence from previous ultrastructural studies, and more recent confocal investigations, has argued for both afferent and efferent innervation of NECs of the gill filaments. However, without a clear understanding of the neurochemistry of NECs and associated nerve fibres, it is difficult to assign specific functions of identified neural pathways and to ascertain how these might contribute to the hypoxic response. Future physiological studies of these neural pathways at the cellular level in an identified O_2-sensing system, such as that of the zebrafish gill, may characterize the function of isolated gill neurons and chemical communication between these cells and NECs and shed light on these matters. In addition, the role of innervation to the lamellae is largely unexplored and it is not known if these contribute to respiratory or ion regulation, or both. The nature of putative MRC innervation, whether afferent or efferent, is also unknown. Clearly, MRCs coordinate ion-regulatory processes in the gill, and so it may be concluded that any innervation of these cells is expected to be efferent. But can MRCs also detect ionic changes in either the serosal or external environments and transmit this information *via* afferent nerves? Such an answer may await confirmation of innervation at the ultrastructural level and functional studies.

Use of the zebrafish as a model system may facilitate studies designed to solve the above outstanding issues. Whole-mount preparation of gill tissue coupled with confocal microscopy has allowed complete morphological characterization of several cell types and innervation patterns in this species. Moreover, with a growing number of commercially available antibodies, mutants and the advent of gene knockdown in zebrafish embryos by morpholino injection (Heasman, 2002), a more complete physiological and molecular characterization of gill innervation may be just around the corner.

List of abbreviations and glossary

aFA	afferent filament artery of the gill
Afferent	travelling towards (e.g. sensory nerves travelling towards the central nervous system or arteries carrying deoxygenated blood toward the respiratory lamellae of the gill)
Bipolar neurons	neurons having two axons or branches
eFA	efferent filament artery of the gill
Efferent	travelling away from (e.g. motor nerves travelling away from the central nervous system or arteries carrying oxygenated blood away from the respiratory lamellae of the gill)
Extrinisic innervation	nerve fibres of the gill filaments or lamellae having parent cell bodies outside of the gill
Filaments	primary structures of the gill, branching from the supportive gill arches and giving rise to secondary lamellae
Intrinsic innervation	nerve fibres of the gill filaments having parent cell bodies also within the gill filaments
Lamellae	secondary structures of the gill, branching from the filaments
MRC	mitochondria-rich cell
Multipolar neurons	neurons having more than two axons or branches
NEC	neuroepithelial cell
Nerve plexus	an interlaced network of nerve fibres
Paraneurons	cells having characteristics similar to that of a neuron, such as retention of neurochemicals and secretory vesicles (see Fujita, 1989)
PBC	pseudobranch cell
Post-trematic ramus	a branch of a cranial nerve that is posterior to the gill slit
Pre-trematic ramus	a branch of a cranial nerve that is anterior to the gill slit
Varicosities	small ‘swellings’ along a nerve fibre that may indicate regions of synaptic contact with other structures or cells

The authors acknowledge grant support to M.G.J. and C.A.N. by the Natural Sciences and Engineering Research Council (NSERC) of Canada.

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