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RESEARCH ARTICLE

Serotonergic neuroepithelial cells of the skin in developing zebrafish: morphology, innervation and oxygen-sensitive properties

Maria L. Coccimiglio and Michael G. Jonz*

Department of Biology, University of Ottawa, Ottawa, ON, K1N 6N5, Canada

*Author for correspondence (mjonz@uottawa.ca)

SUMMARY

In teleost fish, O_2 chemoreceptors of the gills (neuroepithelial cells or NECs) initiate cardiorespiratory reflexes during hypoxia. In developing zebrafish, hyperventilatory and behavioural responses to hypoxia are observed before development of gill NECs, indicating that extrabranchial chemoreceptors mediate these responses in embryos. We have characterised a population of cells of the skin in developing zebrafish that resemble O_2-chemoreceptive gill NECs. Skin NECs were identified by serotonin immunolabelling and were distributed over the entire skin surface. These cells contained synaptic vesicles and were associated with nerve fibres. Skin NECs were first evident in embryos 24–26 h post-fertilisation (h.p.f.), and embryos developed a behavioural response to hypoxia between 24 and 48 h.p.f. The total number of NECs declined with age from approximately 300 cells per larva at 3 days post-fertilisation (d.p.f.) to ~120 cells at 7 d.p.f., and were rarely observed in adults. Acclimation to hypoxia (30 mmHg) or hyperoxia (300 mmHg) resulted in delayed or accelerated development, respectively, of peak resting ventilatory frequency and produced changes in the ventilatory response to hypoxia. In hypoxia-acclimated larvae, the temporal pattern of skin NECs was altered such that the number of cells did not decrease with age. By contrast, hyperoxia produced a more rapid decline in NEC number. The neurotoxin 6-hydroxydopamine degraded catecholaminergic nerve terminals that made contact with skin NECs and eliminated the hyperventilatory response to hypoxia. These results indicate that skin NECs are sensitive to changes in O_2 and suggest that they may play a role in initiating responses to hypoxia in developing zebrafish.

Key words: chemoreceptor, hypoxia, development, O_2, gill, hyperoxia.

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INTRODUCTION

The ability to sense and respond to low levels of oxygen (O_2), or hypoxia, is vital for the development and survival of many organisms, including vertebrates, so as to maintain internal O_2 levels within a normal physiological range to protect cells and tissues. Respiratory chemoreceptors are specialized cells that detect changes in the partial pressure of environmental or arterial O_2 and CO_2 (P_{O_2} and P_{CO_2}) and initiate compensatory changes in ventilation and heart rate (Milsom and Burleson, 2007; López-Barneo et al., 2008; Perry et al., 2009). Respiratory chemoreceptors are well described in mammals (López-Barneo et al., 2008; Nurse et al., 2009; Domnik and Cutz, 2011). In teleost fish, O_2- and CO_2-sensitive neuroepithelial cells (NECs) of the gills are homologues of mammalian chemoreceptors. Zebrafish NECs isolated *in vitro* respond to acute hypoxia and high P_{CO_2} *via* ion channel inhibition and membrane depolarisation (Jonz et al., 2004; Qin et al., 2010). Gill NECs retain synaptic vesicles containing neurotransmitters, such as serotonin, and when stimulated these cells are believed to release their contents across a chemical synapse onto afferent nerve terminals (Dunel-Erb et al., 1982; Jonz and Nurse, 2003; Perry et al., 2009). Chemoreceptor responses to hypoxia are then carried from the gill arches to the central nervous system *via* afferent fibres of the glossopharyngeal and vagus nerves (Milsom and Brill, 1986; Burleson and Milsom, 1993; Sundin and Nilsson, 2002). Within the gill filaments, NECs receive neural innervation from multiple sources. These generally include catecholaminergic nerve endings, with ultrastructural features suggestive of afferent nerve fibres, and serotonergic neurons intrinsic to the gill arches (Dunel-Erb et al., 1982; Bailly et al., 1992; Jonz and Nurse, 2003; Bailly, 2009).

The zebrafish presents an interesting model with which to study the development of O_2 sensing in vertebrates. Zebrafish transition from anoxia-tolerant embryos to hypoxia-sensitive larvae within the first 2 to 3 days of life (Padilla and Roth, 2001; Mendelsohn et al., 2008). By 3 days post-fertilisation (d.p.f.), gill development begins (Kimmel et al., 1995), but functional, innervated gill NECs are not observed until 7 d.p.f. (Jonz and Nurse, 2005), and so these cells cannot contribute to O_2-sensing responses before this time. Nevertheless, the hyperventilatory response to acute hypoxia begins at 3 d.p.f. and increases dramatically before 7 d.p.f. (Jonz and Nurse, 2005). The evidence, therefore, indicates that formation of O_2-chemosensory pathways in zebrafish occurs during the first 7 days of development, and that there must be an extrabranchial population of O_2 chemoreceptors (i.e. located outside of the gills) that mediates behavioural and hyperventilatory responses to hypoxia before the development of gill chemoreceptors. Extrabranchial receptors of the oropharyngeal cavity have been implicated in the hypoxic response in adults of the tambaqui (Milsom et al., 2002); however, these putative receptors do not regulate changes in ventilatory frequency.

In developing fish, the skin is an important site of gas exchange (Rombough, 1988), ion regulation (van der Heijden et al., 1999; Varsamos et al., 2002; Pan et al., 2005) and chemical sensing (Hansen et al., 2002; Northcutt, 2005). In developing zebrafish, cutaneous respiration accounts for nearly all gas exchange and does not become limiting until ~10 d.p.f. (Rombough, 2007). The skin

may then be regarded as an optimal site for O_2 sensing in small organisms, such as zebrafish embryos, that do not require a circulatory system to mediate the transfer of respiratory gases between the external environment and tissues. In the pond snail *Helisoma trivolvis*, for example, serotonergic sensory-motor neurons on the surface of embryos detect changes in O_2 and mediate an adaptive behavioural response to hypoxia (Kuang et al., 2002). In a similar manner, developing zebrafish increase whole-body and pectoral fin movements as early as 2 d.p.f. when confronted with hypoxia (Jonz and Nurse, 2005), a behaviour in developing fish that improves the diffusion gradient for respiratory gas exchange across the skin (Rombough, 1988).

Recently, a population of serotonergic NEC-like cells of the skin was reported in zebrafish embryos (Jonz and Nurse, 2006) and adults of the amphibious fish *Kryptolebias marmoratus* (Regan et al., 2011) that morphologically resembled O_2-sensitive NECs of the gills. It was proposed that these skin cells act as extrabranchial O_2 chemoreceptors. However, the O_2-sensitive properties of skin NECs have not yet been examined.

In this study, we reasoned that skin NECs may display morphological characteristics of O_2 chemoreceptors. We describe the distribution, morphology and innervation of skin NECs in developing zebrafish. We show that skin NECs appear transiently during embryonic development and are lost, for the most part, during early larval stages. In addition, we demonstrate that manipulations that modify chemoreceptor morphology, such as acclimation to hypoxia and nerve terminal ablation, produce changes that correlate with aberrations in the development of peak resting ventilatory frequency and the hyperventilatory response to acute hypoxia. We show that skin NECs are sensitive to changes in O_2 and may be involved in the development of O_2 chemoreception in zebrafish.

MATERIALS AND METHODS

Animals

Adult zebrafish [*Danio rerio* (Hamilton 1822)] were obtained from a commercial supplier (Mirdo Importations, Montreal, QC, Canada) and maintained in a closed re-circulating system at 28.5°C on a 14 h:10 h light:dark cycle (Westerfield, 2000). Embryos were obtained using standard zebrafish breeding techniques (Westerfield, 2000) and placed in embryo medium in an incubator equilibrated with room air at 28.5°C. Embryo medium (E3) contained the following: 5 mmol l^{-1} NaCl, 0.17 mmol l^{-1} KCl, 0.33 mmol l^{-1} $CaCl_2$ and 0.33 mmol l^{-1} $MgSO_4$ at pH 7.8. After 2 days, embryos were transferred to 2 litre plastic tanks filled with dechlorinated water maintained at 28.5°C. All procedures were conducted in accordance with the guidelines established by the Canadian Council for Animal Care.

Immunohistochemistry

Embryos and larvae were prepared as whole mounts for immunohistochemistry following previously established procedures (Jonz and Nurse, 2005). Animals were euthanized with 1 mg ml^{-1} tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada). Specimens were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde and kept at 4°C overnight. PBS contained the following: 137 mmol l^{-1} NaCl, 15.2 mmol l^{-1} Na_2HPO_4, 2.7 mmol l^{-1} KCl and 1.5 mmol l^{-1} KH_2PO_4 at pH 7.8 (Bradford et al., 1994; Jonz and Nurse, 2003). Fish were then rinsed in PBS and placed in a permeabilising solution containing 2% Triton X-100 in PBS (PBS-TX) overnight at 4°C. For experiments in which the skin was isolated from adults, 2–3 mm sections of skin were collected from 3-month-old zebrafish and separated from the underlying connective tissue.

Whole zebrafish, or tissue preparations, were then incubated in primary antibodies diluted with PBS-TX (Table 1) for 24 h at 4°C. In double-labelling experiments, primary antibodies were used in combination (see figure legends). Tissue was then rinsed in PBS and incubated for 1 h in secondary antibodies (Table 1) in PBS-TX in a dark chamber. Following another rinse in PBS, tissue was mounted on glass slides in Vectashield (Vector Laboratories, Burlingame, CA, USA).

Details of antibodies used in the present study are summarized in Table 1. Identification of skin NECs was performed by immunolabelling with anti-serotonin. This antibody has been used to characterise the serotonergic system of gill NECs in several teleost species, including zebrafish (Jonz and Nurse, 2003; Saltys et al., 2006). Polyclonal antibodies were raised in rabbit against a serotonin (5-HT) creatinine sulfate complex conjugated with bovine serum albumin (according to the manufacturer's specifications). In addition, antibodies against the synaptic vesicle protein SV2 were used to characterise skin NECs. Anti-SV2 has previously been shown to label sensory or neurosecretory cells of the gill epithelium (Jonz and Nurse, 2003; Saltys et al., 2006). SV2 antibodies were raised against synaptic vesicles from the elasmobranch electric organ and bound to a transmembrane glycoprotein of ~95 kDa on the cytoplasmic side of synaptic vesicles in endocrine and neurosecretory cells [according to Buckley and Kelly (Buckley and Kelly, 1985) and the manufacturer's specifications].

The neural innervation of the skin was identified using an antibody against a zebrafish-derived neuron-specific antigen, zn-12. zn-12 is a general neuronal marker in zebrafish, and its labelling of neural structures in adults and larvae in this species has been previously characterized (Trevarrow et al., 1990; Jonz and Nurse, 2003; Jonz and Nurse, 2005). zn-12 was raised in mouse against membrane fractions from the adult zebrafish central nervous system and recognises a human natural killer-1-like (HNK-1-like) epitope (manufacturer specifications). Western blot analysis has indicated that both zn-12 and HNK-1 antibodies label similar bands ranging in molecular weight from 60 to 248 kDa (see Metcalfe et al., 1990).

Acclimation to hypoxia and hyperoxia

Developing zebrafish were acclimated at 28.5°C to one of four different levels of water P_{O_2}: normoxia or control (150 mmHg), mild hypoxia (80 mmHg), severe hypoxia (30 mmHg) or hyperoxia (300 mmHg). For mild hypoxia acclimation, fresh embryos were placed in an atmosphere-controlled incubator (Forma 3110, ThermoFisher Scientific, Ottawa, ON, Canada) for 2 days. Hypoxia was produced by injection of 100% N_2 and the incubator P_{O_2} was monitored and stabilized by a thermal conductivity O_2 sensor and feedback system. Embryo medium was replaced daily to minimize mortality. When larvae reached 2 d.p.f. they were placed in small beakers containing 200 ml dechlorinated water bubbled with compressed air and N_2 using a Pegas 4000 gas mixer (Columbus Instruments, Columbus, OH, USA). An O_2 meter (YSI model 55, YSI, Yellow Springs, OH, USA) was used to measure O_2 levels.

For more severe hypoxic treatment, embryos were housed in a hypoxic incubator (as above) until 6 h post-fertilisation (h.p.f.) and then placed in dechlorinated water and bubbled with the above combination of gases to achieve the desired hypoxic level. The earlier transfer of embryos exposed to 30 mmHg hypoxia promotes survival (Vulesevic and Perry, 2006). Chronic hyperoxia exposures were performed in the same manner as above, except that water was bubbled with 100% O_2 to achieve the desired P_{O_2} throughout the exposure and verified with an O_2 meter (as above). Control animals were treated in the same manner but exposed to normoxia in both incubator and beakers.

Nerve terminal ablation by 6-hydroxydopamine exposure

The neurotoxin 6-hydroxydopamine (6-OHDA) (Sigma-Aldrich, Oakville, ON, Canada) has been shown to degrade catecholaminergic nerve terminals in mammalian and teleost preparations, including zebrafish (Bailly et al., 1992; Blum et al., 2001; Parng et al., 2007). Zebrafish were exposed to 6-OHDA following a procedure modified from Parng et al. (Parng et al., 2007). Zebrafish at 2 d.p.f. were placed in 250 μmol l^{-1} 6-OHDA in 50 ml dechlorinated water maintained at 28.5°C. For subsequent immunohistochemical experiments, larvae were exposed to 6-OHDA for up to 5 days. For subsequent behavioural assays, larvae were exposed for 1–5 days. Controls were kept under the same conditions but were untreated.

Behavioural assays

Behavioural responses to hypoxia were observed as previously described (Jonz and Nurse, 2005). Briefly, zebrafish between 1 and 9 d.p.f. were placed in a superfusion chamber constructed using a glass-bottomed Petri dish (MatTek, Ashland, MA, USA) with a 14 mm depression well. A piece of nylon mesh stabilized with a moulded ring made from Sylgard (Dow Corning, Midland, MI, USA) was placed inside the dish to confine the zebrafish to the well. During an experiment, the chamber was continuously superfused with dechlorinated water at 4 ml min^{-1} using a gravity-fed system. Solutions were delivered to the chamber using gas-impermeable tubing (Tygon, Saint-Gobain Performance Plastics, Pittsburgh, PA, USA) and removed with a low-noise vacuum pump (ThermoFisher Scientific). Larvae ≥3 d.p.f. were lightly anaesthetized before and during the assay with 0.05 mg ml^{-1} MS-222 to prevent movement. The chamber was placed on the stage of a stereomicroscope (MZ6, Leica, Wetzlar, Germany).

Zebrafish embryos younger than 3 d.p.f. lack gills but exhibit an increase in the frequency of whole-body movements when confronted with hypoxia, whereas older larvae (≥3 d.p.f.) display increased gill ventilation frequency, as indicated by movement of the operculae and buccal pumping (Jonz and Nurse, 2005). We therefore used the frequency (min^{-1}) of whole-body movements and ventilation frequency (f_V) as measures of a behavioural response to acute hypoxia in embryos and larvae, respectively. Acute hypoxia (25 mmHg) was produced by continuously bubbling dechlorinated water in a reservoir bottle with 100% N_2 and measured using an O_2 meter (as described above). Control solution (150 mmHg) was kept in a separate reservoir. Resting (control) frequencies were recorded 3 min after fish were placed in the chamber to allow the fish to recover from transfer. After hypoxic solution had been introduced to the chamber for 3 min, fish were observed for 30 s and the frequency of movement was recorded using a hand-held counter and stopwatch (ThermoFisher Scientific). Values were expressed as the number of gill beats or body movements per minute.

Behavioural responses to acute hypoxia, as described above, were also recorded in fish acclimated to hypoxia and hyperoxia, and in fish pre-treated with 6-OHDA. In the latter case, during the course of the experiments it was discovered that 6-OHDA reduced resting f_V and had an additional inhibitory effect on the hyperventilatory response to acute hypoxia. We therefore used sodium cyanide (NaCN; ThermoSigma-Aldrich) to verify that the ability of larvae to ventilate was not compromised by 6-OHDA exposure *via* an uncharacterized or non-specific effect of the drug. NaCN concentrations of up to 200 μg ml^{-1} have previously been used to stimulate ventilation in zebrafish (Vulesevic et al., 2006). In our experiments, untreated and 6-OHDA treated fish were raised until 5 d.p.f. Larvae from each group were exposed to a 1 mmol l^{-1} (50 μg ml^{-1}) solution of NaCN dissolved in dechlorinated water for 1 min and transferred to the superfusion chamber. After 3 min, f_V was recorded.

Microscopy and image analysis

Whole-mount preparations processed for immunohistochemistry were viewed using a confocal microscope (Fluoview 200, Olympus, Center Valley, PA, USA) equipped with argon (Ar) and krypton (Kr) lasers with peak outputs of 488 and 568 nm, respectively. Images were captured using Fluoview 2.1 software (Olympus). In some experiments, an epifluorescence microscope was used (Axiophot, Zeiss, Jena, Germany) and images were collected with Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada). Image processing was carried out with ImageJ software (v. 1.42q, National Institutes of Health, Bethesda, MD, USA) and CorelDraw 10 (Corel, Ottawa, ON, Canada).

To identify potential morphological changes in skin NECs during development, and following acclimation to various levels of water P_{O2}, we measured morphometric parameters from micrographs of 5-HT-immunolabelled skin NECs using ImageJ. Cell number was recorded along one side of each zebrafish and this value was multiplied by two to estimate the total number of NECs in the skin at each developmental stage and level of P_{O2}. In addition, the tissue density of skin NECs (number per unit area) was also recorded to account for the increase in size of the specimens with time, and to reduce variability. Density was determined by counting cells in a 200 μm^2 sampling area of tissue for each animal, and this value was converted to no. mm^{-2}.

In experiments where zebrafish were pre-exposed to 6-OHDA, we quantified neural innervation to the skin and NECs. We measured the distance between varicosities (i.e. swellings that represent putative points of contact) along single nerve axons in the skin as an indicator of varicosity density, where a greater distance between varicosities indicated a lower density along the

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

Antibody	Dilution	Antigen	Host	Source	Catalog no.	Secondary
Primary						
5-HT	1:250	Serotonin	Rabbit	Sigma (polyclonal)	S5545	FITC
SV2	1:200	SV2	Mouse	DSHB (monoclonal)	SV2	Alexa 594
zn-12	1:100	Neuron surface	Mouse	DSHB (monoclonal)	zn-12	Alexa 594
Secondary						
Alexa 594	1:100	Mouse	Goat	Invitrogen	A11005	–
FITC	1:50	Rabbit	Goat	Cedar Lane	111-095-003	–

DSHB, Developmental Studies Hybridoma Bank, University of Iowa; FITC, fluorescein isothiocyanate; SV2, synaptic vesicle protein; zn-12, zebrafish-derived neuronal antibody.

axon. Measurements were recorded from zn-12/5-HT immunolabelled micrographs using ImageJ by overlaying a 100 μm^2 sampling grid on the image and measuring the inter-varicosity distance (μm) at pre-defined grid regions to avoid experimenter bias. Values were pooled from multiple individuals. In addition, the number of points of contact of nerve terminals on skin NECs was determined. Points of contact between a NEC and a nerve terminal were identified by co-localization of 5-HT and zn-12 immunoreactivity. The total number of zn-12-immunoreactive nerve terminals on a single NEC was recorded from each zebrafish.

Statistics

Data are presented as means ± s.e.m. for skin NEC number, density and behavioural responses to acute hypoxia. Multiple comparisons within one treatment group were performed using ANOVA followed by the Bonferroni *post hoc* test to compare means. Multiple comparisons between different treatment groups (e.g. normoxia *versus* hypoxia) were performed using a two-way ANOVA followed by the Bonferroni *post hoc* test. Distance between nerve varicosities and points of contact for 6-OHDA treated zebrafish were compared with control values using the Student's *t*-test. All details of statistical analyses, including samples sizes, are presented in the figure legends. Statistical analyses were performed using GraphPad Prism v.5.03 (GraphPad Software, La Jolla, CA, USA).

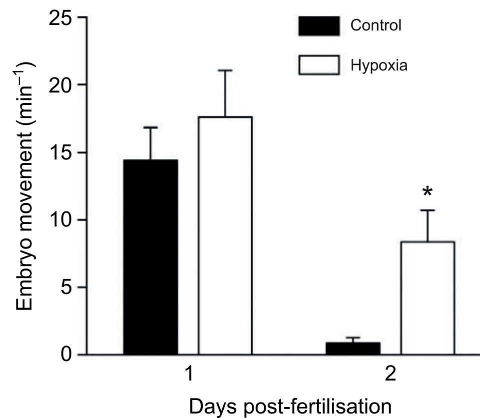

Fig. 1. Development of a behavioural response to acute hypoxia in zebrafish embryos. Measurement of the frequency (min^{-1}) of spontaneous whole-body movements at 1 and 2 days post-fertilisation (d.p.f.). No difference was found at 1 d.p.f., but at 2 d.p.f. embryos exposed to hypoxia (P_{O_2}=25 mmHg) displayed a significantly greater frequency of whole body movements. Asterisk indicates a significant difference from control (P<0.05, two-way ANOVA with Bonferroni *post hoc* test, N=31 for 1 d.p.f. and 33 for 2 d.p.f.).

RESULTS

A behavioural response to hypoxia was present in 2 d.p.f. embryos

We observed the frequency of whole-body movements in embryos to identify early behavioural responses to acute hypoxia (P_{O_2}=25 mmHg) in the absence of gills and gill chemoreceptors. Control embryos displayed spontaneous whole-body movements that decreased in frequency between 1 and 2 d.p.f. (Fig. 1). Zebrafish embryos display stereotyped motor behaviours, which include spontaneous movements that peak in frequency at 19 h.p.f. and then decline; and at 2 d.p.f. swimming contractions become infrequent and occur only in bursts (Drapeau et al., 2002). This may explain the decrease in the frequency of spontaneous movements in our experiments between 1 and 2 d.p.f. At 2 d.p.f., the frequency of spontaneous whole-body movements was 0.9±0.4 min^{-1} in controls and was significantly increased to 8.4±2.5 min^{-1} during application of acute hypoxia. Hypoxia did not induce any significant change at 1 d.p.f.

Resting ventilation frequency and the response to hypoxia were dependent on acclimation P_{O_2}

The response to acute hypoxia at 3 d.p.f. and later was measured as an increase in the frequency of gill ventilation (f_V), as observed by the rate of buccal or opercular movements. Under normal conditions, in which larvae were reared at a P_{O_2} of 150 mmHg, resting f_V increased between 3 and 4 d.p.f. and reached a plateau between 4 and 6 d.p.f., with a mean value of 56±8.4 min^{-1} at 5 d.p.f. (Fig. 2A, arrow). When these same larvae were subjected individually to acute hypoxia, f_V was increased significantly between 4 and 7 d.p.f., with a peak response of 168±12.9 min^{-1} at 5 d.p.f. (Fig. 2B). When larvae were reared under hypoxic (30 mmHg) or hyperoxic (300 mmHg) conditions, development of both resting f_V and the ventilatory response to acute hypoxia was changed. Acclimation to hypoxia produced a delayed, peak resting f_V of 40.0±10.3 min^{-1} at 6 d.p.f. (Fig. 2C, arrow) that was lower than peak resting f_V in unacclimated controls of the same age (Fig. 2A). Surprisingly, hypoxia-acclimated larvae responded to acute hypoxia at 6 d.p.f. with a significant ventilatory depression to 10.5±4.4 min^{-1} (Fig. 2D). By contrast, acclimation to hyperoxia appeared to accelerate development of peak resting f_V, which occurred at 4 d.p.f. (36±8.3 min^{-1}; Fig. 2E, arrow), and was lower than in unacclimated controls. Like chronic hypoxia, acclimation to hyperoxia also induced a significant decrease in f_V (1.3±1.2 min^{-1}) when larvae were confronted with acute hypoxia (Fig. 2F).

Serotonergic NECs were found in the skin of developing zebrafish

In all zebrafish embryos and larvae observed, we identified a population of serotonergic cells of the skin by immunolabelling with a primary antibody directed against 5-HT. These cells were morphologically similar to chemoreceptive NECs of the gills in developing and adult zebrafish (Jonz and Nurse, 2003; Jonz and Nurse, 2005). As shown in the representative confocal images of a 3 d.p.f. larva in Fig. 3, NECs were distributed over the entire surface of the skin, including the head and eyes (Fig. 3A–C), the trunk and yolk sac (Fig. 3D–F) and the tail (Fig. 3G–I). Cells were dispersed throughout the tissue, rather than in clusters, and measured approximately 10 μm in diameter. Skin NECs from all regions were associated with zn-12-immunoreactive nerve fibres (red, Fig. 3). These nerve fibres were organized in a particularly high density network that, in some cases, terminated close to the external environment (Fig. 3H, arrowhead). In regions of skin isolated from adult (3-month-old) zebrafish and labelled as above, 5-HT-positive NECs of the skin were not present along the body but were rarely observed on the tail (Fig. 4).

Higher-magnification imaging revealed that zn-12 nerve fibres displayed varicosities along their length that came in close proximity to skin NECs (Fig. 5A, arrowheads), suggestive of nerve terminals. In addition, NECs were situated in the skin epithelium within 1 or 2 μm of the external environment (Fig. 5B). NECs extended neuron-like process, typical of cells with chemoreceptive properties, for up to 5 μm and these were oriented toward the external environment

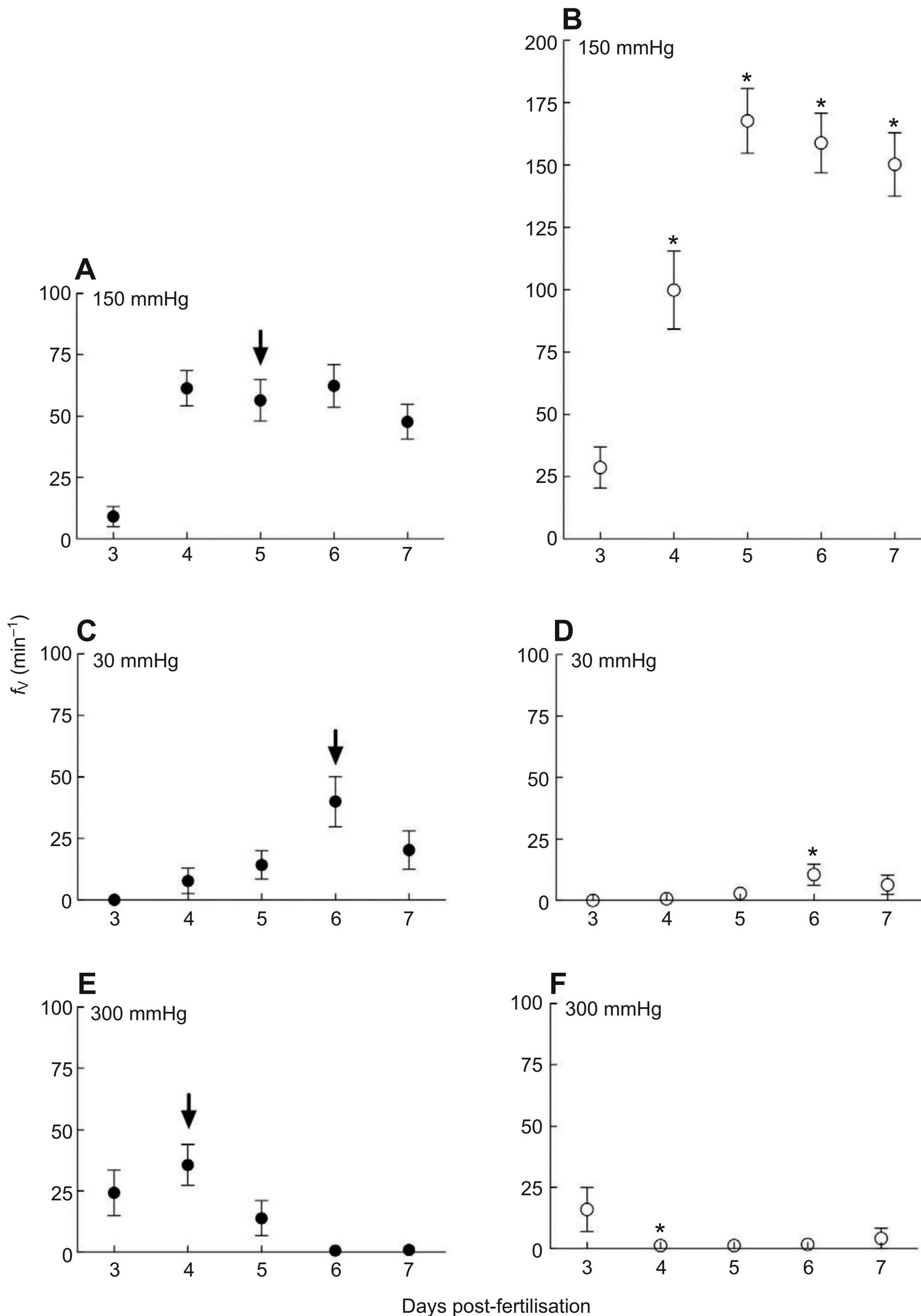

Fig. 2. Effects of acclimation to hypoxia and hyperoxia on ventilatory rate in developing zebrafish. Ventilation frequency (f_V; min^{-1}) is plotted against developmental stage in days post-fertilisation (d.p.f.). Acclimation P_{O_2} (30, 150 or 300 mmHg) is indicated in each panel. (A,C,E) Resting f_V in unstimulated, developing zebrafish. Estimated time of peak resting f_V was dependent on acclimation P_{O_2} and is indicated by arrows. (B,D,F) f_V during exposure to acute hypoxia (25 mmHg). Acclimation to hypoxia and hyperoxia eliminated the hyperventilatory response to acute hypoxia. Asterisks in B, D and F indicate a significant difference from corresponding resting f_V values in A, C and E (P<0.05; two-way ANOVA with Bonferroni *post hoc* test; N=25, 26, 18, 20, 21 for 3–7 d.p.f. in A and B; N=20 for each data point in C–F).

(Fig. 5C). We further demonstrated, using anti-5-HT and anti-SV2 co-labelling, that putative nerve terminals containing the synaptic vesicle protein SV2 surrounded the NEC membrane (Fig. 6, arrowheads). In addition, weak SV2 labelling was found within skin NECs, although intracellular labelling appeared to be variable and was not found in every cell (Fig. 6, arrows).

To determine when skin NECs and their nervous innervation first appeared in developing zebrafish, embryos from 24 to 48 h.p.f. were examined after co-labelling with 5-HT and zn-12 antibodies. These experiments indicated that NECs were not present at 24 h.p.f., although zn-12-immunoreactive nerve fibres had already begun to invade regions where NECs would later be found (Fig. 7A,B). At 26–28 h.p.f., NECs were numerous in the skin and were predominately associated with nerve fibres (Fig. 7C–F). Therefore, these observations indicate that nerve fibres appeared in the skin first and NECs arose between 24 and 26 h.p.f.

The number of serotonergic skin NECs was dependent on acclimation P_{O_2}

Having identified an embryonic response to hypoxia, modulation of this response by acclimation to variable P_{O_2}, and a population of NECs of the skin that resembles O_2 chemoreceptors, we next asked whether skin NECs would display properties typical of O_2 chemoreceptors.

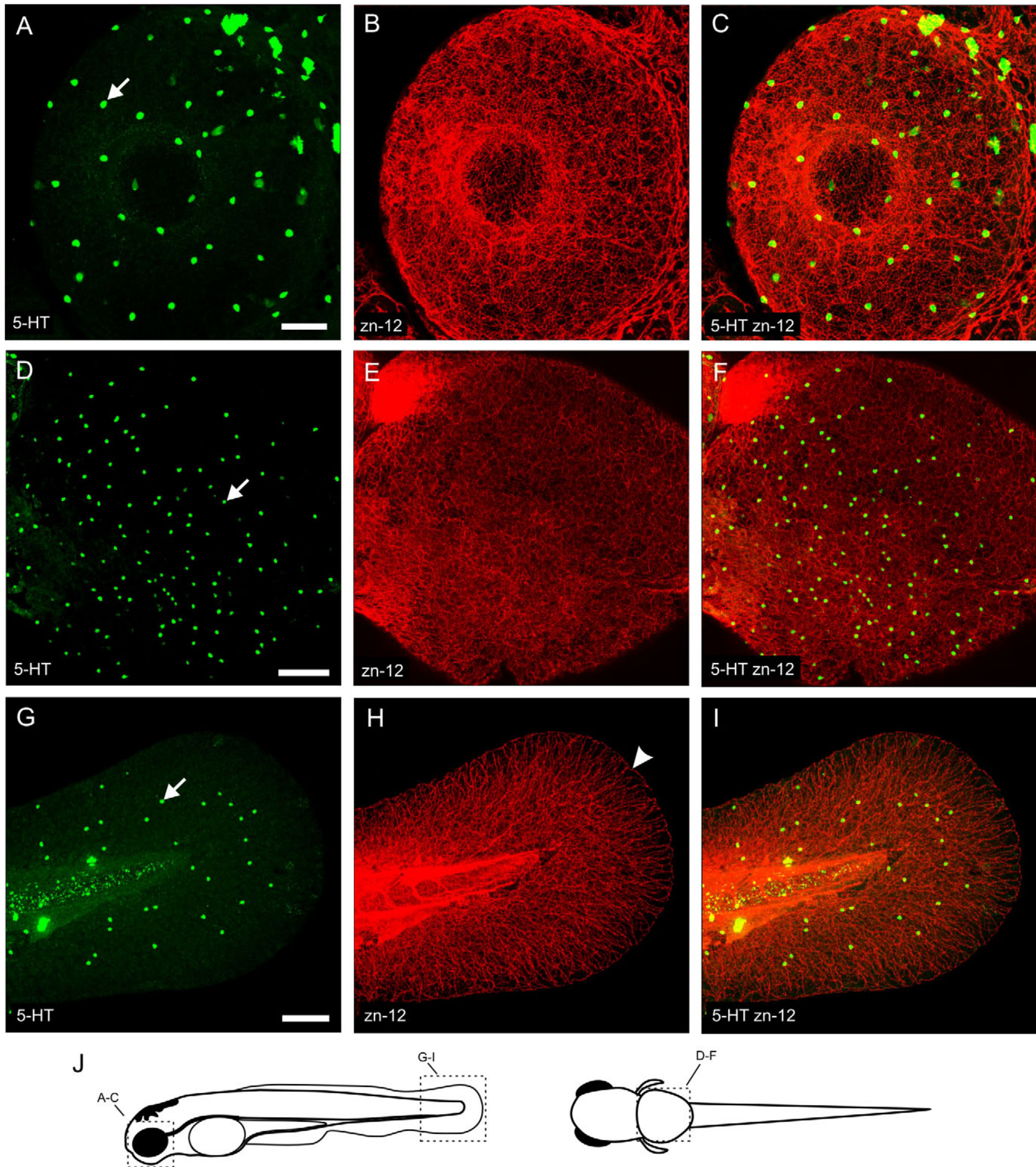

Fig. 3. Confocal imaging of serotonergic skin neuroepithelial cells (NECs) and associated innervation in zebrafish larvae at 3 days post-fertilisation. 5-HT-immunoreactive (IR) NECs (green, arrows) and zn-12-IR nerve fibres (red) were found throughout the skin, including the surface of the eye (A–C), yolk sac (D–F) and tail (G–I). In each case, NECs were intimately associated with a dense network of nerve fibres that, in some cases, terminated close to the external environment (see arrowhead in H). (J) Schematic of larval zebrafish showing corresponding locations of images. Scale bars, 50 μm in A–C; 100 μm in D–F; 100 μm in G–I.

Morphological parameters of skin NECs, such as the number of cells and density per tissue area, were obtained from larvae at different developmental stages acclimated to different levels of water P_{O_2}, including: controls (150 mmHg), mild hypoxia (80 mmHg), severe hypoxia (30 mmHg) and hyperoxia (300 mmHg). Fig. 8A,B illustrates that, under control conditions, both the number of NECs and their density was decreased as development continued. Skin NECs were most numerous at 3 d.p.f., with a mean of 302±21.8 cells per larva,

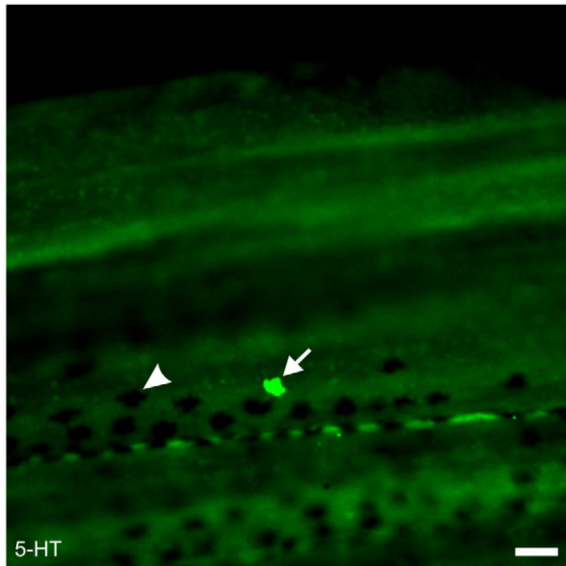

Fig. 4. Immunofluorescence imaging of a serotonergic neuroepithelial cell (NEC) in the skin of adult zebrafish. A 5-HT-immunoreactive NEC (green, arrow) is shown in the skin of the tail of a 3-month-old fish. Arrowhead indicates a pigment cell. Scale bar, 20 μm.

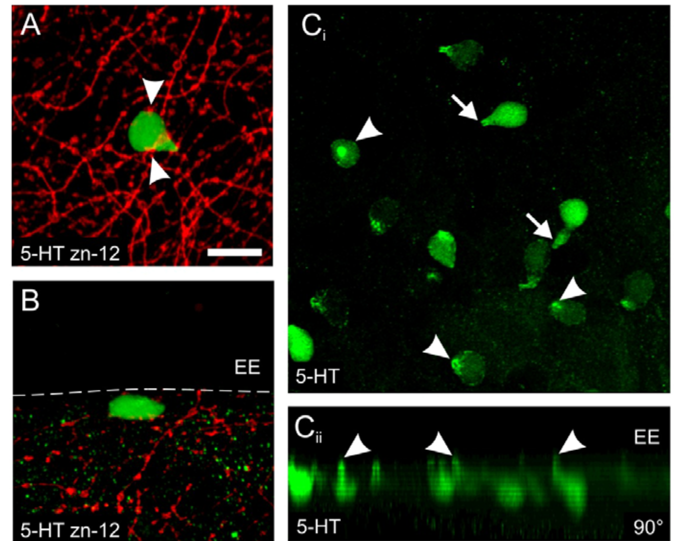

Fig. 5. Skin neuroepithelial cells (NECs) were co-localized with nerve fibre varicosities and extended axon-like processes toward the external environment. Confocal images of whole-mount tissue labelled with anti-5-HT (green) and anti-zn-12 (red). (A) NECs were typically in close proximity to nerve varicosities (arrowheads). (B) NECs were found within close proximity to the epithelial edge (dashed line) and external environment (EE). (C) NECs extended processes measuring up to 5 μm in length. In a field of NECs, arrows indicate processes that extend horizontally, while arrowheads indicate processes that extend to the external environment (i.e. toward the viewer). The confocal image in Ci was tilted back 90 deg in Cii to show that the same processes marked with arrowheads are directed toward the EE. Scale bar, 10 μm (applies to all panels).

and this had significantly declined to 118±12.1 cells per larva by 7 d.p.f. The density of NECs also declined during this same period from 438±32.9 to 262±25.4 cells mm^{-2} (Fig. 8B).

The decline in cell number and density of skin NECs with development was dependent on the P_{O_2} of the water in which zebrafish embryos were acclimated. When zebrafish were acclimated to mild hypoxia, the decline in cell number occurred at a slower rate compared with controls (reaching a level of significance only at 7 d.p.f.) while the density of NECs showed no significant change with development (Fig. 8C). Acclimation to a more severe level of hypoxia appeared to elicit a stabilizing effect on the number and density of skin NECs, with no significant decrease occurring at any of the developmental stages tested (Fig. 8D). In these experiments, between 3 and 9 d.p.f. cell number was maintained between 296±28.6 and 234±19.2 cells per larva, and density was maintained between 621±54.1 and 571±40.0 cells mm^{-2}. It is noteworthy that the density of skin NECs was higher between 3 and 9 d.p.f. following acclimation to severe hypoxia, compared with controls and those acclimated to mild hypoxia (Fig. 8B,C). This difference was likely related to an overall decrease in body size in larvae acclimated to severe hypoxia compared with controls (Table 2). When acclimated to hyperoxia, the number of NECs was markedly lower at 3 d.p.f. (75.9±8.1 cells per larva) and was further reduced at 5 d.p.f., whereas no change in density was evident (Fig. 8E). These data collectively show a significant effect of water P_{O_2} on skin NEC populations during early development, such that the developmental loss of NECs was reduced, or delayed, by acclimation to hypoxia and accelerated by acclimation to hyperoxia.

6-OHDA reduced innervation to skin NECs

It was hypothesized that if skin NECs play role in O_2 sensing and the response to hypoxia, their role will be dependent on neural innervation as it is in the gills of adult zebrafish (Jonz and Nurse, 2005). Gill NECs have been shown to be innervated by at least three different nerve fibre types, including those that are catecholaminergic (Dunel-Erb et al., 1982; Bailly et al., 1992). We therefore used the catecholaminergic nerve toxin 6-OHDA to induce chemical degeneration of nerves of the skin that make contact with serotonergic NECs (Fig. 9). The density of zn-12-positive nerve fibres was reduced in zebrafish treated with 250 μmol l^{-1} 6-OHDA for 5 days (Fig. 9A,B). In addition, we found a greater distance between varicosities along single nerve fibres (an indicator of reduced varicosity density) after 6-OHDA treatment (Fig. 9C,D) and a decreased number of presumed synaptic contacts between zn-12-positive nerve fibres and NECs (Fig. 9E,F). The distance between varicosities along single nerve fibres increased significantly from 2.1±0.2 μm in controls to 2.5±0.2 μm in 6-OHDA-treated zebrafish (Fig. 9G), and the mean number of nerve terminals on NECs decreased from 5.6±0.7 to 4.0±0.4 (Fig. 9H). These data indicate that skin NECs received catecholaminergic innervation that was ablated by 6-OHDA. These experiments also revealed remaining nerve fibres that were not affected by 6-OHDA (Fig. 9B,D,F).

The ventilatory response to acute hypoxia was abolished by 6-OHDA denervation

The consequence of prolonged exposure of 6-OHDA on f_V in developing zebrafish was determined. Behavioural assays indicated two effects of neurotoxin treatment. The first was that 6-OHDA had a variable effect on resting f_V. At some developmental stages, f_V was relatively lower in 6-OHDA-treated larvae (Fig. 10A, closed bars) compared with untreated controls (see Fig. 2A). The second effect was that 6-OHDA abolished the increase in f_V (Fig. 10A, open bars) typically associated with exposure to acute hypoxia (see

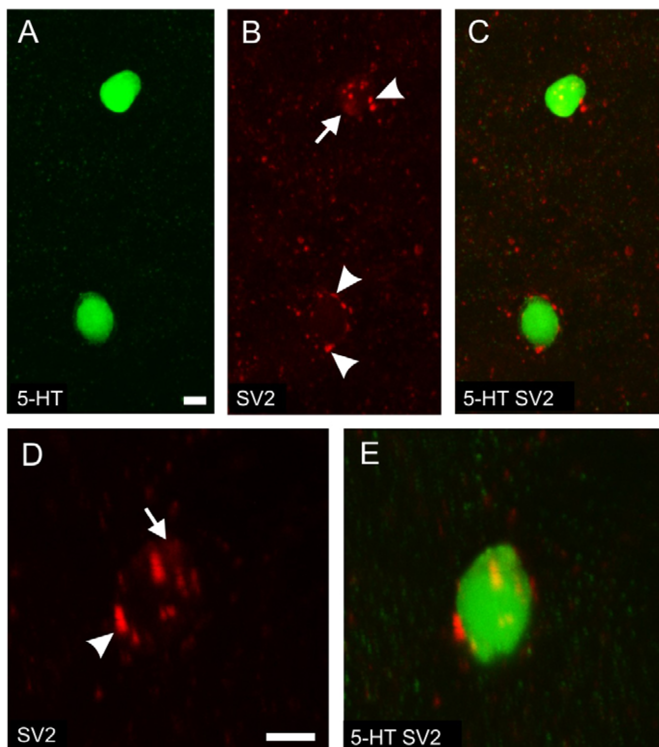

Fig. 6. Localization of a synaptic vesicle protein to skin neuroepithelial cells (NECs) and nerve terminals. Confocal images of whole-mount tissue labelled with anti-5-HT (green) and anti-SV2 (red) in 3 d.p.f. larvae from the head (A–C) and tail (D,E). (A–C) Immunolabelling with anti-SV2 localized vesicle-containing varicosities that appeared to contact skin NECs (arrowheads), but gave variable results for intracellular localization. In the upper cell in B, some intracellular SV2 is visible (arrow) but not in the lower cell. (D,E) Higher magnification of another cell from the tail region indicates weak intracellular labelling of SV2 (arrow) but pronounced labelling of SV2-positive extracellular varicosities (arrowhead). Scale bars, 5 μm (applies to all panels).

Fig. 2B). Because 6-OHDA was applied to whole zebrafish and appeared to itself depress resting f_V, we controlled for the potential non-specific effects of 6-OHDA treatment (such as on the central nervous system or motor ventilatory reflexes). Untreated zebrafish, and those pre-exposed to 6-OHDA, were acutely exposed to NaCN, a potent ventilatory stimulant, to artificially induce hyperventilation. As Fig. 10B demonstrates, both untreated and 6-OHDA-treated zebrafish tested at 5 d.p.f. responded equally well to NaCN in the form of hyperventilation. These results confirm that 6-OHDA treatment did not directly impair the ability of larvae to hyperventilate.

DISCUSSION

The present study has characterized the morphological features of serotonergic NECs of the skin in developing zebrafish, which are most numerous during embryonic and early larval stages, when zebrafish respond to hypoxia but lack O_2-sensitive chemoreceptors of the gills. We have also demonstrated the O_2 sensitivity of skin NECs by showing that the number and tissue density of these cells is dependent on the P_{O_2} of the water in which developing zebrafish were acclimated. Furthermore, we have described changes in the development of behavioural responses to acute hypoxia that are concurrent with changes in the number of skin NECs, and have demonstrated the loss of the acute hypoxic response following chemical denervation.

Morphological characteristics of skin NECs

NECs of the skin in developing zebrafish were identified by their immunoreactivity to antibodies directed against 5-HT. In addition to retention of this neurotransmitter, NECs of the skin also expressed the synaptic vesicle protein SV2, suggesting that these cells were neurosecretory. Moreover, skin NECs were oriented toward the external environment, in some cases extending short neuron-like processes in this direction, and were associated with nerve fibres. These characteristics are consistent with those of serotonergic NECs of the gills (Dunel-Erb et al., 1982; Bailly et al., 1992; Jonz and Nurse, 2003), which are believed to mediate hyperventilation and other responses to hypoxia (Milsom and Burleson, 2007; Perry et al., 2009). The role of 5-HT in skin NECs is not presently clear, but it may evoke afferent nerve activity as it does in the gill (Burleson and Milsom, 1995) and lead to changes in ventilation, or have local vascular effects (Jonz and Nurse, 2003). Although there is no direct evidence in fish for 5-HT release by hypoxia, hypoxic stimulation of O_2-chemosensory carotid body type I cells and pulmonary neuroepithelial bodies (NEBs) induced release of 5-HT (Fu et al., 2002; Nurse, 2010).

Chemoreceptors of the skin in fish, including developing zebrafish, have been described in previous studies. These include taste cells and solitary chemosensory cells (Kotrschal et al., 1997; Hansen et al., 2002). Although these chemosensory cells share similar morphological characteristics with skin NECs, such as proximity to the external environment and apical processes, skin NECs do not appear to be related to these cell types. Skin NECs are evenly distributed throughout the larvae in zebrafish and decrease in number and density with development. By contrast, taste cells and solitary chemosensory cells increase in density in developing zebrafish and have markedly different distributions (Kotrschal et al., 1997; Hansen et al., 2002). In addition, there are no reports that taste or solitary chemosensory cells retain 5-HT.

Nerve fibres labelled by the zn-12 antibody appeared in the skin by 24 h.p.f. and preceded 5-HT-positive NECs. In developing zebrafish, innervation of the skin by sensory axons of cranial nerve fibres coincides with maturation of the basal keratinocyte layer, which precedes many other cell types, and these nerve fibres arborize within the epithelial layers (O'Brien et al., 2012). Pre-exposure of developing zebrafish to the neurotoxin 6-OHDA for up to 5 days chemically degraded nerve fibres in the skin, reduced the density of varicosities along single nerve fibres, and reduced the number of nerve terminals associated with skin NECs. 6-OHDA has been shown to selectively destroy catecholaminergic nerve terminals in fish and mammals (Bailly et al., 1992; Blum et al., 2001; Parng et al., 2007). Our results, therefore, suggest that skin NECs in zebrafish receive catecholaminergic innervation. In a similar manner, chemoreceptive NECs of the gills are innervated by multiple sources, including catecholaminergic nerves with ultrastructural features of sensory nerve terminals (Dunel-Erb et al., 1982; Bailly et al., 1992; Bailly, 2009). Moreover, the rat carotid body receives innervation by catecholaminergic primary sensory neurons of glossopharyngeal and vagal ganglia, including those that display dopaminergic properties and innervate chemosensory type I cells (Katz et al., 1983; Finley et al., 1992).

Some nerve terminals surrounding skin NECs remained in our studies following 6-OHDA treatment. Although some catecholaminergic neurons may not take up 6-OHDA (Blum et al., 2001), this observation suggests that NECs receive innervation

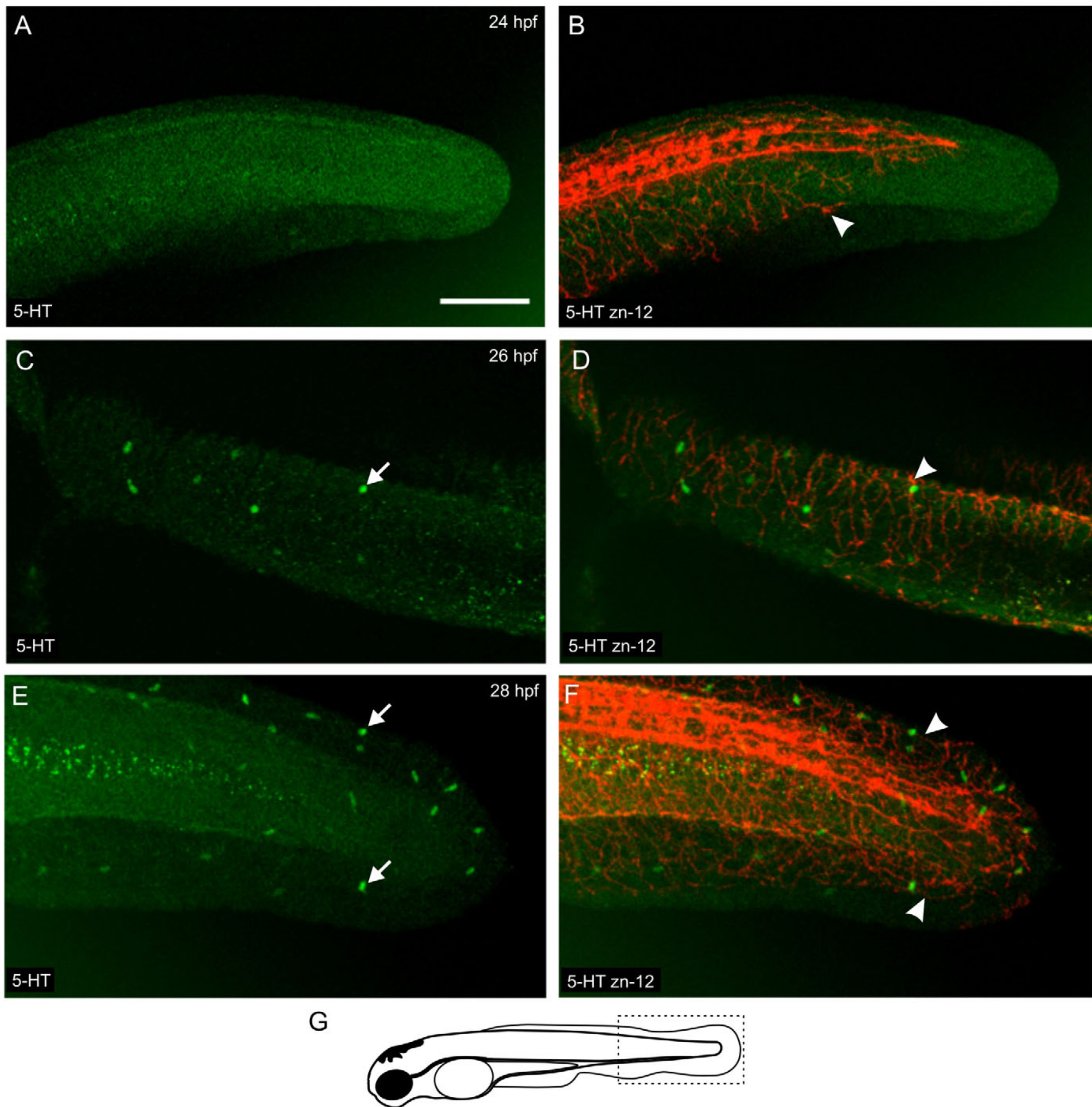

Fig. 7. Development of neuroepithelial cells (NECs) and innervation of the skin in zebrafish embryos. Fluorescence imaging of the tail region of embryos immunolabelled with anti-5-HT (green) and anti-zn-12 (red). (A,B) At 24 h post-fertilisation (h.p.f.), there were no detectable NECs of the skin. However, during this time growing nerve fibres (arrowhead) were observed. (C,D) At 26 h.p.f., several skin NECs were usually observed (arrow) and these were associated with nerve fibres (arrowhead). (E,F) At 28 h.p.f., NECs and their innervation had increased in number. (G) Schematic showing the approximate orientation of embryos shown in all panels. Scale bar, 100 μm (applies to all panels).

from other sources. In teleosts, including zebrafish, gill NECs are also innervated by indolaminergic nerve fibres, and nerve terminals that have ultrastructural features of efferent synapses (Dunel-Erb et al., 1982; Bailly et al., 1992; Jonz and Nurse, 2003; Bailly, 2009). In addition, our results showing localization of SV2 to nerve terminals surrounding skin NECs are also suggestive of efferent innervation onto these cells. The role of efferent innervation in skin or gill NECs is presently unclear, but in the carotid body and pulmonary NEBs in mammals, efferent innervation is important as a source of modulation and inhibitory feedback in chemoreceptor responses to hypoxia (Adriaensen and Scheuermann, 1993; Campanucci and Nurse, 2007). Although characterization of the specific sources of innervation to skin NECs in zebrafish awaits further study, the innervation of the skin in fish may include spinal and cranial (e.g. trigeminal and facial) contributions, which may innervate taste and solitary chemosensory cells (Whitear, 1992; Kotrschal et al., 1993; Reutter and Witt, 1993; O'Brien et al., 2012).

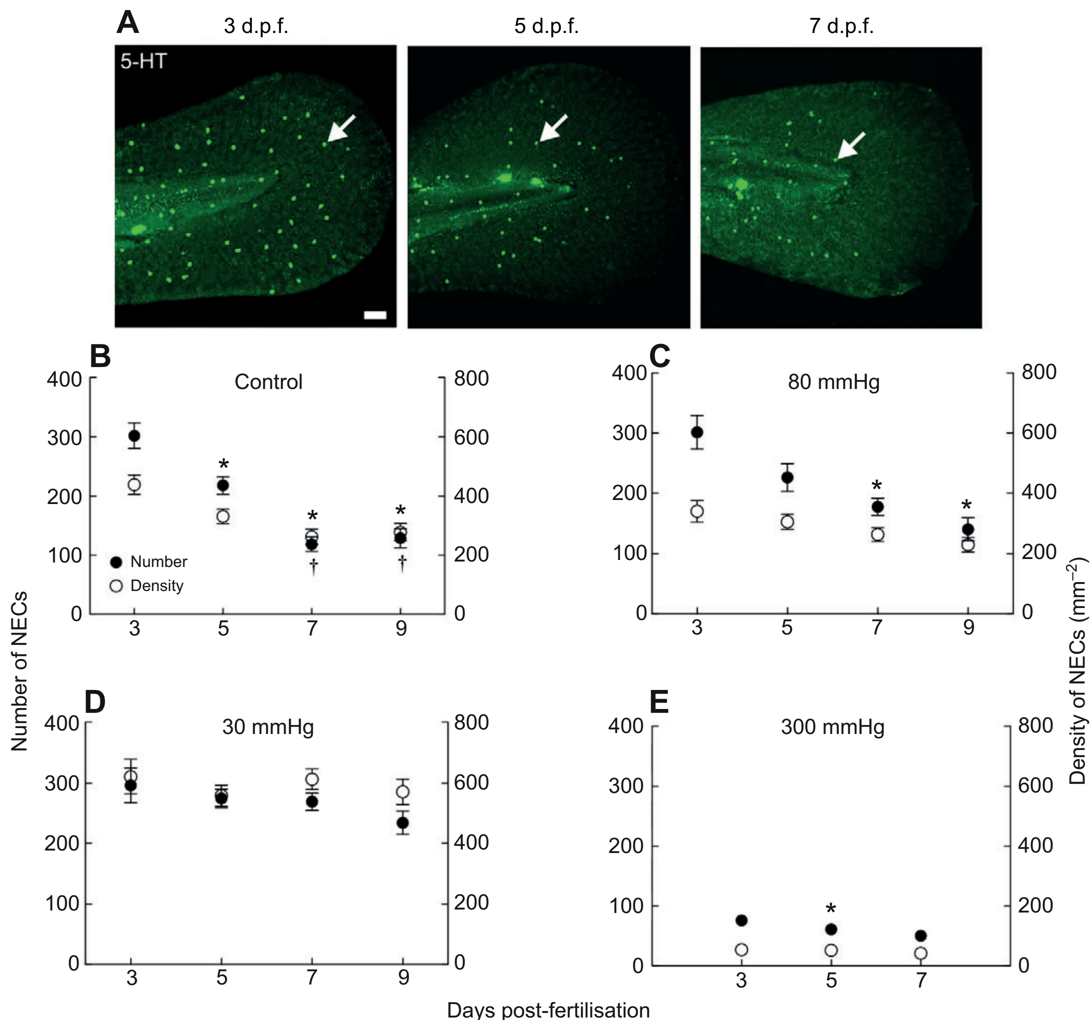

Fig. 8. The number and density (mm^{-2}) of skin neuroepithelial cells (NECs) in zebrafish is dependent on developmental stage and water P_{O_2}. (A) Confocal images of zebrafish larvae ranging from 3 to 7 days post-fertilisation (d.p.f.). Whole larvae were immunolabelled with anti-5-HT (green) but only the tails are shown for clarity. The number of 5-HT-immunoreactive (IR) NECs (arrows) decreased with age. Scale bar, 100 μm. (B) Under control rearing conditions (P_{O_2}=150 mmHg, as in A) the mean ± s.e.m. number (closed circles) and density (open circles) of 5-HT-IR skin NECs significantly decreased with time. For 3, 5, 7 and 9 d.p.f., N=13, 16, 15 and 12, respectively. (C) In zebrafish subjected to chronic exposure of mild hypoxia (P_{O_2}=80 mmHg) there was a significant decrease in NEC number but not tissue density. For 3, 5, 7 and 9 d.p.f., N=9, 12, 12 and 9, respectively. (D) Acclimation to severe hypoxia (P_{O_2}=30 mmHg) prevented any net change in number or density throughout the tested developmental period. N=8 for each data point. (E) Chronic exposure to hyperoxia (P_{O_2}=300 mmHg) led to a more rapid decline in both number and density of skin NECs. For 3, 5 and 7 d.p.f., N=15, 11 and 1, respectively. Asterisks in B, C and E indicate a significant difference in number compared with data points at 3 d.p.f.; daggers in A indicate a significant difference in density compared with 3 d.p.f. Note that error bars are small in E and are not visible. In B, C and E, P<0.01, ANOVA with Bonferroni *post hoc* test.

Our behavioural experiments, in which 6-OHDA was shown to affect f_V, provide a link between the morphological effects of 6-OHDA observed at the tissue level and the potential physiological consequences of skin NEC denervation. In larvae in which nerve terminals associated with skin NECs were degraded by 6-OHDA treatment, the hyperventilatory response to acute hypoxia was eliminated. These data suggest that the hyperventilatory response to hypoxia in developing zebrafish may be dependent upon catecholaminergic innervation to skin NECs.

The application of 6-OHDA alone had a depressive effect on resting f_V. This was a predicted effect of peripheral denervation by 6-OHDA, because in adult teleosts maintenance of normal respiratory rhythm is influenced by input from peripheral receptors (Taylor et al., 1999). Although 6-OHDA does not cross the blood–brain barrier (Blum et al., 2001), we ruled out any potential central or other non-specific effects of the drug on the ability of larvae to hyperventilate by showing that we could chemically stimulate a hyperventilatory reflex with NaCN in 5 d.p.f. larvae after 6-OHDA treatment. These effects did not occur through skin NECs, which were denervated, nor could they have occurred through O_2 chemoreceptors of the gills, which are not fully innervated in zebrafish until 7 d.p.f. (Jonz and Nurse, 2005). There is at least one plausible explanation that may account for the mechanism of NaCN-induced hyperventilation in 6-OHDA-treated larvae. NaCN is a metabolic poison and acts on chemoreceptors as a potent respiratory stimulant in both mammals and teleosts (González et al., 1992; Sun et al., 1992; Vulesevic et al., 2006). However, because there is no direct evidence for central O_2 or CO_2 chemoreceptors in water-breathing fish (Milsom, 2010a; Milsom, 2010b), the action of NaCN on central chemoreceptors is unlikely. In addition to its effects on chemoreceptors, NaCN may have a wide range of non-specific

Table 2. Mean ± s.e.m. length from snout to tail of zebrafish larvae raised under control conditions (150 mmHg) or acclimated to severe hypoxia (30 mmHg)

	Control (150 mmHg)		Hypoxia (30 mmHg)		
Stage (d.p.f.)	Length (mm)	*N*	Length (mm)	*N*	*P*
3	3.4±0.1	11	3.1±0.1	14	*
5	3.8±0.1	14	3.2±0.1	20	*
7	4.0±0.04	15	3.6±0.1	20	*
9	3.9±0.1	16	3.8±0.1	20	n.s.

Samples sizes (*N*) are indicated for each group.
*Significant difference between groups of the same stage (ANOVA followed by Bonferroni *post hoc* test, $P<0.05$).
d.p.f., days post-fertilisation; n.s., not significant.

effects throughout the central nervous system. These include activation of membrane ion channels (Aarts et al., 2003), increased intracellular Ca^{2+} (Dubinsky and Rothman, 1991; Patel et al., 1992) and neurotransmitter release (Sánchez-Prieto and González, 1988; Patel et al., 1991). It is therefore possible that these known central effects of NaCN impinged directly, or indirectly, on the ventilatory network in our experiments, leading to hyperventilation in the absence of an endogenous O_2 chemosensory mechanism.

Development of skin and gill NECs and the hypoxic response in zebrafish

We have shown in the present study that zebrafish become hypoxia-sensitive at 2 d.p.f., as indicated by an increase in the frequency of whole-body movements, and hypoxic hyperventilation was first evident 1 to 2 days later (see also Jonz and Nurse, 2005). Zebrafish embryos are initially tolerant to anoxia before 24 h.p.f. and enter a state of developmental and metabolic arrest. The effects of anoxia, however, become lethal between 48 and 52 h.p.f. (Padilla and Roth, 2001; Mendelsohn et al., 2008). Thus, zebrafish transition from anoxia-tolerant to hypoxia-sensitive embryos between 24 and 48 h.p.f. We have demonstrated that during this period NECs began to appear in the skin after 24 h.p.f. and reached peak number and density by 3 d.p.f. At this point in zebrafish development, gill filament primordia are beginning to form, but neither filament NECs nor respiratory lamellae are present (Jonz and Nurse, 2005). After this time, skin NECs declined markedly until 7 d.p.f. and were rarely observed in adult zebrafish. These trends would seem to suggest that skin NECs are important in developing zebrafish, with potentially limited significance in adults. Furthermore, in the

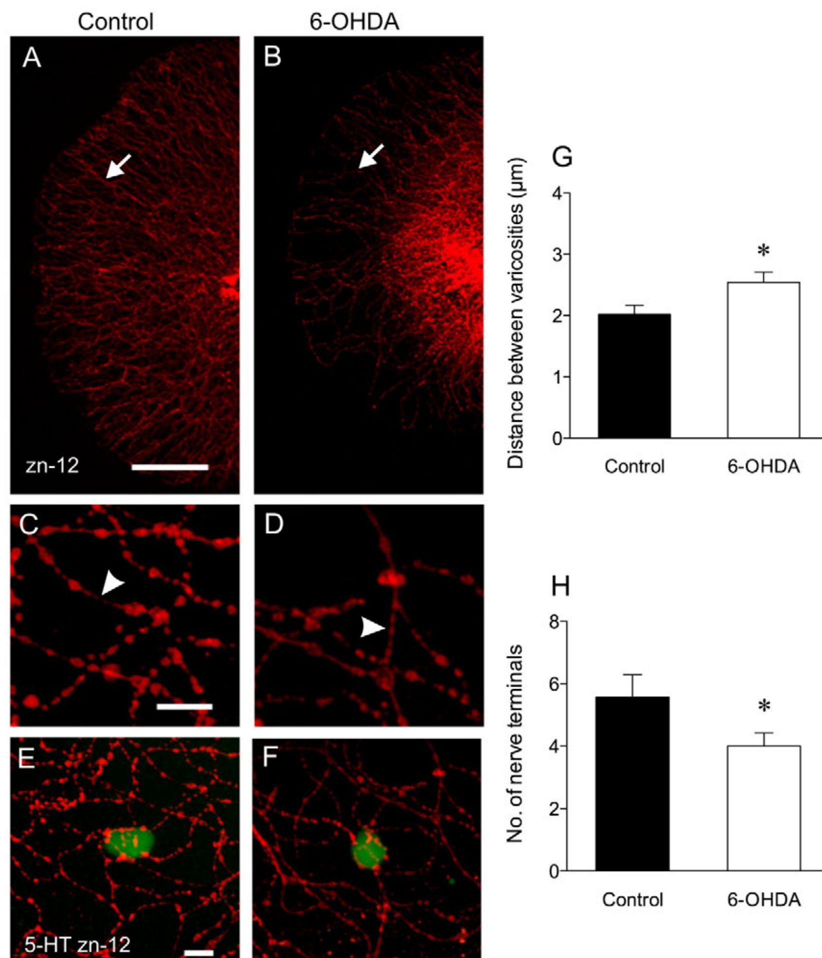

Fig. 9. Ablation of nerve fibres innervating neuroepithelial cells (NECs) in the skin of zebrafish larvae by 6-hydroxydopamine (6-OHDA). Immunofluorescence imaging of anti-5-HT and anti-zn-12 in controls (A,C,E) and larvae pre-exposed to 250 $\mu mol\,l^{-1}$ 6-OHDA (B,D,F). (A,B) Compared with controls, zebrafish treated with 6-OHDA had fewer zn-12-immunoreactive (IR) nerve fibres (arrows). (C,D) In 6-OHDA-treated fish, there was a greater distance between varicosities along sampled nerve fibres (arrowheads), suggesting reduced varicosity density. (E,F) When 5-HT-IR skin NECs were observed with associated nerve fibres, there was a reduced number of zn-12-IR varicosities surrounding skin NECs (presumed nerve terminals) in fish treated with 6-OHDA. (G) Mean ± s.e.m. distance between nerve varicosities was greater in larvae pre-treated with 250 $\mu mol\,l^{-1}$ 6-OHDA (open bar) compared with controls (closed bar). Asterisk indicates a significant difference from control ($P<0.05$, *t*-test, $N=60$). (H) Mean ± s.e.m. number of nerve terminals surrounding skin NECs was reduced in larvae pre-treated with 250 $\mu mol\,l^{-1}$ 6-OHDA (open bar, $N=10$) compared with controls (closed bar, $N=7$). Asterisk indicates a significant difference from control ($P<0.05$, *t*-test). Scale bars, 100 μm in A and B; 5 μm in C and D; 5 μm in E and F.

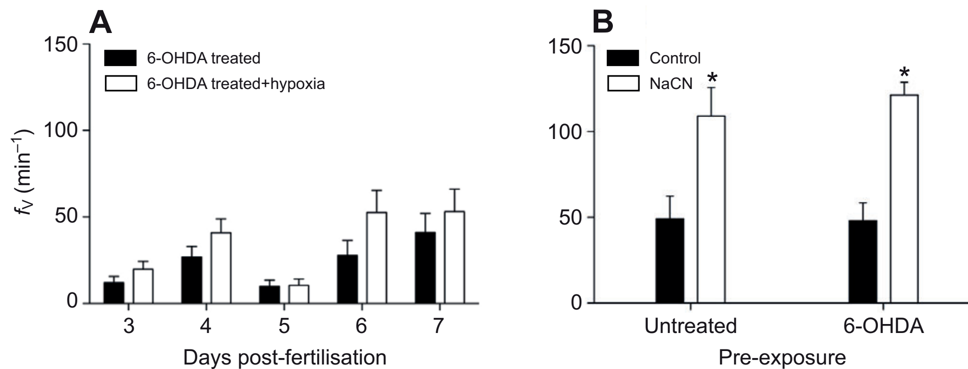

Fig. 10. Treatment with 6-hydroxydopamine (6-OHDA) eliminated the hyperventilatory response to acute hypoxia. (A) In zebrafish larvae pre-treated with 250 μmol l^{-1} 6-OHDA, mean ± s.e.m. ventilatory frequency (f_V; min^{-1}) did not increase significantly between 3 and 7 days post-fertilisation (d.p.f.) during exposure to acute hypoxia (P_{O_2}=25 mmHg). For 3, 4, 5, 6 and 7 d.p.f., *N*=66, 72, 50, 30 and 20, respectively. (B) In 5 d.p.f. larvae, mean ± s.e.m. f_V increased significantly, in both untreated controls and larvae pre-treated with 250 μmol l^{-1} 6-OHDA, following acute exposure to 1 mmol l^{-1} NaCN. Asterisks indicate significant differences from control (*P*<0.01, two-way ANOVA with Bonferroni *post hoc* test, *N*=8 for control, *N*=9 for NaCN).

absence of functional O_2-chemoreceptive NECs of the gills before 7 d.p.f. (Jonz and Nurse, 2005), our results further suggest that skin NECs are good candidates for the extrabranchial chemosensors that mediate behavioural responses to acute hypoxia during this time.

The concurrent increase in the number of gill NECs (Jonz and Nurse, 2005) and the decrease in skin NECs during embryonic and larval development may be related to a shift in the site of respiration, and potentially O_2 sensing, from the skin in developing zebrafish to the gills in juveniles and adults. A similar redistribution occurs with epithelial ionoregulatory mitochondria-rich cells, as ion regulatory mechanisms shift from the skin to the gills during development and precede the change in respiratory surfaces (Rombough, 2002; Fu et al., 2010). Like NECs, mitochondria-rich cells are numerous in the skin of fish during embryonic and early larval stages, and then decrease in number while they begin to populate the developing gills (van der Heijden et al., 1999; Varsamos et al., 2002; Pan et al., 2005; Jonz and Nurse, 2006).

In the present study, we found that chronic changes in water P_{O_2} for the first 7 d.p.f. altered the development of peak resting f_V, such that exposure to hyperoxia accelerated the establishment of peak f_V to 4 d.p.f., and hypoxia delayed peak f_V to 6 d.p.f. These shifts in the timing of peak resting f_V in acclimated larvae were suggestive of underlying morphological changes in O_2-chemoreceptive pathways. Indeed, we observed morphological changes in skin NECs that corresponded with shifts in peak f_V. Skin NECs remained abundant in zebrafish acclimated to hypoxia and did not decrease with age, as in controls. This relative increase in both number and density with severe hypoxic treatment indicates that the number of skin NECs may be upregulated during chronic hypoxia, a response typical of proliferating NECs in fish (Jonz et al., 2004) and O_2 chemoreceptors mammals (Nurse and Vollmer, 1997; Wang and Bisgard, 2002) following exposure to chronic hypoxia. Furthermore, it is interesting to note that chronic hypoxia slows the development of NECs of the gills in zebrafish during early larval stages (K. Shakarchi, P. C. Zachar and M.G.J., unpublished), so the persistence of more skin NECs (with putative O_2 chemosensitivity) during this time may be beneficial.

Hypoxia may have a variety of negative effects on development in fish, including a decrease in the rate of embryonic development, overall length and swimming activity (Rombough, 1988; Wu, 2009). Consistent with a previous study in zebrafish (Shang and Wu, 2004), we found in our experiments that acclimation to severe hypoxia (30 mmHg) reduced body length up to 7 d.p.f.; this appears to account for the increased density of skin NECs observed in severely hypoxic zebrafish relative to those acclimated to mild hypoxia and controls (compare Fig. 8B,C with 8D). However, despite the general impact of hypoxia on embryonic and larval development, the absolute number of skin NECs reached the same value (~300 cells per larva) at 3 d.p.f. regardless of water P_{O_2}. This indicates that the ability of zebrafish to produce the appropriate number of skin NECs is not compromised during development by a reduction in available O_2. This also suggests that, in our experiments, changes in skin NEC numbers that occurred between 5 and 9 d.p.f. were induced directly by changes in water P_{O_2}, rather than arising indirectly from the otherwise non-specific effects of prolonged hypoxia during development.

In contrast to our results with hypoxia, chronic hyperoxia had a predictably opposite effect on skin NECs. Acclimation of zebrafish to hyperoxia rapidly diminished the number and density of skin NECs, even at the earliest stages observed. Similar results have been previously reported in adult zebrafish, where hyperoxia decreased the density of NECs in the gill and blunted the hyperventilatory response to acute hypoxia (Vulesevic et al., 2006). Furthermore in rat, chronic exposure to hyperoxia leads to a reduced number of carotid body type I cells, loss of chemoafferent neurons, and attenuation of the carotid body response to hypoxia (Erickson et al., 1998; Bavis, 2005). Although it is possible that the effects of hyperoxia on reducing skin NEC populations in the present study may have been due to the production of reactive oxygen species (ROS) that may be associated with hyperoxia (Horowitz, 1999), impairment of carotid body chemoreceptor development does not appear to be related to ROS-mediated toxicity (Bavis et al., 2008).

An unexpected result from our experiments was that zebrafish reared in either hyperoxia or hypoxia displayed acute ventilatory depression, instead of hyperventilation, when challenged with acute hypoxia. In mammals, both hyperoxia and hypoxia during postnatal development may, paradoxically, result in an attenuated or absent hyperventilatory response to acute hypoxia (Bavis, 2005). Hyperoxia induces a reduction in the number of carotid body chemoreceptors and a decreased capacity for remaining chemoreceptors to respond normally to hypoxic stimuli, while hypoxia affects other aspects of respiratory development (Bavis, 2005). Likewise in zebrafish, chronic exposure to hyperoxia during development induces a persistent reduction in the hyperventilatory response to hypoxia at

maturity (Vulesevic and Perry, 2006), indicating a long-lasting effect of hyperoxia on respiratory development. The adaptive role of decreased f_V in response to acute hypoxia in developing zebrafish acclimated to abnormal levels of water P_{O_2} is presently unclear. However, given the sensitivity of skin NECs to changes in O_2 demonstrated in the present work, future physiological studies may investigate the potential role of these cells in respiratory development in zebrafish.

Conclusions and perspectives

The present results have shown that extrabranchial O_2 chemoreceptors mediate behavioural responses to acute hypoxia in developing zebrafish, and that serotonergic skin NECs may fulfill this role and contribute to O_2 sensing during embryonic and early larval stages. Future investigations will be aimed at elucidating the specific intracellular mechanisms by which O_2 regulates cell number, and obtaining physiological evidence for the role of skin NECs in chemosensing.

An interesting question that arises from this study is that of the importance of a regulatory mechanism to control hyperventilation in response to acute hypoxia in an embryonic organism, such as the zebrafish, that does not require branchial gas exchange or a circulatory system to survive (Rombough, 2007; Schwerte, 2009). Putative roles for extrabranchial O_2 chemoreceptors in embryonic zebrafish may include coordination of hatching time, which is dependent upon water P_{O_2} in other species (Wu, 2009), or initiation of the development of functional ventilatory patterns responsive to hypoxia before the gills and gill O_2 sensors have matured. Physiological regulatory systems, such as that of respiration, may be composed of multiple components that do not necessarily develop at the same time (Spicer and Burggren, 2003). The early appearance of extrabranchial chemosenors may therefore contribute to preparing for the development of a functional ventilatory and chemosensory system in the gills.

It is clear that in vertebrates, transitions in sites of O_2 chemoreception from one tissue to another occur during development and represent important adaptive or metamorphic events. In mammals, chromaffin cells of the adrenal medulla are sensitive to hypoxia during the perinatal period and facilitate adaptation to extrauterine life (Nurse et al., 2009). As development continues, chromaffin cells lose their sensitivity to hypoxia as the carotid body, the primary peripheral O_2-sensing organ, begins to mature (Donnelly, 2005). Pulmonary NEBs are also O_2 sensitive and are believed to be important during the perinatal period (Domnik and Cutz, 2011). In anamniotic vertebrates, such as amphibians, O_2 sensing occurs in the gills of water-breathing larvae (reviewed by Jonz and Nurse, 2006), as it does in fish, but after the gills degenerate during metamorphosis, a carotid body-like organ, called the carotid labyrinth, develops and takes over as the primary peripheral chemosensory organ in adults (Kusakabe, 2002). Thus, the present study indicates that such developmental transitions in O_2-sensing sites, such as from an extrabranchial site to the gills, may have evolved first in fish.

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
6-OHDA	6-hydroxydopamine
d.p.f.	days post-fertilisation
h.p.f.	hours post-fertilisation
NaCN	sodium cyanide
NEB	neuroepithelial body
NEC	neuroepithelial cell
P_{O_2}	partial pressure of oxygen
P_{CO_2}	partial pressure of carbon dioxide
ROS	reactive oxygen species
SV2	synaptic vesicle protein
f_V	ventilation frequency
zn-12	zebrafish neuron-specific antigen

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