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Development of oxygen sensing in the gills of zebrafish

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Summary

Previous studies have described the morphology, innervation and O_2-chemoreceptive properties of neuroepithelial cells (NECs) of the zebrafish gill filaments. The present work describes the ontogenesis of these cells, and the formation of functional O_2-sensing pathways in developing zebrafish. Confocal immunofluorescence was performed on whole-mount gill preparations using antibodies against serotonin (5-HT) and a zebrafish-derived neuronal marker (zn-12) to identify the appearance and innervation of gill NECs during larval stages. NECs were first expressed in gill filament primordia of larvae at 5 days postfertilization (d.p.f.) and were fully innervated by 7 d.p.f. *In vivo* ventilation frequency analysis revealed that a behavioural response to hypoxia (11.2±2.8 min^{-1}) developed in embryos as early as 2 d.p.f., and a significant increase ($P<0.05$) in the ventilatory response to hypoxia (200.8±23.0 min^{-1}) coincided with innervation of NECs of the filaments. In addition, exogenous application of quinidine, a blocker of O_2-sensitive background K^+ channels in NECs, induced hyperventilation in adults in a dose-dependent manner and revealed the development of a quinidine-sensitive ventilatory response in 7 d.p.f. larvae. This study shows that NEC innervation in the gill filaments may account for the development of a functional O_2-sensing pathway and the hyperventilatory response to hypoxia in zebrafish larvae. At earlier stages, however, O_2-sensing must occur through another pathway. The possibility that a new type of 5-HT-positive NEC of the gill arches may account for this earlier hypoxic response is discussed.

Key words: O_2 chemoreceptor, development, gill, hypoxia, neuroepithelial cells, zebrafish, *Danio rerio*.

Introduction

In vertebrates, cardioventilatory changes are driven by input from peripheral O_2-chemoreceptors, exemplified in mammals by type I cells of the carotid body (González et al., 1994; Peers, 1997; López-Barneo et al., 2001) and neuroepithelial bodies (NEBs) of the lung (Youngson et al., 1993; Fu et al., 2002). Neuroepithelial cells (NECs) of the fish gill have long been considered O_2-chemoreceptors (Dunel-Erb et al., 1982; Burleson et al., 1992). Recent studies in zebrafish have indicated directly that isolated NECs of the gill filaments respond to hypoxia with inhibition of quinidine-sensitive background K^+ channels and membrane depolarization (Jonz et al., 2004), similar to several mammalian O_2-sensitive cells (Buckler et al., 2000; López-Barneo et al., 2001; Campanucci et al., 2003; Kemp et al., 2004). The characterization of O_2-sensitive NECs has therefore established the cellular basis for the initiation of the hypoxic response in fish (Milsom and Brill, 1986; Burleson et al., 1992; Burleson and Milsom, 2003). Furthermore, zebrafish gill NECs are innervated and contain synaptic vesicles with the neurotransmitter serotonin (5-HT; Dunel-Erb et al., 1982; Jonz and Nurse, 2003). Thus, hypoxic stimulation of NECs is presumed to cause activation of sensory pathways in the gill, leading to hyperventilation (for reviews, see Burleson et al., 1992; Burleson and Milsom, 2003).

In mammals, developmental shifts occur in functional O_2-sensing pathways. For example, NEBs of the lung and adrenal chromaffin cells are O_2-sensitive during late fetal and neonatal stages, respectively (Youngson et al., 1993; Thompson et al., 1997). However, during the postnatal period and following the onset of aerial respiration, NEBs decrease in number (Reddick and Hung, 1984; Cho et al., 1989) and chromaffin cells lose their hypoxic chemosensitivity (Thompson et al., 1997). Furthermore, during postnatal life there is an increase in the number of sensory nerve fibres innervating the carotid body, the primary O_2-chemosensory organ in adults, and this coincides with a rise in carotid body sensitivity to hypoxia (for reviews, see González et al., 1994; Donnelly, 2000). Although much is known about respiratory development in fish and the effects of hypoxia during early life (Rombough, 1988; Burggren and Pinder, 1991), there is currently little information regarding the ontogenesis of peripheral O_2-sensing mechanisms in aquatic vertebrates. An investigation of the development of O_2 chemoreception in the fish gill is of interest because a functional respiratory system develops much faster than in mammals.

In teleost fish, four gill arches are innervated by the glossopharyngeal (first arch only) and vagus nerves, and bear numerous gill filaments, where O_2-sensitive NECs reside (Jonz and Nurse, 2003; Jonz et al., 2004), and respiratory lamellae. Despite the relatively late formation of the gills, however, fish in embryonic and early larval stages respond to hypoxia (for a review, see Rombough, 1988). In zebrafish, the pharyngeal arches produce gill filament primordia at 3 days postfertilization (d.p.f.) but the gills, which lack respiratory lamellae, do not become functional until 14 d.p.f. (Kimmel et al., 1995; Rombough, 2002). However, the hyperventilatory response to hypoxia (Turesson et al., 2003), and changes in cardiac activity in larvae raised under chronic hypoxia (Jacob et al., 2002), appear to develop before this time. This suggests the presence of an O_2-sensing mechanism before complete development of the gills.

Based on previous studies that have described the chemoreceptive properties of O_2-sensitive NECs in adult zebrafish and a response to hypoxia in larvae, we sought to describe the development of the hyperventilatory response to hypoxia in zebrafish larvae, and to determine if these events were correlated with the appearance of O_2-sensitive NECs and associated neural pathways in gill filament primordia. Using confocal immunofluorescence techniques and ventilation frequency analysis, we describe the development of a quinidine-sensitive, hypoxic response in zebrafish larvae that coincided with O_2-sensitive NEC innervation. In addition, a quinidine-insensitive response to hypoxia that preceded the appearance and innervation of NECs of the gill filaments was identified.

Materials and methods

Animals

Zebrafish *Danio rerio* embryos (wild type, AB strain) were obtained from the Zebrafish International Resource Center (University of Oregon, USA) at 2 or 3 d.p.f. in embryo medium. Developing zebrafish were raised on a diet of *Paramecium multimicronucleatum* (Ward's Natural Science, St Catharines, ON, Canada), and fed brine shrimp *Artemia salina* (San Francisco Bay Brand Inc., Newark, CA, USA) once yolk sac resorption began. Staging of zebrafish embryos and larvae was performed following Kimmel et al. (1995). Adult zebrafish (150–400 mg) were obtained from local commercial sources and fed standard commercial flake food. All fish were maintained in dechlorinated system water at 28°C on a 14 h:10 h light:dark cycle (Westerfield, 2000). Procedures for animal use were carried out according to institutional guidelines, which adhere to those of the Canadian Council on Animal Care (CCAC).

Light microscope observations

Zebrafish larvae were lightly anaesthetized with 0.05 mg ml^{-1} MS 222 (ethyl 3-aminobenzoate methanesulfonate, Sigma) in dechlorinated system water and placed in the well of modified culture dishes (see below) on the stage of an inverted microscope (IM-35, Zeiss, Jena, Germany). Phase-contrast images of developing gill arches and filaments were captured using a digital camera (Retiga, QImaging, Burnaby, BC, Canada) and imaging software (Northern Eclipse, Empix Imaging Inc., Mississauga, ON, Canada). For images of circulating red blood cells in the gill filaments, a rapid exposure time of 10 ms was used.

Confocal immunofluorescence

Neuroepithelial cells of the gill filaments are O_2-sensitive (Jonz et al., 2004), and were identified in developing and adult zebrafish using confocal immunofluorescence and criteria similar to those used by Jonz and Nurse (2003). These NECs were: (1) located within the gill filaments, (2) epithelial, (3) serotonin (5-HT) immunoreactive (IR) and (4) innervated. Other NECs were identified using criteria (2–4). Zebrafish were killed by overdose with 1 mg ml^{-1} MS 222. Techniques for immunolabelling and confocal imaging of adult gill tissue were similar to those previously described (Jonz and Nurse, 2003). Larvae between 3 and 9 d.p.f. were fixed by immersion in phosphate-buffered solution (PBS) containing 4% paraformaldehyde at 4°C overnight. PBS contained the following: (in mmol l^{-1}) NaCl, 137; Na_2HPO_4, 15.2; KCl, 2.7; KH_2PO_4, 1.5; pH 7.8 (Bradford et al., 1994). Fixed larvae were rinsed in PBS and permeabilized for 48–72 h at 4°C with a solution (PBS-TX) containing 1% fetal calf serum (FCS) and 0.5% Triton X-100 in PBS (pH 7.8). NECs of developing gill filaments were identified in whole-mount preparations using antibodies directed against serotonin (5-HT; Dunel-Erb et al., 1982; Jonz and Nurse, 2003). Neurons and nerve fibres of the gill arches and developing filaments were identified using antibodies against a zebrafish-derived neuron-specific antigen (zn-12). zn-12 is a general neuronal marker in zebrafish (Trevarrow et al., 1990), and its labelling of neural structures of the gill has been previously characterized (Jonz and Nurse, 2003). Polyclonal rabbit 5-HT antibodies (Sigma) were used at a dilution of 1:200 and localized with goat anti-rabbit secondary antibodies conjugated to fluorescein isothiocyanate (FITC, 1:50, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Monoclonal mouse anti-zn-12 (Developmental Studies Hybridoma Bank, University of Iowa, USA) was used at a dilution of 1:100 and localized with goat anti-mouse secondary antibodies conjugated with Alexa 594 (1:100, Molecular Probes, Eugene, OR, USA). All antibodies were diluted with PBS-TX. Fixed larvae were incubated in primary antibodies for 24–48 h at 4°C and in secondary antibodies at room temperature (22–24°C) for 1 h in darkness. Gill baskets or individual gill arches were removed from larvae with fine forceps and prepared as whole mounts on glass microscope slides in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) to reduce photobleaching. Whole-mount gill preparations were examined in the longitudinal plane using an upright microscope (Eclipse E800, Nikon, Melville, NY, USA) and a confocal scanning system (Radiance 2000, BioRad, Hercules, CA, USA) equipped with argon (Ar) and helium-neon (HeNe) lasers with peak outputs of 488 nm and 543 nm, respectively. Images were

detected using a photomultiplier tube and photodiode array, and were collected using confocal graphics software (LaserSharp 2000, BioRad). Each image is presented as a composite projection of serial optical sections. Image processing and manipulation was performed using Corel Draw 9 (Corel Corp., Ottawa, ON, Canada).

Ventilation frequency measurements

Adult zebrafish were lightly anaesthetized with 0.1 mg ml^{-1} MS 222 dissolved in dechlorinated system water. Initial tests were performed to determine if quinidine, a blocker of the O_2-sensitive background K^+ current in chemoreceptive NECs of the zebrafish gill (Jonz et al., 2004), could produce changes in ventilation frequency, and therefore be used to mimic the effects of hypoxia in whole-animal experiments. Adult zebrafish were immersed in 500 ml of system water with or without (control) 1 mmol l^{-1} quinidine. In addition, adults were exposed to voltage-dependent K^+ channel blockers, i.e. 20 mmol l^{-1} tetraethylammonium (TEA) plus 5 mmol l^{-1} 4-aminopyridine (4-AP), or equimolar NaCl (control). TEA plus 4-AP did not inhibit the hypoxic sensitivity of isolated zebrafish NECs (Jonz et al., 2004). The frequency (min^{-1}) of buccal or opercular movements was visually determined after the application of each drug for 5 min. Some experiments were performed in the absence of MS 222 to observe general changes in behaviour induced by these drugs. In experiments where the effects of different concentrations of quinidine were tested on ventilation frequency in adults, a fast-flow (30 ml min^{-1}) continuous perfusion chamber was constructed. A small well was carved out of a plastic Petri dish (60 mm) coated with Sylgard (Dow Corning Corp., Midland, MI, USA) and filled with ~4 ml of solution. Anaesthetized zebrafish were transferred to the chamber and held lightly in place with a fine nylon mesh pinned to the bottom of the dish to restrict excessive movements during experiments. The dish was placed on the stage of a dissecting microscope (M6C-10, Lomo, Prospect Heights, IL, USA) and the frequency of buccal or opercular movements was observed during a 3 min perfusion of 0.1 mmol l^{-1}, 0.5 mmol l^{-1} or 1 mmol l^{-1} quinidine. All solutions contained 0.1 mg ml^{-1} MS 222 at room temperature (22–24°C) and were adjusted to pH 7.4. Drugs were purchased from Sigma.

The effects of hypoxia and 1 mmol l^{-1} quinidine on behaviour and ventilation frequency were further tested on embryos and larvae of various developmental stages (2–10 d.p.f.). For these experiments, developing zebrafish were placed in a small chamber and perfused continuously at 4 ml min^{-1}. Embryos and larvae were lightly anaesthetized with 0.05 mg ml^{-1} MS 222 dissolved in dechlorinated system water. Zebrafish were transferred in a Pasteur pipette containing a small volume of system water (100 μl) to the central well (~8 mm in diameter) of modified culture dishes. Polystyrene culture dishes (Falcon, BD Biosciences, San Jose, CA, USA) were modified by drilling a small central hole in the bottom and attaching a glass coverslip to the underside with Sylgard. A piece of fine nylon mesh was placed over the bottom of the dish on the inside, which confined the embryos/larvae to the well. The dish was fitted with a stainless steel collar that held the mesh in place and formed a perfusion chamber. The dish was fixed to the stage of an inverted microscope (Axiovert S 100, Zeiss). Responses of developing zebrafish to hypoxia and quinidine were determined by observing the rate of body/pectoral fin movements or buccal/opercular movements, depending on the development stage. Embryonic and larval fish rely on cutaneous respiration during early developmental stages and exhibit increased movement of the pectoral fins and body when exposed to hypoxia to facilitate gas exchange (for a review, see Rombough, 1988). In older larvae (≥3 d.p.f.), movement of the gills and operculum, and buccal pumping, developed and were instead used to determine ventilation frequency. Hypoxia (P_{O_2}=25 mmHg) was produced by bubbling the solution in the perfusion reservoir with 100% N_2 for at least 30 min prior to the experiment. The P_{O_2} of solution in the perfusion chamber was verified using a carbon fibre electrode (10 μm, Dagan Corporation, Minneapolis, MN, USA) and an EPC 9 amplifier (Heka Electronik, Lambrecht, Germany) as described previously (Jonz et al., 2004). Control solution (P_{O_2}=150 mmHg) was contained in another reservoir and bubbled with compressed air. Tubing used to transfer the perfusate to the chamber was gas impermeable (Tygon, Saint-Gobain Performance Plastics Corporation, Akron, OH, USA). The responses of zebrafish to hypoxia, 1 mmol l^{-1} quinidine, or 1 mmol l^{-1} quinidine plus hypoxia were determined after perfusing the chamber with each solution for 3 min. All solutions contained 0.05 mg ml^{-1} MS 222 at room temperature (22–24°C) and were adjusted to pH 7.4. Preliminary experiments indicated that higher doses of MS 222 (e.g. 0.1 mg ml^{-1}) reduced the response of larvae to hypoxia, but 0.5 mg ml^{-1} did not. In all experiments reported in the present study, ventilation or response frequency measurements were determined several minutes after placing the animals in the observation chamber to ensure that subsequent responses were not affected by handling or confinement with the nylon mesh.

Statistical analysis

Ventilation frequencies and behavioural responses from all experiments are reported as mean ± S.E.M. Student's *t*-test was used to compare the means of two groups. For data analysis necessitating multiple comparisons, analysis of variance (ANOVA) followed by the Bonferroni post-test was employed.

Results

NECs of the gill in adult zebrafish

Confocal immunofluorescence of isolated gill preparations indicated the presence of many serotonin (5-HT)-immunoreactive (IR) neuroepithelial cells (NECs) of the gill filaments and respiratory lamellae (Fig. 1A). Both lamellar NECs and O_2-sensitive NECs of the filaments received innervation from zn-12-IR nerve fibres, as described previously (Jonz and Nurse, 2003). In addition, 5-HT-IR cells were observed in the epithelium of the gill arches and were

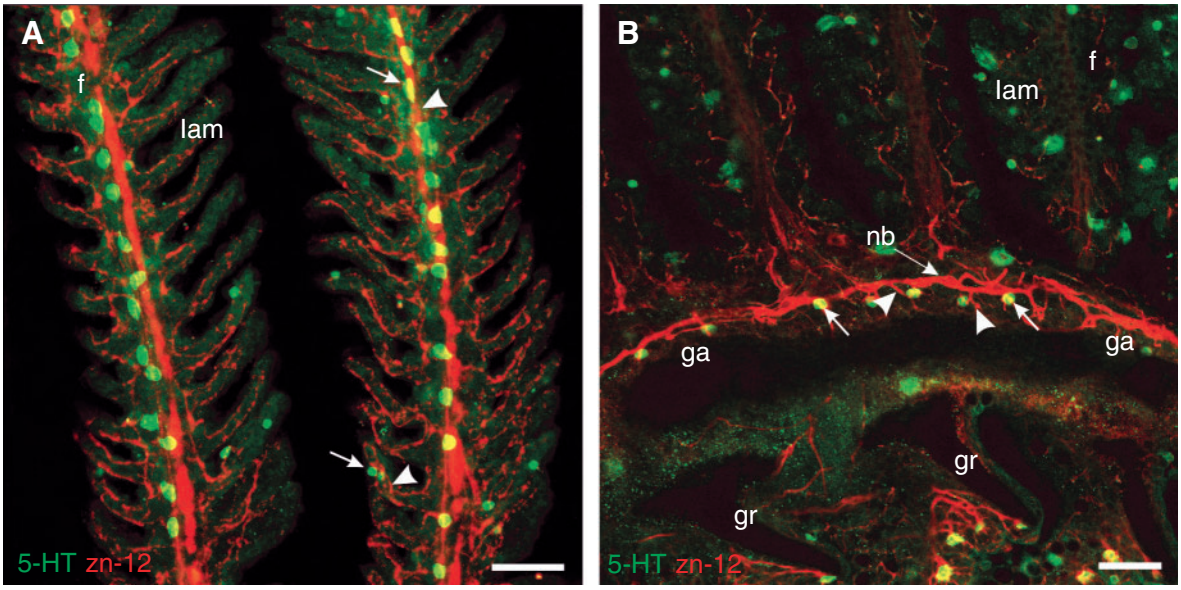

Fig. 1. Neuroepithelial cells and associated innervation in the gills of adult zebrafish. (A) Confocal image of two adjacent gill filaments (f) with respiratory lamellae (lam). Neuroepithelial cells (NECs, green) were serotonin (5-HT)-immunoreactive (IR) and located in the filaments (i.e. O_2-sensitive NECs) and lamellae (arrows). Both NEC populations were innervated by zn-12-IR nerve fibres (red; arrowheads) that emanated from nerve bundles. Scale bar, 50 μm. (B) Serotonin (5-HT)-immunoreactive (IR) NECs (green; arrows) of the gill arch (ga). NECs of the gill arch were organized along a zn-12-IR (red) nerve bundle (nb) and were innervated by zn-12-IR nerve fibres (arrowheads). Also visible are 5-HT-IR NECs of the respiratory lamellae (lam) near proximal regions of the gill filaments (f), and 5-HT-IR Merkel-like basal cells of the gill rakers (gr). Scale bar, 50 μm.

organized in a linear pattern along a zn-12-IR branch of the branchial nerve from which they received innervation (Fig. 1B). These morphological characteristics indicate that 5-HT-IR cells of the gill arches are a new type of NEC (see Discussion). Although these cells resembled O_2-sensitive NECs of the gill filaments, they were limited to the gill arches and did not receive intrinsic innervation (see Jonz and Nurse, 2003), suggesting that they may be of a distinct cell type.

NECs of the gill in developing zebrafish

The gill basket in developing zebrafish is ventrally-situated and is composed of four bilateral pharyngeal arches that bear gill filament primordia (Fig. 2A–C). Gill arches were observed in live specimens at 3 and 5 d.p.f., and red blood cells could be seen moving through the early vasculature of developing filaments (Fig. 2B). Isolated gills were examined using confocal immunofluorescence in larvae between 3 and 9 d.p.f. Developing gill filaments were observed as early as 3 d.p.f. and did not contain 5-HT-IR NECs at this stage (Fig. 3A,B); however, other 5-HT-IR NECs of the gill arches were present at 3 d.p.f. and appeared to be innervated by zn-12-IR nerve fibres (Fig. 3A,B). This organization of gill arch NECs continued throughout larval development (Figs 4A,B, 5C,D) and resembled that seen in the adult stage (Fig. 1B). In addition, zn-12-IR neurons were also observed in the gill arch at 3 d.p.f. (Fig. 3B), and at later developmental stages (Figs 4B,D, 5B). It is noteworthy that although filament primordia did not contain NECs at 3 d.p.f., zn-12-IR nerve fibres were observed in these regions (Fig. 3B–D). These may form free nerve endings in the gill filaments. At 5 d.p.f., 5-HT-IR NECs were observed in developing gill filaments (Fig. 4A,C) that resembled those of adults (Fig. 1A). At this stage, innervation of NECs of the filaments by zn-12-IR nerve fibres was variable, indicating that formation of contacts between filament NECs and nerve fibres may begin around this time. Fig. 4B illustrates an example of a zn-12-IR nerve fibre emanating from the branchial nerve that did not reach 5-HT-IR NECs of developing filaments. By contrast, Fig. 4D depicts the close association or innervation of a NEC by a zn-12-IR nerve fibre at 5 d.p.f. After 7 d.p.f., gill filaments were longer and primordia of respiratory lamellae were first observed (Fig. 5A). By this time, NECs of the gill filaments clearly received innervation from nerve fibres of the branchial nerve or gill arch neurons (Fig. 5B), as was also observed at 9 d.p.f. (Fig. 5C,D). NECs of the respiratory lamellae, however, as described in adult zebrafish (Jonz and Nurse, 2003), were not observed in lamellar primordia during these developmental stages. The major developmental events described in this section are summarized in Fig. 9.

Effects of quinidine on ventilation in adult zebrafish

Preliminary experiments (not shown) indicated that unanaesthetized adult zebrafish immersed in 1 mmol l^{-1} quinidine or 20 mmol l^{-1} TEA plus 5 mmol l^{-1} 4-AP displayed unbalanced and erratic swimming behaviour. This suggests that these K^+ channel blockers were taken up across the gills. Interestingly, only 1 mmol l^{-1} quinidine had the additional effects of inducing hyperventilation and surface-skimming

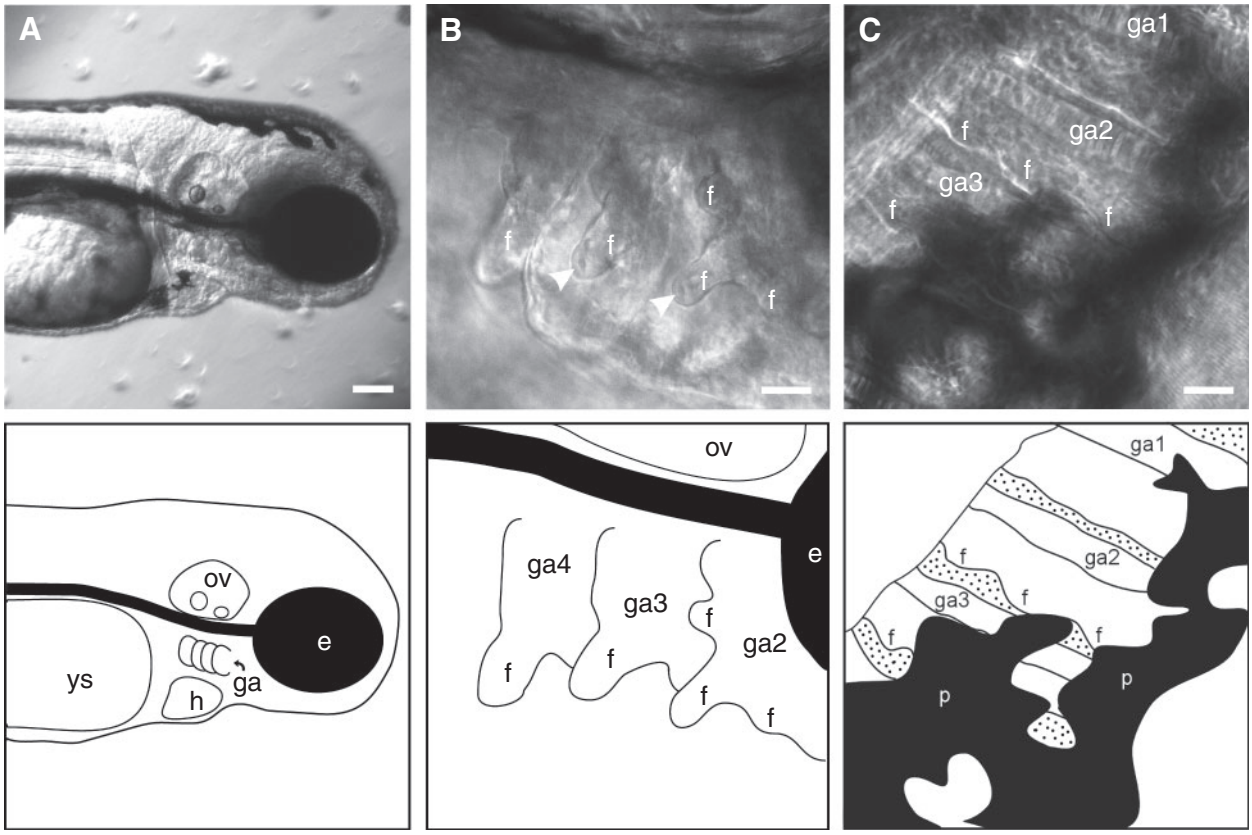

Fig. 2. Gill arches and developing filaments in zebrafish larvae. Corresponding schematic drawings are shown below. (A) Phase-contrast image of the anterior end of a 3 d.p.f. larva showing the ventrally positioned gill arches from a lateral view. Scale bar, 100 μm. (B) Lateral view of developing gill filaments in a 5 d.p.f. larva. Same orientation as in A. Filaments are visible on gill arches 2, 3 and 4. In addition, red blood cells (arrowheads) were seen moving through filament primordia. Scale bar, 25 μm. (C) Ventral view of the left side of the gill basket of a 5 d.p.f. larva. The first three gill arches are visible and each gave rise to several filament primordia. Pigment of the overlying tissue partially obscures arch 3. Scale bar, 25 μm. Stippling in the diagram below indicates the space (i.e. buccal cavity) between gills. e, eye; f, filament; ga, gill arch; h, heart; ov, otic vesicle; p, pigment; ys, yolk sac.

behaviour in larvae, reminiscent of hypoxia-like responses. As shown in Fig. 6, in anaesthetized adults, 1 mmol l^{-1} quinidine significantly increased ventilation frequency from 161.1±13.4 min^{-1} to 207.8±5.1 min^{-1} (P<0.005). In contrast, zebrafish exposed to TEA plus 4-AP or an equimolar substitution of NaCl (control) showed no significant change in ventilation frequency (P>0.05; ANOVA–Bonferroni test). The dose-dependent effect of quinidine on ventilation frequency is illustrated in Fig. 7 for adult zebrafish studied in continuous-perfusion experiments. Doses of 0.5 and 1 mmol l^{-1} quinidine significantly increased ventilation frequency above control values (P<0.05; ANOVA–Bonferroni test).

Effects of hypoxia and quinidine on ventilation in developing zebrafish

Experiments were performed in which a behavioural response or ventilation frequency was quantified in zebrafish embryos and larvae (2–10 d.p.f.) after stimulation with hypoxia or quinidine, to determine the time during development when O_2-sensitive pathways become functional. Based on data indicating that gill NECs were consistently innervated by 7 d.p.f. (Fig. 5), we predicted that this developmental event would have a significant physiological impact on the ventilatory response. In a continuously perfused chamber, zebrafish embryos responded to hypoxia as early as 2 d.p.f. Embryos at this stage exhibited no observable behaviour under control conditions (normoxia), but after exposure to hypoxic solution embryos responded with a significant increase in the frequency of pectoral fin and body movements (11.2±2.8 min^{-1}; N=11; P<0.05; Student's t-test). Buccal or opercular movements were not observed at this stage. As shown in Fig. 8A, at 3 d.p.f. the response to hypoxia included a significant increase in the frequency of buccal and opercular movements (i.e. hyperventilation) from 2.8±1.0 min^{-1} during normoxia to 42.5±12.5 min^{-1} during hypoxia (P<0.05; Student's t-test). This response was irregular in frequency, but synchronous with movement of the pectoral fins, suggesting that the latter response may indeed act to improve cutaneous gas exchange in larvae, as previously reported (see Rombough, 1988). This coordinated ventilatory response to hypoxia continued throughout development and became regular in frequency at 8 d.p.f. An increase in basal ventilatory frequency and a

dramatic rise in the hyperventilatory response to hypoxia occurred at 7 d.p.f. (Fig. 8A). In normoxia, ventilation frequency was 45.6±15.4 min^{-1}, whereas in hypoxia ventilation significantly increased to 200.8±23.0 min^{-1} ($P<0.05$; Student's *t*-test). This rise in the hyperventilatory response to hypoxia at 7 d.p.f. was significantly greater than the response at earlier stages ($P<0.001$; ANOVA–Bonferroni test), and did not increase further at 9 d.p.f. These results are consistent with the innervation and involvement of a greater number of O_2-sensitive chemoreceptors.

Based on previous work, which established that quinidine mimics the hypoxic response in isolated gill NECs from adult zebrafish by inhibiting background K^+ channels and inducing depolarization (Jonz et al., 2004), and the present data indicating that quinidine induced hyperventilation in adult zebrafish (Fig. 7), we asked if development of a quinidine-sensitive hypoxic response could be demonstrated at the whole-animal level in zebrafish larvae. In a continuously perfused chamber, the ventilatory response of zebrafish larvae to 1 mmol l^{-1} quinidine was absent at 3 d.p.f. (Fig. 8B) in

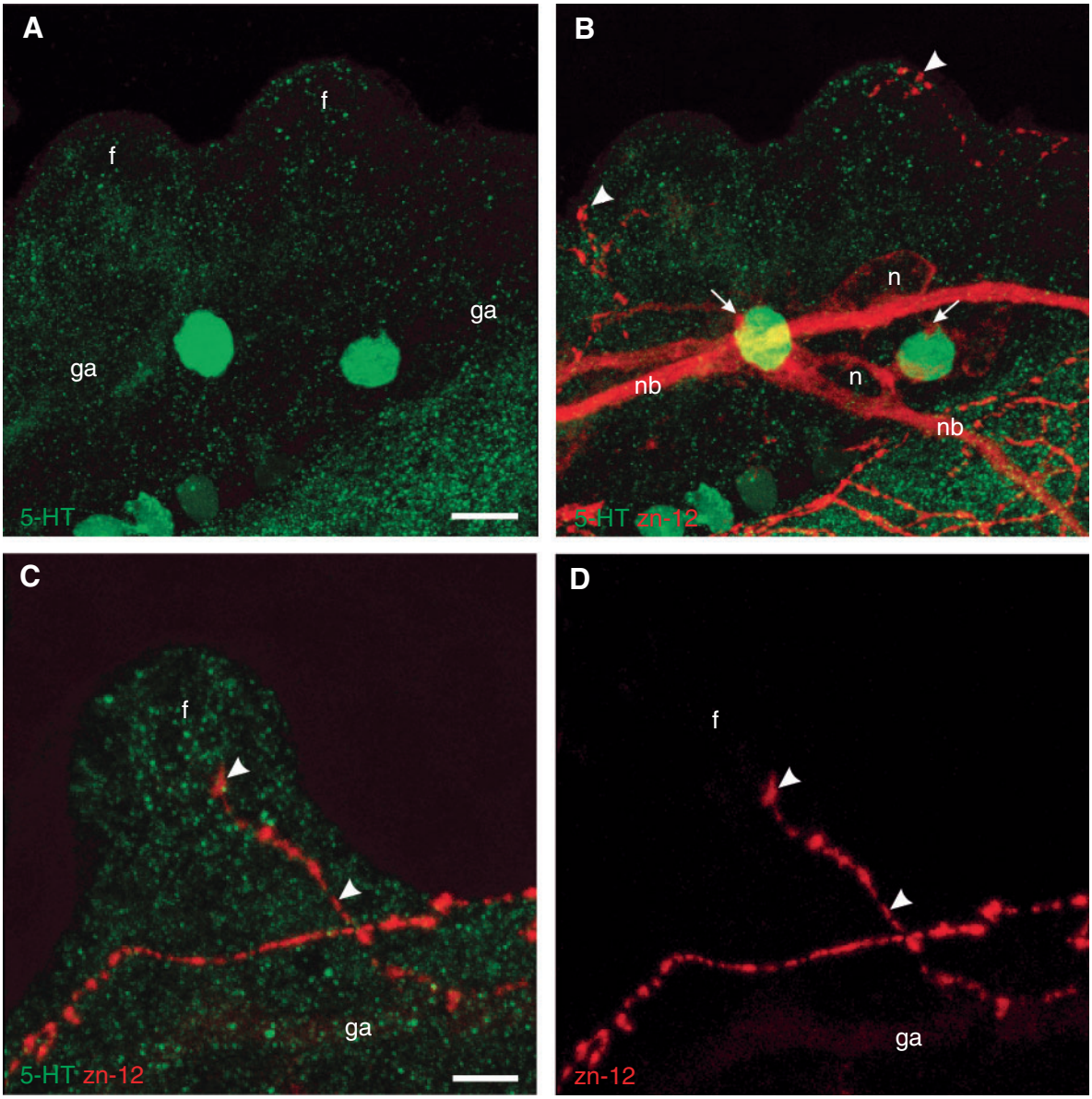

Fig. 3. Confocal image of an isolated zebrafish gill at 3 d.p.f. (A) Labelling with antibodies against serotonin (5-HT) demonstrated that O_2-sensitive neuroepithelial cells (NECs) were absent in gill filament primordia (f), but two NECs (green) of an unidentified type were located in the gill arch (ga). Scale bar, 10 µm. (B) Image in A shown with zn-12-IR (red). 5-HT-immunoreactive (IR) NECs of the gill arch were associated with nerve fibres (arrows) of zn-12-IR nerve bundles (nb). Two zn-12-IR neurons (n) are also visible. Although NECs were not found in developing gill filaments at this stage, zn-12-IR nerve fibres (arrowheads) were present. (C) Higher magnification image of a single developing gill filament primordium (f) from another larva showing the absence of filament NECs and the presence of a zn-12-IR nerve fibre (arrowheads) as it entered the filament from the gill arch (ga). Scale bar, 5 µm. (D) Image in C shown with only zn-12-IR.

larvae that responded to hypoxia (not shown), indicating the absence of a quinidine response when NECs of the gill filaments were absent. However, at 7 d.p.f., ventilation frequency significantly increased from 45.6±15.4 min^{-1} in controls to 122.4±35.6 after quinidine application ($P<0.05$; Student's *t*-test), and a similar response was observed at 10 d.p.f. (Fig. 8B). It is also noteworthy that the ventilatory response of 7 d.p.f. larvae to quinidine plus hypoxia (208.5±69.6 min^{-1}), compared to hypoxia (Fig. 8A) or quinidine (Fig. 8B) alone, did not differ significantly ($P>0.05$; ANOVA–Bonferroni test). Taken together, these data suggest that the effects of hypoxia and quinidine may act through overlapping pathways in the gill to modulate ventilation, and this parallels our previous demonstration of the occlusive effect of quinidine on the hypoxic sensitivity of isolated zebrafish NECs (Jonz et al., 2004). Thus, these results indicate that the appearance of a maximal response to hypoxia, and quinidine sensitivity, at 7 d.p.f. approximately coincides with innervation

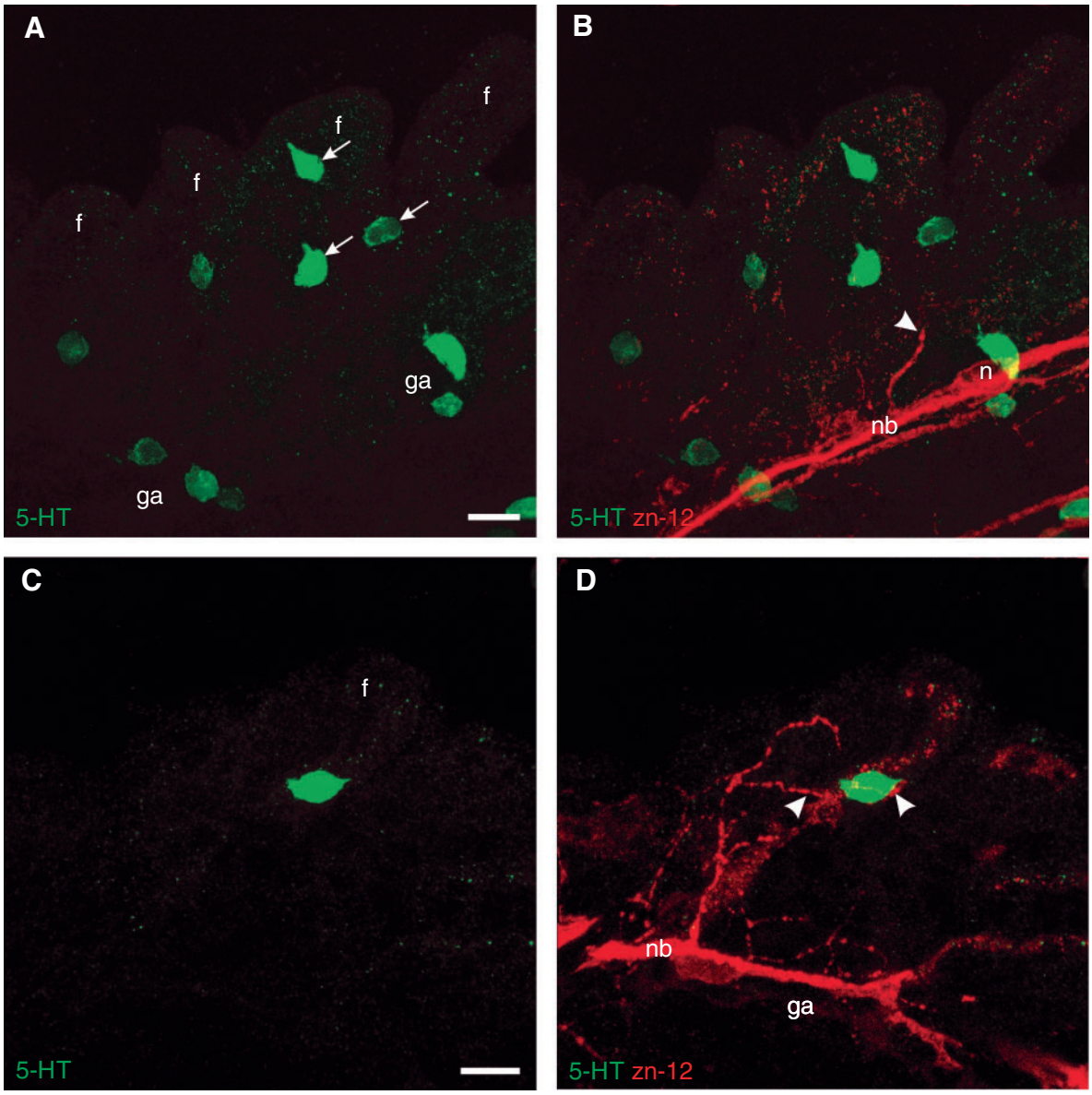

Fig. 4. Innervation of neuroepithelial cells of the gill filaments in zebrafish larvae first appeared at 5 d.p.f. (A) Confocal image of an isolated gill from a 5 d.p.f. larva giving rise to four adjacent developing filaments (f). Serotonin (5-HT)-immunoreactive (IR) neuroepithelial cells (NECs, arrows) are shown in green and were present in gill filaments. 5-HT-IR NECs of the gill arch (ga) were also present. Scale bar, 10 µm. (B) Image in A shown with zn-12-IR. A zn-12-IR nerve fibre (arrowhead) is seen emanating from a nerve bundle (nb) of the gill arch in the direction of developing filaments but does not appear to make contact with filament NECs. In addition, 5-HT-IR NECs of the gill arch are shown associated with zn-12-IR nerve fibres. A zn-12-IR neuron (n) is also visible. (C) A developing gill filament (f) of another 5 d.p.f. larva contained a single 5-HT-IR NEC (green). Scale bar, 10 µm. (D) Image in C shown with zn-12-IR. Unlike the NECs depicted in A and B, this NEC was intimately associated with zn-12-IR nerve fibres (arrowheads) that arose from a nerve bundle (nb) of the gill arch (ga).

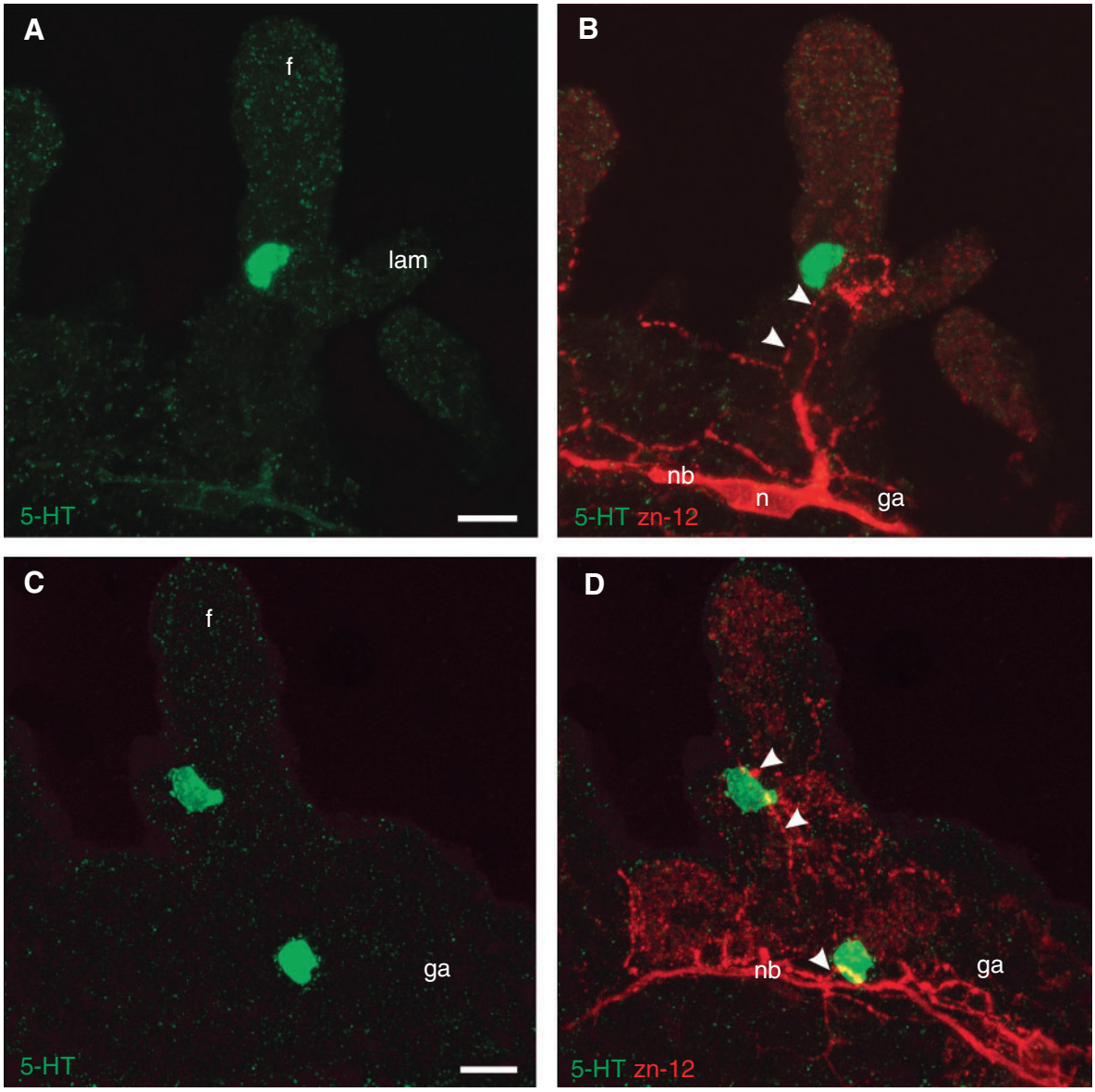

Fig. 5. Innervation of neuroepithelial cells in developing gill filaments of 7 and 9 d.p.f. larvae. (A) Serotonin (5-HT)-immunoreactivity (IR) of an isolated gill from a larva at 7 d.p.f. At this stage, filaments (f) were longer and primordium of respiratory lamellae (lam) were observed. A single 5-HT-IR neuroepithelial cell (NEC, green) resided within the developing filament. Scale bar, 10 µm. (B) Image in A shown with zn-12-IR indicates that the NEC was associated with zn-12-IR nerve fibres (arrowheads) that arose from a nerve bundle (nb) of the gill arch (ga). A zn-12-IR neuron (n) is also visible. (C) 5-HT-IR of an isolated gill from a larva at 9 d.p.f. A 5-HT-IR NEC (green) of the filament (f) and a 5-HT-IR NEC of the gill arch (ga) were present. Scale bar, 10 µm. (D) Image in C shown with zn-12-IR reveals that both of these cells were associated with zn-12-IR (red) nerve fibres (arrowheads) arising from a nerve bundle (nb) of the gill arch (ga).

of filament NECs in developing zebrafish. Before this time, the response to hypoxia is quinidine-insensitive and appears to be independent of NECs of the gill filaments. The above results are summarized in Fig. 9.

Discussion

Appearance and innervation of gill NECs in developing zebrafish

The present confocal immunofluorescence studies, using the expression of serotonin (5-HT) and a zebrafish-derived neuronal marker to identify NECs and nerve fibres of the gill (Dunel-Erb et al., 1982; Jonz and Nurse, 2003) at various developmental stages, established that significant growth of the NEC-nerve fibre system in the gills takes place during early larval stages in zebrafish. These changes occur at a time when zebrafish larvae rely entirely on cutaneous gas exchange for O_2 uptake, and before branchial respiration begins at 14 d.p.f. (Rombough, 2002; see also Fig. 9).

The first 5-HT-immunoreactive (IR) cells to appear during

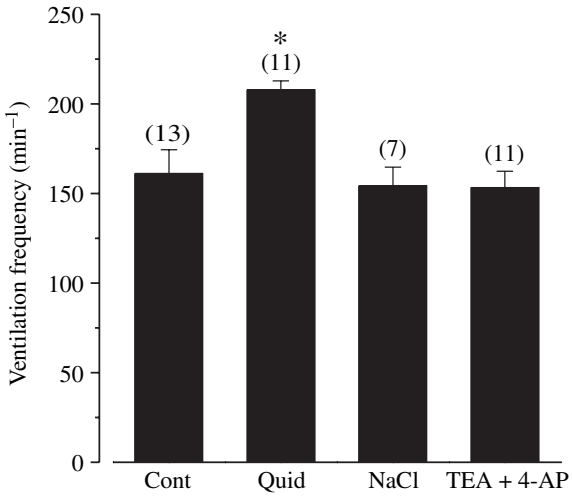

Fig. 6. Effects of K^+ channel blockers on ventilation frequency in adult zebrafish. Adults were immersed in control system water (Cont), 1 mmol l^{-1} quinidine (Quid), 25 mmol l^{-1} NaCl, or 20 mmol l^{-1} TEA + 5 mmol l^{-1} 4-AP. Only quinidine induced a significant increase (asterisk) in ventilation frequency (mean ± S.E.M.; $P<0.005$; ANOVA–Bonferroni test). Sample sizes are indicated in the figure.

development were those of the pharyngeal or gill arches, which were present on or before 3 d.p.f., and appeared to receive innervation from a bundle of zn-12-IR nerve fibres. These cells displayed morphological features characteristic of NECs, such as their epithelial location, storage of a neurotransmitter (i.e. 5-HT) and innervation. However, their location in the gill arches instead of the gill filaments, and their lack of innervation from neurons intrinsic to the filaments in adults, preclude their designation as O_2-sensitive NECs (Jonz and Nurse, 2003; Jonz et al., 2004) at this point. In addition, NECs of the gill arches were distinct from 5-HT-IR Merkel-like cells of the gill rakers that associate with taste receptor cells (M. G. Jonz and C. A. Nurse, unpublished observations; see also Zaccone et al., 1994; Hansen et al., 2002). However, since NECs of the gill arches are innervated and persist in adults, it is plausible that they may play an important physiological role in the gill, perhaps similar to O_2-sensitive NECs of the filaments. Alternatively, gill arch NECs may well be progenitors or precursors of O_2-sensitive NECs of the filaments.

While gill filament primordia were observed as early as 3 d.p.f. (see also Kimmel et al., 1995), NECs were not observed in these structures until 5 d.p.f. By this time, filament NECs resembled the adult morphology and were close to the external medium and arterial blood supply, as indicated by the presence of circulating red blood cells in the filaments. In addition, at 5 d.p.f. some NECs of the filaments appeared to receive innervation from zn-12-IR nerve fibres emanating from the branchial nerve of the gill arch, the major supply of sensory innervation to the gill filaments from the glossopharyngeal and vagus nerves (Nilsson, 1984; Sundin and Nilsson, 2002). The present findings confirm a previous report that the cranial nerves innervating the gill reach the gill arches by 3 d.p.f. (Higashijima et al., 2000). NEC innervation in the filaments was more common in 7 and 9 d.p.f. larvae and, although not investigated in this study, probably continued to increase with the number of NECs throughout development. In adult zebrafish, many zn-12-IR nerve fibres of the branchial nerve originate from a source extrinsic to the gills and course distally through the filaments *via* a nerve bundle and plexus, where they innervate NECs of both the filaments and respiratory lamellae (Jonz and Nurse, 2003). While we did not observe formation of the nerve plexus in this study, this source of innervation appeared to resemble that of the extrinsic nerve supply in adults. In addition, zn-12-IR neurons that were found in the gill arches at all larval stages examined may also contribute to the innervation of

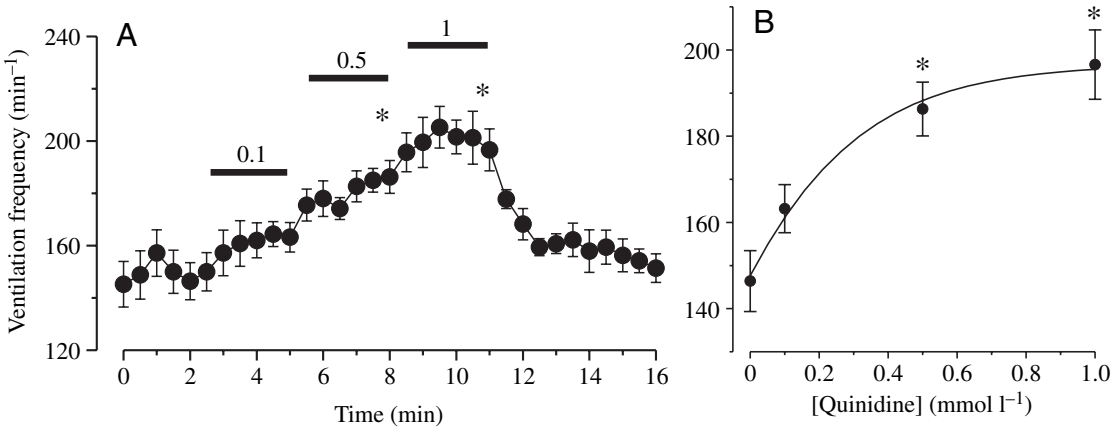

Fig. 7. Effects of quinidine on ventilation frequency in adult zebrafish. (A) Zebrafish were exposed to 0.1 mmol l^{-1}, 0.5 mmol l^{-1} and 1 mmol l^{-1} quinidine in a continuously perfused chamber. Bars represent the duration of quinidine application at each concentration. While 0.1 mmol l^{-1} quinidine had no effect, the addition of 0.5 mmol l^{-1} and 1 mmol l^{-1} significantly increased (asterisks) ventilation frequency (min^{-1}; mean ± S.E.M.) compared to the control (N=6; $P<0.05$; ANOVA–Bonferroni test). This effect was fully reversible. (B) Dose–response curve of data in A. Data points from 2, 5, 8 and 11 min are plotted. Asterisks indicate a significant increase from control (0 mmol l^{-1}). N=6; $P<0.05$; ANOVA–Bonferroni test.

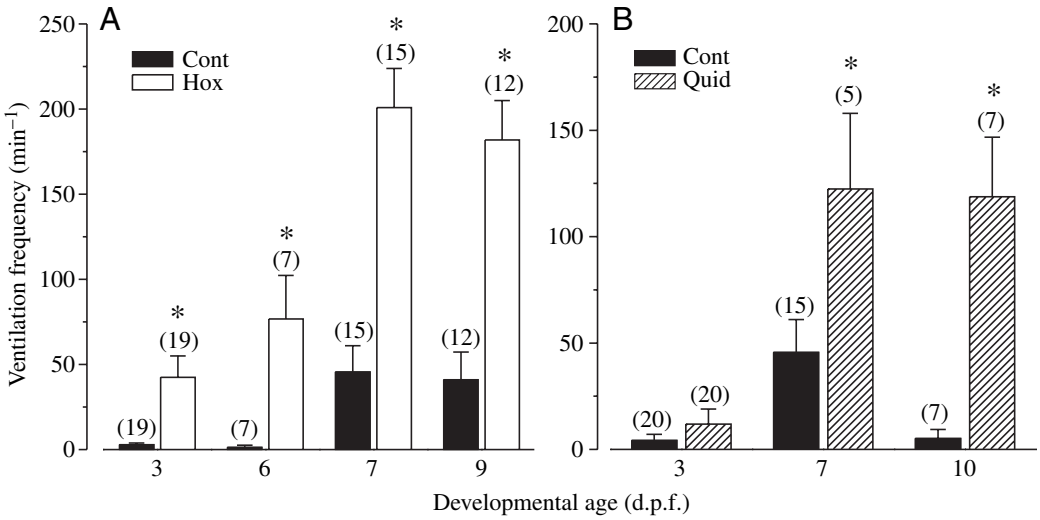

Fig. 8. Effects of hypoxia and quinidine on ventilation frequency in developing zebrafish. (A) Larvae 3–9 d.p.f. were subjected to control solution (Cont; P_{O_2}=150 mmHg; filled bars) or hypoxia (Hox; P_{O_2}=25 mmHg; open bars) in a continuously perfused chamber. Larvae responded to the hypoxic challenge with an increase in ventilation frequency (min^{-1}; mean ± S.E.M.) as early as 3 d.p.f. This response increased to a maximum at ~7 d.p.f. Asterisks denote a significant increase in ventilation frequency compared to control at each developmental stage (P<0.05; Student's t-test). In addition, the ventilatory response to hypoxia was significantly greater at 7 d.p.f. compared to the response at previous stages (P<0.001; ANOVA–Bonferroni test). Sample sizes are indicated in the figure. (B) Larvae 3–10 d.p.f. were exposed to control solution (Cont; filled bars) or 1 mmol l^{-1} quinidine (Quid; hatched bars) in the same perfusion system as in A. Ventilation frequency (mean ± S.E.M.) in 3 d.p.f. larvae was not significantly affected by the application of quinidine, but was quinidine-sensitive at 7 and 10 d.p.f. Asterisks indicate a significant difference from control at each developmental stage (P<0.05; ANOVA–Bonferroni test). Sample sizes are indicated in the figure.

NECs of the filaments. In adults, such neurons intrinsic to the gills are located within the filaments, where they innervate filament NECs and extend processes that terminate at a proximal region of the efferent filament artery (Jonz and Nurse, 2003). It is possible that the neurons of the gill arch in larvae may be pioneering neurons that migrate into the filaments and innervate these structures later in development. Therefore, filament NEC morphology and innervation patterns observed in larvae at approximately 7 d.p.f. are reminiscent of those observed in adults, and may represent afferent sensory pathways involved in O_2 sensing in the gill (see Jonz and Nurse, 2003).

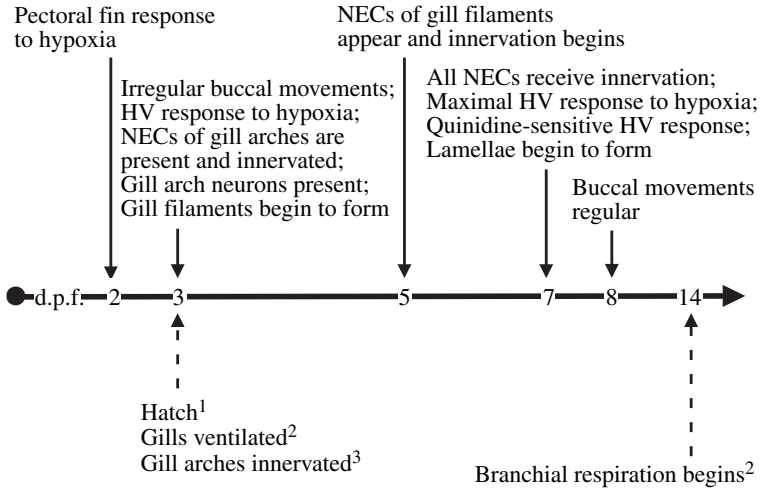

Fig. 9. Chronology of the development of ventilatory events and structures in zebrafish. Solid arrows correspond to results obtained from the present study; dotted arrows indicate the work of previous authors. [1]Kimmel et al., 1995; [2]Rombough, 2002; [3]Higashijima et al., 2000. 5-HT, serotonin; d.p.f., days postfertilization; IR, immunoreactive; NECs, neuroepithelial cells; HV, hyperventilatory

Development of a hyperventilatory response to hypoxia and quinidine

A behavioural response to hypoxia developed relatively early in zebrafish and presented itself as an increase in frequency of pectoral fin and whole-body movements in 2 d.p.f. embryos. Such a response has previously been observed in embryonic and larval fish and may facilitate gas exchange across the skin before the gills develop (for a review, see Rombough, 1988). Zebrafish larvae can rely completely on cutaneous respiration until at least 7 d.p.f. (Rombough, 2002). In addition, the present study also demonstrates the coordinated activity of gill ventilation with pectoral fin and body movements in larvae. Therefore, these data suggest that the latter behaviours in embryos and larvae may indeed improve cutaneous gas exchange and can be used to identify a response to hypoxia before the hyperventilatory response develops. At 3 d.p.f., a ventilatory response to hypoxia developed. Since NECs of the gill filaments were not observed at these stages, this O_2-sensitive response must have originated from elsewhere.

Although the primary O_2-sensitive chemoreceptors involved in the hypoxia response in fish are located within the gills, several studies have suggested the existence of other populations of extrabranchial O_2 chemoreceptors (Burleson et al., 1992; Milsom et al., 2002; Burleson and Milsom, 2003). Given that convective O_2 transport is not needed in zebrafish larvae until ~14 d.p.f. (Jacob et al., 2002), approximately the time that the gills are needed for respiration (Rombough, 2002), the early response to hypoxia observed in the present work is not likely to be the result of stimulation of central chemoreceptors. Such a central O_2-sensing mechanism would require a functional system of O_2 transport to detect blood hypoxia. In addition, there is currently no convincing evidence to indicate a role for central O_2 chemoreceptors in respiratory regulation. Interestingly, Milsom et al. (2002) reported that only sectioning of the trigeminal and facial nerves, in addition to glossopharyngeal and vagus denervation, completely abolished the ventilatory response to hypoxia in the tambaqui, and suggested that an additional group of O_2-sensitive chemoreceptors may be present in the orobranchial cavity. Since a sensory component of the facial nerve also innervates the gill arches in fish (Nilsson, 1984; Sundin and Nilsson, 2002), our results may further suggest that NECs of the gill arches (which also face the orobranchial cavity) may contribute to O_2 chemoreception and detect changes in O_2 tension in embryos and larvae before O_2-sensing pathways in the gill filaments develop. In mammals, for example, neuroepithelial bodies (NEBs) of the lung and adrenal chromaffin cells are O_2-sensitive during late fetal and neonatal stages, and may play a significant role in the transition to postnatal life and adaptation to hypoxia during development (Youngson et al., 1993; Thompson et al., 1997; Cutz and Jackson, 1999). Likewise, development of the hyperventilatory response to hypoxia before complete formation of the gills in zebrafish may act to ensure that O_2-sensing pathways are functional by the time the larvae become completely dependent on branchial respiration. Moreover, during mammalian development there is an increase in the number of sensory nerve fibres innervating type I cells of the carotid body, the primary O_2-chemosensory organ in adults, and this coincides with an increase in type I cell and carotid body sensitivity to hypoxia (for reviews, see González et al., 1994; Donnelly, 2000). Similarly, we observed that after the first appearance of NECs in the gill filaments in 5 d.p.f. zebrafish larvae, their innervation was not consistently observed until 7 d.p.f. This increase in innervation of NECs of the gill filaments corresponded to an increase in basal ventilatory frequency and a rise in sensitivity of the ventilatory response to hypoxia that reached a maximum at 7 d.p.f. These changes may have been due to an increase in input to the central nervous system from activation of more peripheral chemoreceptive pathways.

We further showed that filament NECs are indeed functional at 7 d.p.f., and that an increase in innervation could account for the rise in the response to hypoxia, by using the background K^+ channel blocker, quinidine, to inhibit O_2-sensitive ion channels of gill NECs *in vivo*. Quinidine has previously been shown to produce the same effects as hypoxia, such as background K^+ channel inhibition and membrane depolarization, in several O_2-sensitive cells (O'Kelly et al., 1999; Buckler et al., 2000; Campanucci et al., 2003), including NECs of the zebrafish gill filaments (Jonz et al., 2004). The present experiments performed on adult zebrafish established that whole-animal application of quinidine elicited a hyperventilatory response in a dose-dependent manner. Although the specificity of the effects of quinidine are difficult to determine when applied in this manner, the present data lead us to propose that quinidine, like hypoxia, can stimulate NECs of the gill filaments by inhibiting the O_2-sensitive background K^+ current when exogenously applied, leading to activation of sensory pathways. This may have occurred *via* direct stimulation of NECs exposed to quinidine in the external water, or secondarily, following uptake across the gills. NECs reside within a permeable epithelium, where they are exposed to water and the arterial blood supply, and may be capable of responding to changes in both environments. Furthermore, although exogenous application of quinidine and the voltage-dependent K^+ channel blockers, tetraethylammonium (TEA) and 4-aminopyridine (4-AP), clearly had other behavioural effects on unanaesthetized adult zebrafish, only quinidine produced the additional response of hyperventilation. In parallel with these findings, quinidine, but not TEA or 4-AP, mimicked and occluded the hypoxic response in isolated O_2-sensitive NECs (Jonz et al., 2004). In developing zebrafish, application of quinidine did not induce hyperventilation in 3 d.p.f. larvae, indicating the absence of a non-specific quinidine response when quinidine-sensitive NECs were not present, but induced hyperventilation in 7 and 10 d.p.f. larvae once innervated NECs had appeard. Moreover, because the hyperventilatory response of larvae to quinidine plus hypoxia did not differ from the response to quinidine or hypoxia alone, quinidine appeared to function through the same pathway as hypoxia to modulate ventilation.

Thus, the present results, showing the development of a ventilatory response sensitive to both hypoxia and quinidine, corroborate our findings of the later appearance and innervation of O_2-sensitive NECs of the gill filaments, and suggest that this innervation is sensory and is required to produce the ventilatory response to hypoxia. Interestingly, the behavioural response of pectoral fin and buccal movements remained coordinated during the stages examined, but did not become regular in frequency until 8 d.p.f. This may relate more to central rather than peripheral pathways that appear to develop later still in zebrafish larvae (Turesson et al., 2003). Results from this study may also suggest that since significant development of the ventilatory response to hypoxia in zebrafish takes place in the absence of NECs of the respiratory lamellae, which are similar to NECs of the filaments in both their morphology and innervation (Jonz and Nurse, 2003), lamellar NECs may not play a major role in O_2 sensing, or are more important during later developmental stages once the lamellae have completely formed.

The present study describes the ontogenesis of peripheral O_2

chemoreception in the gills of zebrafish and the hyperventilatory response to hypoxia, and is the first account of the correlation between such developmental events and identified functional O_2-chemoreceptors in an aquatic vertebrate. We show that the development of an elevated response to hypoxia, and a quinidine-sensitive ventilatory response, occurred within the first week following fertilization and may be attributable to the appearance and innervation of O_2-sensitive neuroepithelial cell (NECs) of the gill filaments. Before the appearance of filament NECs, zebrafish responded to hypoxia *via* another O_2-sensing pathway, suggesting that shifts in O_2-sensing sites occur with development in fish, as they do in mammals. Moreover, it appears that these developmental changes in functional O_2-sensing pathways are not unique to air-breathing mammals, but may have appeared earlier in vertebrate evolution.

The results from this study form a foundation for future investigations in O_2 chemoreception involving the use of mutagenesis and large-scale genetic screens. Since larvae can survive without the need for branchial respiration for many days, mutations affecting the gill are not expected to be lethal during this time. Behavioural assays designed to test the ventilatory responses of mutagenized zebrafish larvae to hypoxia and quinidine may facilitate the identification and characterization of mutations that affect the function of O_2 sensing in NECs. Such advances may lead to a greater understanding of O_2 chemoreception at the cellular level.

List of abbreviations

4-AP	4-aminopyridine
5-HT	5-hydroxytryptamine (serotonin)
ANOVA	analysis of variance
d.p.f.	days postfertilization
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
IR	immunoreactive
MS 222	ethyl 3-aminobenzoate methanesulfonate
NEB	neuroepithelial body
NEC	neuroepithelial cell
PBS	phosphate-buffered solution
PBS-TX	phosphate-buffered solution plus Triton X-100
P_{O_2}	partial pressure of O_2
TEA	tetraethylammonium
zn-12	zebrafish neuron-specific antibody

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