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## RESEARCH ARTICLE

# Serotonergic and cholinergic elements of the hypoxic ventilatory response in developing zebrafish

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**SUMMARY**

**The chemosensory roles of gill neuroepithelial cells (NECs) in mediating the hyperventilatory response to hypoxia are not clearly defined in fish. While serotonin (5-HT) is the predominant neurotransmitter in $O_2$-sensitive gill NECs, acetylcholine (ACh) plays a more prominent role in $O_2$ sensing in terrestrial vertebrates. The present study characterized the developmental chronology of potential serotonergic and cholinergic chemosensory pathways of the gill in the model vertebrate, the zebrafish (*Danio rerio*). In immunolabelled whole gills from larvae, serotonergic NECs were observed in epithelia of the gill filaments and gill arches, while non-serotonergic NECs were found primarily in the gill arches. Acclimation of developing zebrafish to hypoxia ($P_{O_2}$=75 mmHg) reduced the number of serotonergic NECs observed at 7 days post-fertilization (d.p.f.), and this effect was absent at 10 d.p.f. *In vivo* administration of 5-HT mimicked hypoxia by increasing ventilation frequency ($f_V$) in early stage (7–10 d.p.f.) and late stage larvae (14–21 d.p.f.), while ACh increased $f_V$ only in late stage larvae. In time course experiments, application of ketanserin inhibited the hyperventilatory response to acute hypoxia ($P_{O_2}$=25 mmHg) at 10 d.p.f., while hexamethonium did not have this effect until 12 d.p.f. Cells immunoreactive for the vesicular acetylcholine transporter (VAChT) began to appear in the gill filaments by 14 d.p.f. Characterization in adult gills revealed that VAChT-positive cells were a separate population of neurosecretory cells of the gill filaments. These studies suggest that serotonergic and cholinergic pathways in the zebrafish gill develop at different times and contribute to the hyperventilatory response to hypoxia.**

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## INTRODUCTION

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 physiological changes, such as hyperventilation (Milsom and Burleson, 2007; López-Barneo et al., 2008; Perry et al., 2009). This ability to sense low levels of $O_2$, or hypoxia, is important for the development and survival of many organisms, so as to maintain internal $O_2$ levels within a normal physiological range to protect cells and tissues. In teleost fish, $O_2$/$CO_2$-sensitive serotonergic neuroepithelial cells (NECs) of the gills are homologues of the peripheral respiratory chemoreceptors of mammals (Milsom and Burleson, 2007; López-Barneo et al., 2008; Jonz and Nurse, 2009). In zebrafish, isolated NECs respond to acute hypoxia and hypercapnia (high $P_{CO_2}$) with ion channel inhibition and membrane depolarization (Jonz et al., 2004; Qin et al., 2010). Gill NECs are characterized by having cytoplasmic synaptic vesicles containing neurotransmitters and 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 are then carried from the gills to the central nervous system by afferent fibres of the glossopharyngeal and vagus nerves (Milsom and Brill, 1986; Burleson and Milsom, 1993; Sundin and Nilsson, 2002).

Serotonin (5-hydroxytryptamine, 5-HT) is the predominant neurotransmitter stored in NECs of the gills (Perry et al., 2009) and is an important mediator of hypoxic responses in $O_2$-sensitive pulmonary neuroepithelial bodies (NEBs) and carotid body type I cells in mammals (Fu et al., 2002; Nurse, 2010). However, in the carotid body, acetylcholine (ACh) is a primary excitatory neurotransmitter, while 5-HT acts as a neuromodulator (Milsom and Burleson, 2007; Shirahata et al., 2007; Nurse, 2010). In isolated gills of trout, both ACh and 5-HT induced chemosensory discharge recorded from afferent fibres of the glossopharyngeal nerve, and perfusion of these chemicals in intact animals increased ventilatory rate (Burleson and Milsom, 1995a; Burleson and Milsom, 1995b). Furthermore, serotonergic NECs and cells containing the vesicular acetylcholine transporter (VAChT) were found in the gill filaments and skin in the amphibious fish *Kryptolebias marmoratus*, and the behavioural response to hypoxia was mediated by a serotonergic and cholinergic system (Regan et al., 2011). Additional VAChT-positive neurons were described in the gill filaments of trout and goldfish (Porteus et al., 2013). Thus, it appears that the hypoxic response initiated by $O_2$ chemoreceptors may include both serotonergic and cholinergic mechanisms. What is lacking in the literature, however, is information about which neurotransmitter(s) is actually released by NECs during hypoxic stimulation, the receptor types at pre- and post-synaptic membranes at the NEC–nerve synapse, and a detailed understanding of the chemosensory mechanisms that mediate the cellular response to hypoxia.

The zebrafish (*Danio rerio*) presents an advantageous model with which to study $O_2$ chemosensory mechanisms and their

development in vertebrates. Zebrafish embryos are initially anoxia tolerant but become hypoxia-sensitive larvae within the first 2–3 days of life (Padilla and Roth, 2001; Mendelsohn et al., 2008). Embryos and early larvae are completely reliant on cutaneous gas exchange and are not dependent on branchial (gill) respiration until after 10 days post-fertilization (d.p.f.) (Rombough, 2007). Nevertheless, the hyperventilatory response to hypoxia is first observed at 3 d.p.f. and increases dramatically by 7 d.p.f. (Jonz and Nurse, 2005). Coincident with these changes is the development of gill primordia at 3 d.p.f. (Kimmel et al., 1995), a transition in $O_2$ chemosensitivity from an extrabranchial site to the gills (Jonz and Nurse, 2006; Coccimiglio and Jonz, 2012), and innervation of serotonergic NECs of the gill filaments at 7 d.p.f. (Jonz and Nurse, 2005). Given this dramatic series of developmental events, examination of respiratory and chemosensory development in embryonic and post-embryonic zebrafish may reveal important clues about neurochemical pathways in the gill and the response to hypoxia.

The objectives of the present study were to characterize NEC types in the gills of developing zebrafish, and to describe the chronology of potential chemosensory pathways that may contribute to the hypoxic ventilatory response. We demonstrate that serotonergic, non-serotonergic and putative cholinergic cells initially occupy different regions of the developing gills. Furthermore, *in vivo* drug application and assessment of ventilatory responses indicate that serotonergic control of the hypoxic ventilatory response develops in the gills before a cholinergic mechanism. This study suggests that 5-HT and ACh are important neurotransmitters for $O_2$ sensing in zebrafish, but ACh is not required during early development.

## MATERIALS AND METHODS

### Animals

Wild-type adult zebrafish, *Danio rerio* (F. Hamilton 1822), were obtained from a local commercial supplier and held in a closed re-circulated facility at the University of Ottawa. The animals were maintained at 28.5°C on a 14 h:10 h light:dark cycle. All handling and care was conducted in accordance with the guidelines set out by the Canadian Council on Animal Care (CCAC). Embryos were bred as previously described (Westerfield, 2000) and transferred to Petri dishes containing embryo medium (5 mmol $l^{-1}$ NaCl, 0.17 mmol $l^{-1}$ KCl, 0.33 mmol $l^{-1}$ $CaCl_2$, 0.33 mmol $l^{-1}$ $MgSO_4$ at pH 7.8) and placed in an incubator at 28.5°C. After hatching, larvae were placed in 1 l aquaria filled with dechlorinated water and maintained at 28.5°C.

### Acclimation to chronic hypoxia

Zebrafish embryos were placed immediately after collection in an incubator (Forma 3110, ThermoFisher Scientific, Ottawa, ON, Canada) at 28.5°C, in which atmospheric $P_{O_2}$ was reduced to 75 mmHg by injection of 95% $N_2$ and measured with a thermal conductivity $O_2$ sensor and feedback system. This level of hypoxia was selected for acclimation as it resulted in a low level of mortality. Critical $P_{O_2}$ for zebrafish under 10 d.p.f. is about 70–75 mmHg (Barrionuevo et al., 2010). At 5 d.p.f., larvae were transferred to aquaria in which dechlorinated water was maintained at 28.5°C and bubbled with a mixture of compressed air and $N_2$ to achieve a $P_{O_2}$ of 75 mmHg using a Pegas 4000 gas mixer (Columbus Instruments, Columbus, OH, USA). Water $P_{O_2}$ was measured daily with an $O_2$ meter (Model 55, YSI Inc., Yellow Springs, OH, USA). Control larvae were maintained under similar conditions but in a normoxic (150 mmHg) atmosphere and in water bubbled with compressed air. Once larvae had reached 7 or 10 d.p.f., they were removed and processed for immunohistochemical labelling and image analysis.

### Immunohistochemistry

NECs and other cell types of the gills in zebrafish were identified and studied using previously established procedures (Jonz and Nurse, 2003; Jonz and Nurse, 2005). Zebrafish used for imaging were killed with 1 mg $ml^{-1}$ MS-222 (tricaine methanesulphonate; Syndel Laboratories, Vancouver, BC, Canada) or rapidly stunned and decapitated. Whole larvae and gill complexes (i.e. containing four bilateral gill pairs) from adults were then fixed by immersion in a phosphate-buffered solution (PBS: 137 mmol $l^{-1}$ NaCl, 15.2 mmol $l^{-1}$ $Na_2HPO_4$, 2.7 mmol $l^{-1}$ KCl, 1.5 mmol $l^{-1}$ $KH_2PO_4$ at pH 7.8) (Bradford et al., 1994; Jonz and Nurse, 2003) containing 4% paraformaldehyde at 4°C overnight. After rinsing, whole larvae or gill complexes from adults were permeabilized for 24–48 h using 2% Triton X-100 in PBS at 4°C.

The use of primary and secondary antibodies for immunohistochemistry is detailed in Table 1. Larvae were placed in primary antibodies diluted in 0.5% Triton X-100 solution at 4°C for 24–72 h. Tissue was then rinsed in PBS and treated with secondary antibodies at room temperature for 1–2 h in the dark. Larvae were rinsed and individual gill arches were isolated on the stage of a stereomicroscope (MZ6, Leica, Wetzlar, Germany). For adult tissue, gill complexes were first placed in anti-VAChT for 24 h, followed by a rinse in PBS and incubation in anti-5-HT and anti-SV2 (synaptic vesicle protein, SV2) for 24 h. The tissue was then rinsed, treated with secondary antibodies (as described above) and the gill arches were isolated.

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

| Antibody | Dilution | Antigen | Host | Source | Cat. no. | Secondary |
|---|---|---|---|---|---|---|
| Primary | | | | | | |
| 5-HT | 1:250 | Serotonin | Rabbit | Sigma (polyclonal) | S5545 | FITC(a) or Alexa 405 |
| SV2 | 1:200 | SV2 | mouse | DSHB (monoclonal) | SV2 | Alexa 594 |
| VAChT | 1:200 | Vesicular transporter | Guinea pig | Millipore (polyclonal) | AB1588 | FITC(b) |
| zn-12 | 1:100 | Neuron surface | Mouse | DSHB (monoclonal) | zn-12 | Alexa 594 |
| Secondary | | | | | | |
| Alexa 405 | 1:100 | Rabbit | Goat | Invitrogen | A31556 | – |
| Alexa 594 | 1:100 | Mouse | Goat | Invitrogen | A11005 | – |
| FITC(a) | 1:50 | Rabbit | Goat | Cedar Lane | 111-095-003 | – |
| FITC(b) | 1:50 | Guinea pig | Goat | Millipore | AQ108F | – |

5-HT, 5-hydroxytryptamine (serotonin); FITC, fluorescein isothiocyanate; SV2, synaptic vesicle protein; DSHB, Developmental Studies Hybridoma Bank, University of Iowa; VAChT, vesicular acetylcholine transporter; zn-12, zebrafish-derived neuronal antibody.

Identification of serotonergic NECs was performed by immunolabelling with anti-5-HT. This antibody has been used to characterize 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 5-HT creatinine sulphate complex conjugated with BSA (manufacturer specifications). In addition, antibodies against SV2 were used to identify 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 bind to a transmembrane glycoprotein of ~95 kDa on the cytoplasmic side of synaptic vesicles in endocrine and neurosecretory cells (Buckley and Kelly, 1985) (manufacturer specifications).

Neural innervation of the gills 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). In the present study, we used zn-12 to identify the branchial nerve in isolated gill arches. This aided in discrimination between NECs of the gill arch *versus* NECs of the filaments. zn-12 was raised in mouse against membrane fractions from adult zebrafish central nervous system and recognizes a human natural killer-1-like (HNK-1-like) epitope (manufacturer specifications). Western blot analysis has indicated that zn-12 and HNK-1 antibodies label similar bands ranging in molecular mass from 60 to 248 kDa (see Metcalfe et al., 1990).

Antibodies against VAChT were used to identify cells in the gill with putative cholinergic activity. This same antibody was used to label nerve terminals of motor neurons in zebrafish (Houser et al., 2011). Anti-VAChT recognizes a synthetic peptide corresponding to the C-terminus of the predicted rat VAChT protein (Roghani et al., 1994) (manufacturer specifications). VAChT is expressed in the membranes of cytoplasmic synaptic vesicles and mediates vesicular loading of ACh (Prado et al., 2002). For controls, zebrafish gill tissue was pre-incubated with the VAChT control peptide (cat. no. AG223, EMD Millipore Corp., Billerica, MA, USA) before treatment with VAChT antibodies. This procedure effectively blocked immunolabelling by anti-VAChT. In addition, as a positive control for the VAChT antibody, we removed whole nodose ganglia from adult zebrafish and labelled VAChT-immunoreactive neurons (supplementary material Fig. S1). The nodose ganglion in both mammals and fish contains cholinergic neurons (Gauda et al., 2004; Jonz and Zaccone, 2009).

### Microscopy and image analysis

Isolated whole gills prepared for immunolabelling were laid flat on microscope slides and immersed in Vectashield (Vector Laboratories, Burlingame, CA, USA) to reduce photobleaching. Tissue was observed using a confocal microscope (Fluoview 200, Olympus, Center Valley, PA, USA; or Zeiss LSM META510, Thornwood, NY, USA). Images were collected using Fluoview 2.1 (Olympus) or Zeiss Zen software. Analysis and manipulation of images was performed using Image J (v. 1.42q, National Institutes of Health) and CorelDraw 10 (Corel Corp., Ottawa, ON, Canada). Each image is a composite projection of serial optical sections. Optical sections were imaged in tissue up to 30 μm deep and separated by 0.5–2 μm.

To quantify changes in the number of NECs produced by acclimation to hypoxia, one gill (i.e. the first or second gill arch) from each of several larva at 7 and 10 d.p.f. in both control and hypoxic groups was examined. The total number of serotonergic NECs per gill arch (i.e. the lateral aspect of the arch excluding the filaments) was recorded. In addition, the total number of NECs of the gill filaments was recorded and divided by the total number of filaments, giving the number of NECs per filament. Statistical analysis was conducted using a two-way ANOVA (i.e. control *versus* hypoxia and 7 *versus* 10 d.p.f.) and Bonferroni *post hoc* test with GraphPad Prism v.5.03 software (GraphPad Software Inc., San Diego, CA, USA).

### Ventilation frequency measurements

Ventilation frequency ($f_V$) experiments were performed as described previously (Jonz and Nurse, 2005). Larval zebrafish between 7 and 21 d.p.f. were lightly anaesthetized with 0.05 mg ml$^{-1}$ MS-222 dissolved in dechlorinated water. Larvae were transferred to a superfusion chamber constructed from a 35 mm polystyrene dish fitted with a Sylgard (Dow Corning Corporation, Midland, MI, USA) mould that created a well ~8 mm in diameter. A fine nylon mesh was then placed over the well to prevent the larvae from being washed away during superfusion. The chamber was placed under a stereomicroscope (MZ6, Leica) and continuously superfused (4 ml min$^{-1}$) by a gravity-fed system. Solutions were delivered to the chamber using gas-impermeable tubing (Tygon, Saint-Gobain Performance Plastics Corp., Pittsburgh, PA, USA) and removed with a low-noise vacuum pump (Fisher Scientific, Ottawa, ON, Canada). All specimens were given several minutes to recover from transfer before experiments began.

Responses of larvae at 7, 10, 12, 14 and 21 d.p.f. to 25 mmHg hypoxia and exogenously applied 5-HT (50 μmol l$^{-1}$), ACh (50 μmol l$^{-1}$) and dopamine (DA, 50 μmol l$^{-1}$) were quantified by recording $f_V$, as observed by the number of buccal and opercular movements per minute. Exogenous application of these neurotransmitters at similar concentrations was previously used to study hypoxic responses in trout and the amphibious fish *K. marmoratus* (Wood and Shelton, 1980; Burleson and Milsom, 1995b; Regan et al., 2011). In the present study, the hypoxic solution was generated by bubbling a reservoir of dechlorinated water with $N_2$ (95%) and measured as described above.

In these experiments, larvae were transferred to the superfusion chambered and left for 2 min to recover from handling. Larvae were then exposed to a normoxic solution (150 mmHg) for 3 min and $f_V$ was recorded to establish a reliable baseline measurment. Hypoxia, 5-HT, ACh or DA was then administered for an additional 3 min and $f_V$ was recorded again to obtain a maximal response. To reduce variability, a control group was tested for each treatment at each developmental stage. The results of these experiments are detailed in Table 2 as means ± s.e.m. for each stage and were analysed by two-way ANOVA and Bonferroni *post hoc* test ($P$<0.05). The data were also pooled and presented as means ± s.e.m. of early stage larvae (7–10 d.p.f.) and late stage larvae (14–21 d.p.f.) to summarize developmental trends. These data were also analysed by two-way ANOVA.

In a second set of time course experiments, larvae were exposed to hypoxia for a total of 8 min to allow time for a response to ketanserin (a 5-HT$_2$ receptor antagonist, 100 μmol l$^{-1}$) or hexamethonium (a nicotinic ACh receptor antagonist, 100 μmol l$^{-1}$) and recovery during hypoxic exposure. These drugs were introduced to the chamber for 2 min to observe changes in the hyperventilatory response induced by hypoxia. They have previously been used to modify the emersion response to hypoxia in *K. marmoratus*, when applied exogenously at the same

Table 2. Effects of *in vivo* application of acute hypoxia ($P_{O_2}$=25 mmHg) and neurochemicals on ventilation frequency in zebrafish larvae

| Treatment | 7 d.p.f. | 10 d.p.f. | 12 d.p.f. | 14 d.p.f. | 21 d.p.f. |
|---|---|---|---|---|---|
| Control | 74.7±10.8 (20) | 75.5±11.0 (20) | 99.0±8.6 (20) | 105.0±8.9 (20) | 102.7±12.1 (18) |
| Hypoxia | **144.3±8.2** (20) | **136.5±16.0** (20) | **161.6±12.0** (20) | **168.3±8.5** (20) | **156.7±16.8** (18) |
| Control | 46.9±7.5 (10) | 27.6±6.5 (10) | 32.0±11.9 (9) | 50.8±10.0 (10) | 62.9±7.5 (10) |
| 5-HT | **106.9±23.8** (10) | 63.6±10.7 (10) | **84.7±15.1** (9) | **113.4±8.6** (10) | 109.1±18.7 (10) |
| Control | 110.0±12.2 (20) | 60.5±10.1 (20) | 103.7±12.4 (20) | 69.6±8.5 (20) | 104.9±10.6 (20) |
| ACh | 115.1±14.0 (20) | 70.0±11.9 (20) | 145.4±16.5 (20) | **103.9±12.1** (20) | **156.1±12.5** (20) |
| Control | 75.2±7.3 (10) | 61.6±14.9 (10) | 78.3±12.4 (8) | 83.8±8.9 (10) | 123.6±19.4 (10) |
| DA | **22.0±9.1** (10) | **13.1±7.5** (10) | 25.5±12.9 (8) | **32.6±11.2** (10) | **76.8±14.5** (10) |

5-HT, serotonin; ACh, acetylcholine; DA, dopamine.
Developmental stages are indicated in days post-fertilization (d.p.f.). The concentration of each chemical was 50 μmol $l^{-1}$.
Mean ± s.e.m. ventilation frequencies ($f_V$, $min^{-1}$) are indicated for each group with sample size (*N*) in parentheses.
Values in bold indicate a significant difference from control at that stage (two-way ANOVA, $P<0.05$).

concentration (Regan et al., 2011). Data are presented as means ± s.e.m. and were analysed by repeated measures ANOVA ($P<0.05$). All drugs used in $f_V$ experiments were purchased from Sigma-Aldrich (Oakville, ON, Canada) and were dissolved in system water containing 0.05 mg $ml^{-1}$ MS-222 adjusted to pH 7.8 at room temperature. Ketanserin is poorly soluble in water and was first dissolved in DMSO (0.1% final concentration). This concentration of DMSO alone did not affect $f_V$ in our experiments or in a previous study (Jonz and Nurse, 2005).

## RESULTS

### Serotonergic and non-serotonergic NECs of the developing gills have different distributions

In isolated whole gills of developing zebrafish, we identified three groups of 5-HT-immunoreactive NECs that have been previously characterized in both larvae and adults (Jonz and Nurse, 2003; Jonz and Nurse, 2005; Zachar and Jonz, 2012): the gill arch NECs, which are organized along the axis of the gill arch adjacent to the branchial nerve, the $O_2$ chemoreceptive NECs that occupy the developing filament primordia, and Merkel-like basal cells of the taste buds, which are located along the oral aspect of the gill arch epithelium and the gill rakers (Fig. 1, Fig. 2A–D). In Fig. 2, the branchial nerve is indicated by zn-12 immunoreactivity and outlines the approximate position of gill arch NECs. Double labelling with antibodies against 5-HT and SV2 demonstrated that at 7, 12 and 21 d.p.f. all NECs were immunoreactive for SV2, but not all NECs were serotonergic (Fig. 1). SV2-immunoreactive non-serotonergic NECs were almost exclusively found in the gill arches and occasionally observed in the gill filaments. By contrast, serotonergic NECs were more prominent in the gill filaments than in the gill arches (Figs 1, 2).

### Acclimating developing zebrafish to hypoxia reduces the number of serotonergic gill NECs at 7 d.p.f.

Serotonergic NECs of the gill filaments are $O_2$ sensitive and receive neural innervation by 7 d.p.f. (Jonz et al., 2004; Jonz and Nurse, 2005). We therefore assessed the number of serotonergic NECs of the gill filaments and gill arches in larvae raised in hypoxia (75 mmHg), compared with those in controls raised in normoxia (150 mmHg), at 7 and 10 d.p.f. In gills isolated from 7 d.p.f. larvae acclimated to hypoxia, there were fewer NECs observed in the gill arches and gill filaments compared with controls (Fig. 2A,B). The number of NECs decreased significantly from 2.9±0.3 to 1.0±0.2 per gill arch, and from 1.0±0.1 to 0.6±0.1 per gill filament, i.e. approximately one NEC per two gill filaments (Fig. 2E). By contrast, when zebrafish were raised from embryos in hypoxia and examined at 10 d.p.f., there were no significant changes in the number of NECs in the gill arch (2.9±0.4 to 3.6±0.4) or gill filament (0.9±0.1 to 1.0±0.1) compared with controls (Fig. 2C,D,F). We quantified the ratio of the mean number of NECs following hypoxic acclimation to the mean number in controls ($NEC_{Hyp}/NEC_{Cont}$; Fig. 2G). It was evident that the ratio for both the filaments and gill arches had increased between 7 and 10 d.p.f., indicating that hypoxia had induced a relative increase in NEC number.

### Cholinergic control of the hyperventilatory response develops after the serotonergic pathway

Table 2 summarizes experiments in which zebrafish larvae (7–21 d.p.f.) were exposed *in vivo* to acute hypoxia (25 mmHg), 5-HT, ACh or DA in behavioural assays, and changes in $f_V$ were recorded. DA is an inhibitory neurotransmitter in the carotid body (Nurse, 2010) and fish gill (Burleson and Milsom, 1995a; Burleson and Milsom, 1995b), and was used in the present study to demonstrate inhibition of $f_V$ in our assays. These data were pooled and grouped into early stage larvae (7–10 d.p.f.) and late stage larvae (14–21 d.p.f.) and are presented in Fig. 3. As has been shown previously in early stage larvae up to 10 d.p.f. (Jonz and Nurse, 2005; Turesson et al., 2006), acute hypoxia induced a significant increase in $f_V$, and late stage larvae were equally affected (Fig. 3A). We next screened for the effects of exogenous application of 50 μmol $l^{-1}$ 5-HT, ACh or DA upon $f_V$. Early larvae produced hyperventilatory responses when exposed to 5-HT (Fig. 3B), but reduced $f_V$ when confronted with DA (Fig. 3D). Application of ACh had no effect on younger larvae (Fig. 3C). Late stage larvae, within the range of 14–21 d.p.f., responded to 5-HT and DA in the same manner as younger larvae (i.e. with increased and decreased $f_V$, respectively; Fig. 3B,D), with the additional effect of a significant rise in $f_V$ in response to exogenous application of ACh (Fig. 3C). Thus, at the concentration tested, the stimulatory effects of 5-HT and ACh on $f_V$ mimicked those of hypoxia, with ACh having effects only on late stage larvae, while DA inhibited $f_V$ in both early and late stage larvae.

To further demonstrate that a cholinergic mechanism for controlling the hyperventilatory response to hypoxia develops in the gills later than a serotonergic pathway, we performed time course experiments at three developmental stages (10, 12 and 21 d.p.f.) in which acute hypoxia (25 mmHg) was first used to stimulate NECs in the gill *in vivo*. At all stages of development, $f_V$ was significantly increased by hypoxia within 3 min (Fig. 4). Subsequent addition of

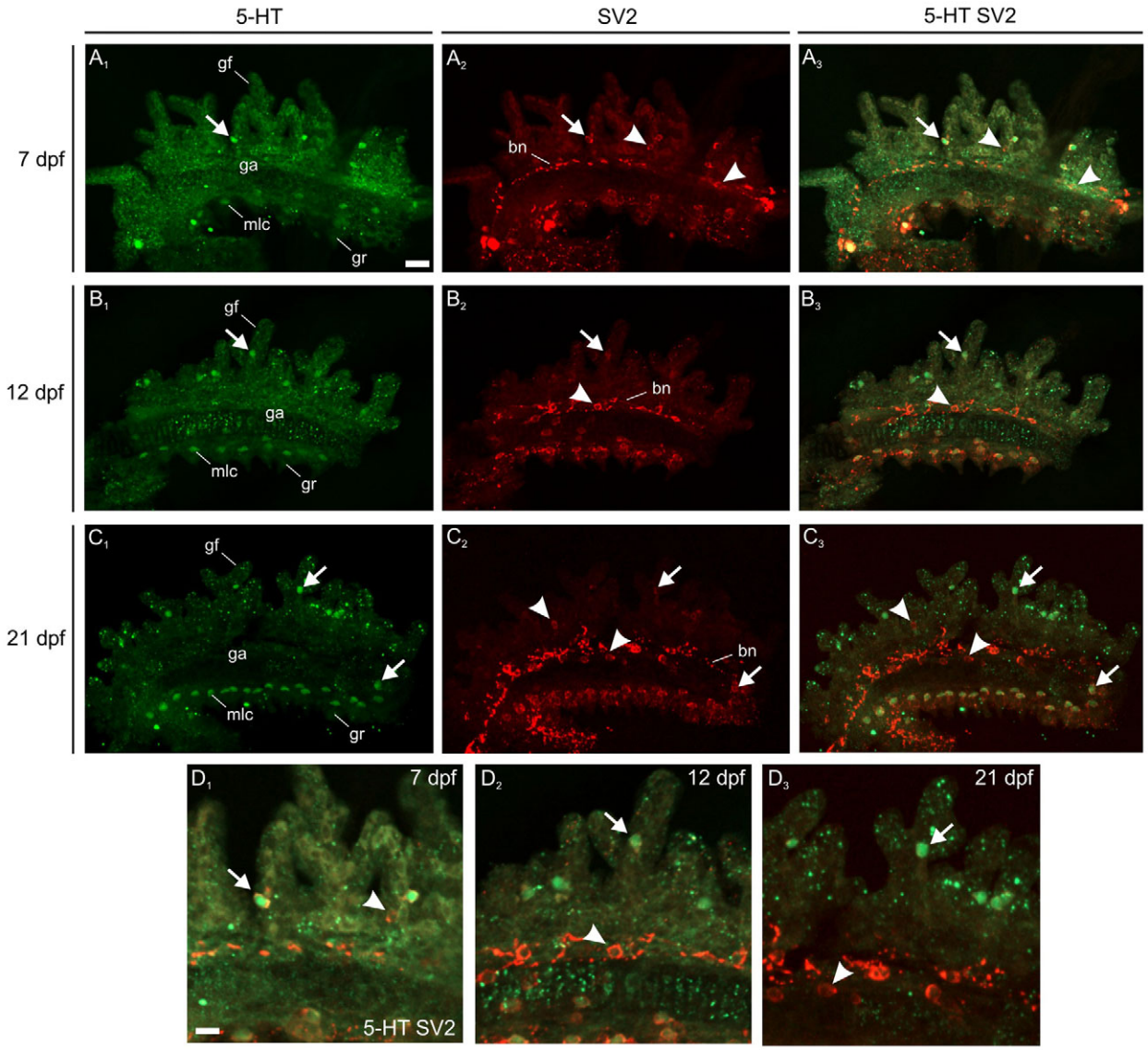


Fig. 1. Distribution of serotonergic and non-serotonergic neuroepithelial cells (NECs) of the gills in developing zebrafish. Confocal micrographs demonstrate double immunohistochemical labelling with antibodies against serotonin (5-HT, green) and the synaptic vesicle protein SV2 (red). Panels labelled 1, 2 and 3 in A–C show 5-HT, SV2 and 5-HT with SV2 labelling, respectively. Zebrafish larvae were raised until 7 days post-fertilization (d.p.f., A), 12 d.p.f. (B) and 21 d.p.f. (C). Serotonergic NECs were primarily observed in the gill filaments, while non-serotonergic NECs were predominantly found in the gill arches. Enlargements of A3, B3 and C3 are shown in D1, D2 and D3, respectively. bn, branchial nerve; ga, gill arch; gf, gill filament; gr, gill raker; mlc, Merkel-like cell. Arrows indicate serotonergic NECs; arrowheads indicate non-serotonergic NECs. Scale bar in A1 is 20 μm and applies to all panels in A–C. Scale bar in D1 is 10 μm and applies to all panels in D.

the 5-$HT_2$ receptor antagonist ketanserin (100 μmol $l^{-1}$) to the superfusate reversibly reduced $f_V$ at all stages tested (Fig. 4A–C). Note that at 21 d.p.f. a significant effect due to ketanserin was observed only after washout of the drug and return to hypoxia (Fig. 4C). The source of this effect is unknown, but we speculate that because larvae at 21 d.p.f. (Fig. 4C) are considerably larger than those at 10 d.p.f. (Fig. 4A), the permeability of ketanserin is relatively reduced and a longer period of time is required to reach a maximal ventilatory response. Consistent with the ACh experiments described in Fig. 3, addition of the nicotinic ACh receptor antagonist hexamethonium (100 μmol $l^{-1}$) in similar time course experiments had no effect upon $f_V$ at 10 d.p.f. (Fig. 4D). At 12 and 21 d.p.f., however, hexamethonium significantly reduced the hypoxia-stimulated rise in $f_V$ (Fig. 4E,F).

### Immunolocalization of VAChT in the developing gill corresponds with development of a cholinergic pathway

Immunohistochemical labelling was performed in isolated gills in order to establish a putative morphological basis for the onset of the effects of ACh and hexamethonium on $f_V$ in late stage larvae. Double labelling with anti-VAChT and a neuronal marker (zn-12) in larvae at 10, 12, 14 and 21 d.p.f. indicated that VAChT was not present in the gill filaments or gill arches up to 12 d.p.f., although the branchial nerve was immunoreactive for both markers during these stages (Fig. 4A,B). At 14 d.p.f., weak immunolabelling of VAChT and zn-12 was present in the gill filaments (Fig. 4C), indicating putative cells. At 21 d.p.f., distinct VAChT/zn-12-immunoreactive cells were observed in the filaments and gill arch.

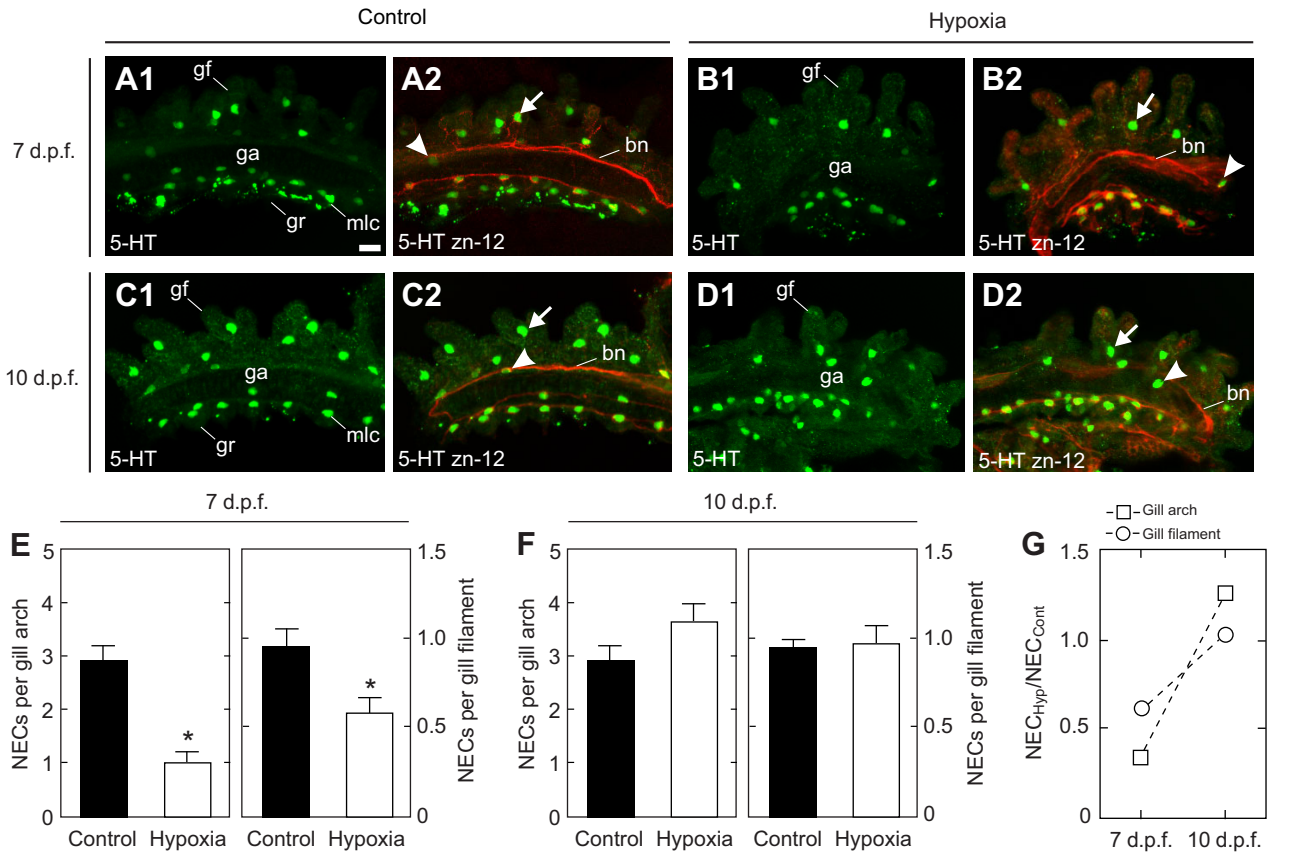


Fig. 2. Acclimation to hypoxia reduced the number of serotonergic NECs of the gills at 7 d.p.f. but not at 10 d.p.f. (A–D) Confocal micrographs showing immunohistochemical labelling with antibodies against 5-HT (green) and the general neuronal marker zn-12 (red) in isolated gills. Panels labelled 1 and 2 in A–D show 5-HT and 5-HT with zn-12 labelling, respectively. Zebrafish larvae were raised for 7 days (A,B) or 10 days (C,D) in normoxia (control; A,C) or hypoxia (75 mmHg; B,D). Hypoxia reduced the number of gill NECs only at 7 d.p.f. bn, branchial nerve; ga, gill arch; gf, gill filament; gr, gill raker; mlc, Merkel-like cell of taste bud. Arrows indicate gill filament NECs; arrowheads indicate gill arch NECs. Scale bar in A1 is 20 μm and applies to all panels. (E,F) Mean and s.e.m. number of NECs per gill arch (left) and NECs per gill filament (right) are shown for 7 d.p.f. (E) and 10 d.p.f. (F) larvae. Asterisks indicate a significant difference from control at 7 d.p.f. (two-way ANOVA, Bonferroni, $P<0.01$). Sample sizes were as follows: E, $N=11$ control and $N=13$ hypoxia; F, $N=7$ control and $N=5$ hypoxia. (G) Ratio of the mean number of NECs following acclimation to hypoxia (NEC$_{Hyp}$) compared with the mean number of NECs in controls (NEC$_{Cont}$). The data are replotted from E and F and show an increase in NEC number between 7 and 10 d.p.f.

### Characterization of VAChT-positive cells in the gill

We used gills from adult zebrafish to map the distribution of VAChT cells in the filaments and to indicate any potential overlap with markers of serotonergic and non-serotonergic NECs that have been described in the adult (Jonz and Nurse, 2003). In isolated gills triple labelled with antibodies against VAChT, 5-HT and SV2, VAChT-positive cells were immunonegative for 5-HT and SV2 (Fig. 6). This indicates that VAChT cells comprise a population of cells separate from serotonergic and non-serotonergic NECs. VAChT-positive cells were less than 10 μm in diameter and did not appear to extend any processes or membrane extensions (Fig. 5D, Fig. 6B). VAChT cells often appeared eccentric in shape, perhaps owing to the pattern of vesicular labelling within the cytoplasm. In addition, in adults it was evident that these cells were found in the efferent filament epithelium (i.e. facing the incident flow of water during ventilation), organized along the midline near serotonergic NECs (Fig. 6A). Although not quantified in this study, the distribution of VAChT cells suggests they were potentially as numerous as serotonergic NECs.

## DISCUSSION

The present study has described temporally defined populations of neurosecretory cells in the gills of developing zebrafish. These include serotonergic NECs that are present in the gills during early larval stages, and a population of cells expressing VAChT, a marker of cholinergic cells (Prado et al., 2002), that develops in later stage larvae. Development of these cell types corresponded with the onset of two potential chemosensory mechanisms that affect the hypoxic ventilatory response. Our results indicate that, despite early development of the hyperventilatory response to hypoxia in embryos, new chemosensory pathways involved in $O_2$ sensing are still forming between 14 and 21 d.p.f. The results of our study are summarized chronologically in Fig. 7 and show the developmental stage at which these observations occurred.

### Development of serotonergic and SV2-positive NECs

NECs immunoreactive for the transmembrane glycoprotein SV2, some of which were also serotonergic, were present in the gills at the earliest stages tested (7 d.p.f.). It was evident that non-serotonergic SV2-positive NECs were predominantly confined to the gill arches, while serotonergic NECs were found primarily in the gill filaments. Both of these cell types are found in the gills of adult zebrafish, but establish a different distribution pattern from that observed in larvae: both serotonergic and non-serotonergic NECs occupy the gill filaments in adults (and respiratory lamellae), while only serotonergic NECs have been observed in the gill arches

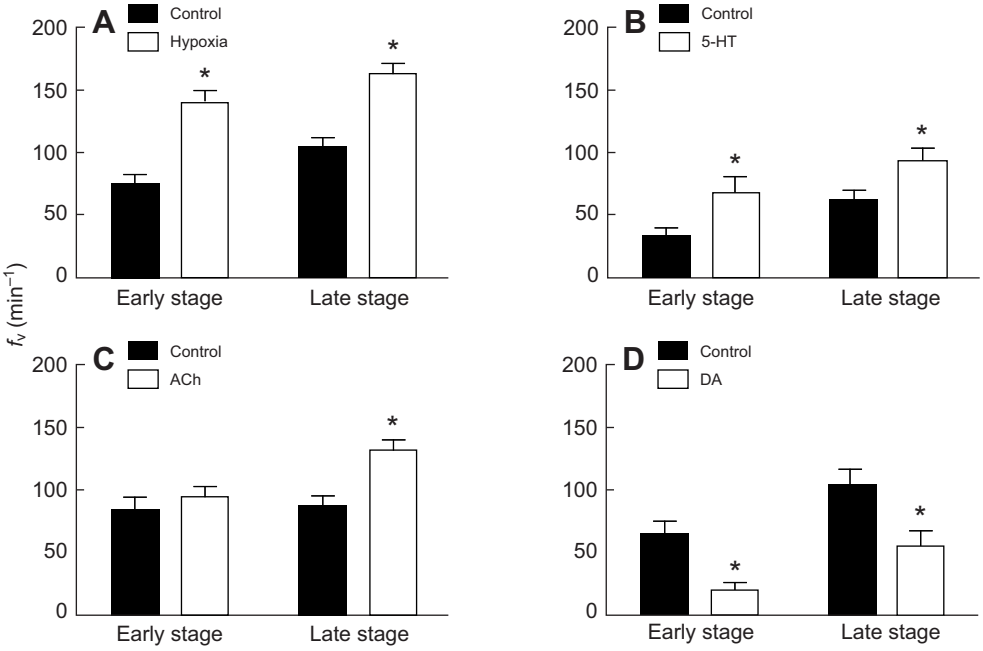


Fig. 3. Effects of hypoxia and application of neurotransmitters on ventilation frequency in zebrafish larvae *in vivo*. Ventilation frequency ($f_V$) is indicated for control and treated groups in early stage larvae (7–10 d.p.f.) and late stage larvae (14–21 d.p.f.). These data were taken from Table 2 and pooled. Larvae were exposed by superfusion to (A) 25 mmHg hypoxia, (B) 50 µmol l$^{-1}$ 5-HT, (C) 50 µmol l$^{-1}$ acetylcholine (ACh) or (D) 50 µmol l$^{-1}$ dopamine (DA). Means and s.e.m. are indicated. *Significantly different from control values (two-way ANOVA, Bonferroni, $P$<0.05).

(Jonz and Nurse, 2003; Jonz and Nurse, 2005). Presumably, this change in distribution of non-serotonergic NECs from the gill arches to the filaments reflects a developmental programme. It has been proposed that NECs of the gill filaments that do not express 5-HT may be part of a proliferative population of cells that differentiate into serotonergic NECs (Bailly et al., 1992; Jonz and Nurse, 2003). Similar SV2-positive NECs lacking 5-HT have also been found in the gills of goldfish (*Carassius auratus*), trout (*Oncorhynchus mykiss*), trairão (*Hoplias lacerdae*), traira (*Hoplias malabaricus*) and larvae of *Xenopus laevis* (Saltys et al., 2006; Coolidge et al., 2008). In addition, when adult zebrafish were acclimated to chronic hypoxia for 60 days, only the SV2-positive non-serotonergic NECs proliferated (i.e. increased in number) (Jonz et al., 2004). Thus, SV2-positive cells of the gill arches in developing zebrafish may reflect a population of progenitor cells that migrate into gill filament primordia and differentiate into serotonergic NECs. We did, however, observe occasional SV2-positive non-serotonergic NECs in the filaments and a lower total number of serotonergic NECs of the gill arches in larvae in the present study. This perhaps indicates a transitional state of migration of these cells. In adult zebrafish, where the gill filaments are mature and serotonergic NECs are confined to the distal regions, putative SV2-positive progenitors reside adjacent to serotonergic NECs (Jonz and Nurse, 2003) and may contribute to maintaining this cell population. A similar arrangement is found in the adult carotid body, where new type I cells can derive from adjacent progenitor type II cells (Pardal et al., 2007). While the carotid body is formed by migration of sympathoadrenal progenitors from the superior cervical ganglion (Kameda, 2005), the origin of gill NECs is not yet understood.

We examined the effects of acclimation to hypoxia (75 mmHg) on the number of serotonergic NECs of the gill filaments at 7 and 10 d.p.f. for three reasons: (1) only these cells have been shown to possess chemoreceptor properties in the gill (Jonz et al., 2004; Qin et al., 2010); (2) NECs appear to be functional as chemoreceptors *in vivo* beginning at 7 d.p.f. (Jonz and Nurse, 2005); and (3) this time frame represents an important developmental period when the systems of gas exchange (Rombough, 2002) and $O_2$ sensing appear to be transferred to the gills from the skin (Jonz and Nurse, 2006; Coccimiglio and Jonz, 2012). Our results indicate that the addition of new NECs in the gill filaments during development is inhibited by hypoxia up to 7 d.p.f. In a recent study (Coccimiglio and Jonz, 2012), it was shown that zebrafish larvae acclimated to hypoxia displayed an increased number of serotonergic NECs of the skin, as well as delayed development of peak resting $f_V$ and an altered ventilatory response to hypoxia. The results of this and the present study suggest that hypoxic acclimation during development may postpone the transition of $O_2$ sensing from an extrabranchial site to the gills and perhaps gill development. We further show that this developmental plasticity, in which the number of gill NECs is susceptible to change by hypoxia, may occur for only a brief period of time, as by 10 d.p.f. the number of NECs in the gill is not reduced (or has increased) following acclimation to hypoxia.

### Serotonergic and dopaminergic pathways are present in early stage larvae

Previously, it was demonstrated in zebrafish that innervation of serotonergic NECs of the gill filaments between 5 and 7 d.p.f. corresponds with a dramatic rise in the hyperventilatory response to hypoxia (Jonz and Nurse, 2005). We confirm, in the present study, that application of 5-HT has a stimulatory effect on $f_V$ in early stage larvae (7–10 d.p.f.). Moreover, by using hypoxia to first increase $f_V$ in time course experiments, we also demonstrated that ketanserin (a 5-HT$_2$ receptor antagonist) abolishes the hyperventilatory response to hypoxia as early as 10 d.p.f. This suggests that hypoxia stimulated the endogenous release of 5-HT, and that 5-HT is an important mediator of the hypoxic ventilatory response during early stages. The findings are in accordance with those of previous studies. 5-HT stimulated receptors within the gills of trout and increased glossopharyngeal nerve discharge and $f_V$ (Burleson and Milsom, 1995a; Burleson and Milsom, 1995b). In addition, in the amphibious fish *K. marmoratus*, pre-exposure to 5-HT increased the sensitivity of emersion behaviour (performed to promote a transition from gill to cutaneous respiration) when confronted with hypoxia, while ketanserin had the opposite effect (Regan et al., 2011). At 7, 12 and 21 d.p.f., 5-HT-immunoreactive NECs were found primarily in the gill filaments, the site of $O_2$-chemosensitive NECs in adult zebrafish

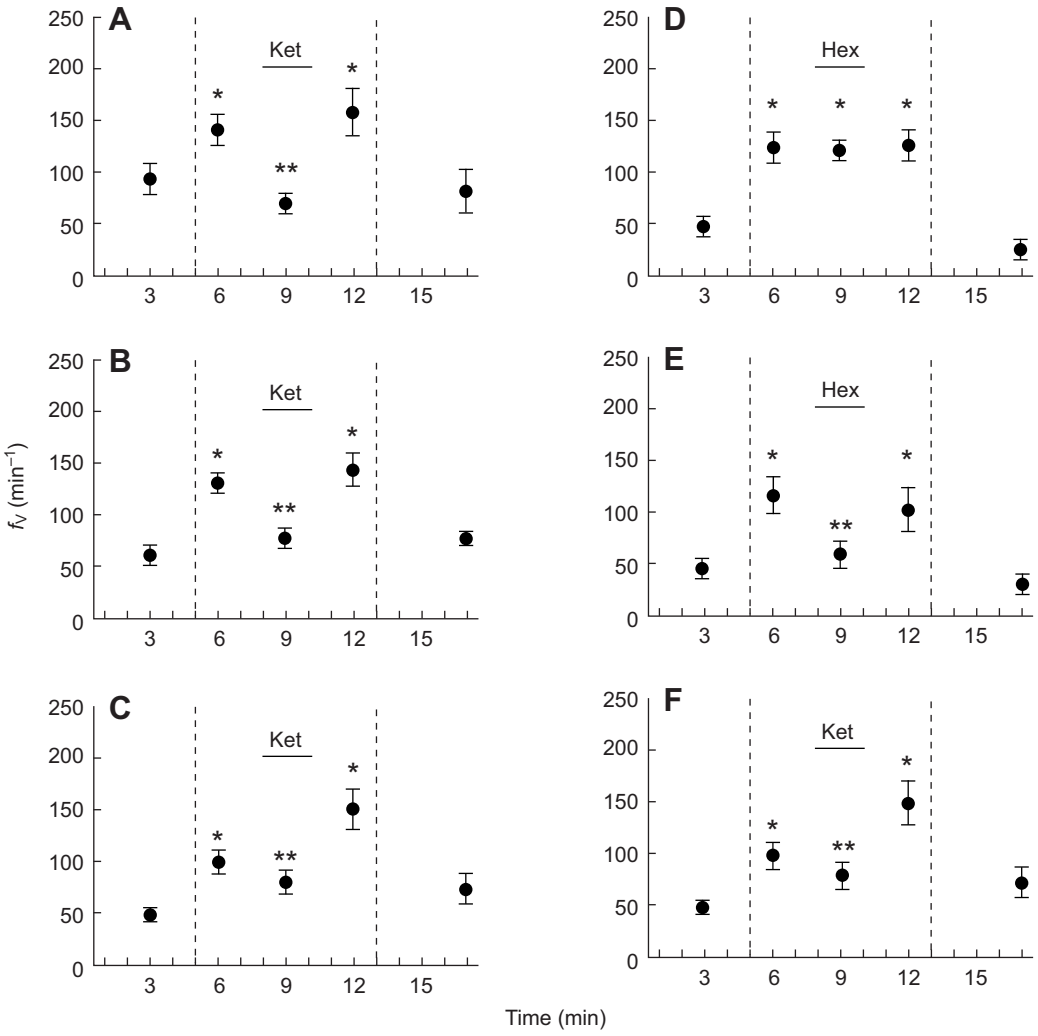


Fig. 4. *In vivo* inhibition of the hyperventilatory response to hypoxia by ketanserin and hexamethonium in zebrafish larvae. In time course experiments, larvae at 10 d.p.f. (A,D), 12 d.p.f. (B,E) and 21 d.p.f. (C,F) were subjected to hypoxia (25 mmHg) for 8 min (indicated by dashed vertical lines) and 100 μmol l$^{-1}$ ketanserin (Ket; A–C) or 100 μmol l$^{-1}$ hexamethonium (Hex; D–F) was subsequently applied for 2 min (horizontal bar). Means ± s.e.m. are indicated. *Significantly different from control values; **significantly different from hypoxic values (at 6 or 12 min) (repeated measures ANOVA and Bonferroni, $P<0.05$). Sample sizes were as follows: A, $N=7$; B, $N=10$; C, $N=10$; D, $N=8$; E, $N=10$; F, $N=10$.

(Jonz et al., 2004). This suggests that NECs of the gill filaments were the likely source of 5-HT release in the gill.

Thus, a serotonergic mechanism, in which 5-HT is released in the gill and acts through G-protein-coupled (metabotropic) 5-HT$_2$ receptors to increase $f_V$, may be present early in larval development in zebrafish. In the developing carotid body, 5-HT is also present during embryonic development in type I cells (Kameda, 2005). In addition, there is evidence that 5-HT is released by the carotid body upon chemostimulation, and that ketanserin inhibits both pre-synaptic (type I cell) and post-synaptic (petrosal neuron) 5-HT$_{2A}$ receptors, which participate in neuromodulation of the chemosensory response (Nurse, 2010). However, ionotropic 5-HT$_3$ receptors are also present on post-synaptic nerve terminals innervating type I cells, and may mediate fast neurotransmission in the carotid body (Zhong et al., 1999; Nurse, 2010).

We also demonstrated that during these early larval stages zebrafish were sensitive to exogenous application of DA. Though only a minor addition to this study, an inhibitory dopaminergic mechanism in the gill may be present at the earliest stages of development. In the carotid body, tyrosine hydroxylase (TH), an enzyme involved in DA production, is present during embryonic development (Kameda, 2005), and in newborns DA is already the main amine of the carotid body (Bairam and Carroll, 2005). A common view is that DA is an inhibitory neuromodulator in the carotid body and acts through pre- and post-synaptic metabotropic receptors (Nurse, 2010). TH was co-localized *in vitro* with 5-HT in $O_2$-sensitive NECs of the catfish (*Ictalurus punctatus*) (Burleson et al., 2006), suggesting that serotonergic NECs of the gill filaments may also release DA. By contrast, immunohistochemical labelling indicated the absence of TH in the gills of goldfish and trout (Porteus et al., 2013).

### VAChT-positive cells are a separate population of neurosecretory cells in the gill

The cells of the gill filaments labelled by the VAChT antibody were not serotonergic, nor were they immunoreactive for anti-SV2. These characteristics suggest that VAChT-positive cells in zebrafish are of a separate population from serotonergic and non-serotonergic NECs, which are both SV2 positive. While SV2 appears to be widely conserved in vertebrates (Buckley and Kelly, 1985), it may not necessarily label all neurosecretory cells (e.g. Pumplin and Getschman, 2000). Indeed, the expression of the VAChT membrane protein in these cells indicates that they probably retain synaptic vesicles that actively store ACh.

Cells immunoreactive for the VAChT protein have also been described in the gill filaments of *K. marmoratus* (Regan et al., 2011) and in gill filament neurons of trout and goldfish (Porteus et al., 2013). In the latter study, VAChT-positive neurons contained 5-HT and were similar in morphology to the serotonergic filament neurons of zebrafish (Jonz and Nurse, 2003). In the present study, however,

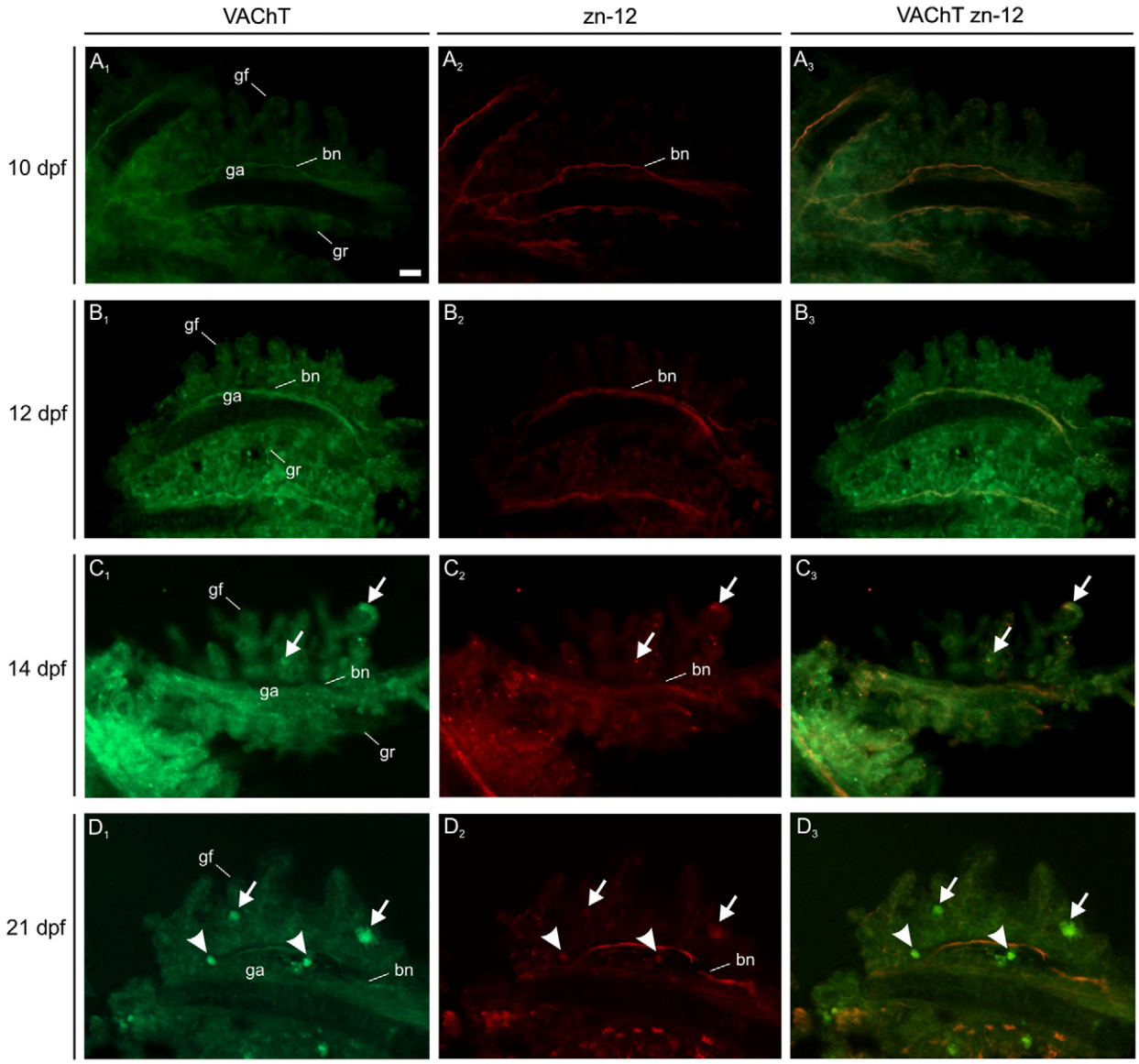


Fig. 5. Immunolocalization of vesicular acetylcholine transporter (VAChT) in isolated gills of developing zebrafish. Confocal micrographs demonstrate double immunohistochemical labelling with antibodies against VAChT (green) and zn-12 (red). Panels labelled 1, 2 and 3 in A–D show VAChT, zn-12 and VAChT with zn-12 labelling, respectively. Zebrafish larvae were raised until 10 d.p.f. (A), 12 d.p.f. (B), 14 d.p.f. (C) and 21 d.p.f. (D). VAChT/zn-12-immunoreactive cells were found only at 14 d.p.f. and 21 d.p.f. bn, branchial nerve; ga, gill arch; gf, gill filament; gr, gill raker. Arrows indicate cells of the gill filament; arrowheads indicate cells of the gill arch. Scale bar in A1 is 20 μm and applies to all panels.

VAChT-positive cells were not serotonergic and did not resemble filament neurons of zebrafish in their morphology or distribution. In zebrafish, neurons of the gill filaments are located beneath the basal lamina and filament arteries, and course along the midline of the filament (Jonz and Nurse, 2003). It is difficult to predict where VAChT cells might fit in with a putative scheme of chemosensing in the gill without a detailed knowledge of the distribution and orientation of these cells. Future studies may reveal whether VAChT cells are innervated, as are NECs, and whether they have a neural or paracrine role in the gill. Closer examination of these cells may also determine whether they should be considered 'paraneurons', which share structural and functional properties of neurons (Fujita, 1989; Zaccone et al., 1997), as do $O_2$-sensitive NECs.

In larvae, VAChT-positive cells were also immunoreactive for the neuronal marker zn-12. This antibody labels neurons and nerve fibres in the zebrafish gill (Jonz and Nurse, 2003; Vulesevic et al., 2006). The significance of this observation is not yet fully understood, but in larvae of *X. laevis*, zn-12-immunoreactive cells that did not contain 5-HT or SV2 were localized to the terminal branches of the gills, where neighbouring NECs were found (Saltys et al., 2006). In addition, as labelling of neurons and processes by zn-12 in zebrafish is dependent on developmental stage (Trevarrow et al., 1990), it is possible that zn-12 labelling of VAChT-positive cells may be restricted to early development. Embryonic zn-12 immunoreactivity of VAChT-positive cells may, therefore, provide some information as to the embryonic origin of these cells.

### A cholinergic pathway in the gill develops later in larvae

A putative cholinergic component of the $O_2$ chemosensory system in the zebrafish gill appears to develop at a later stage. Unlike 5-

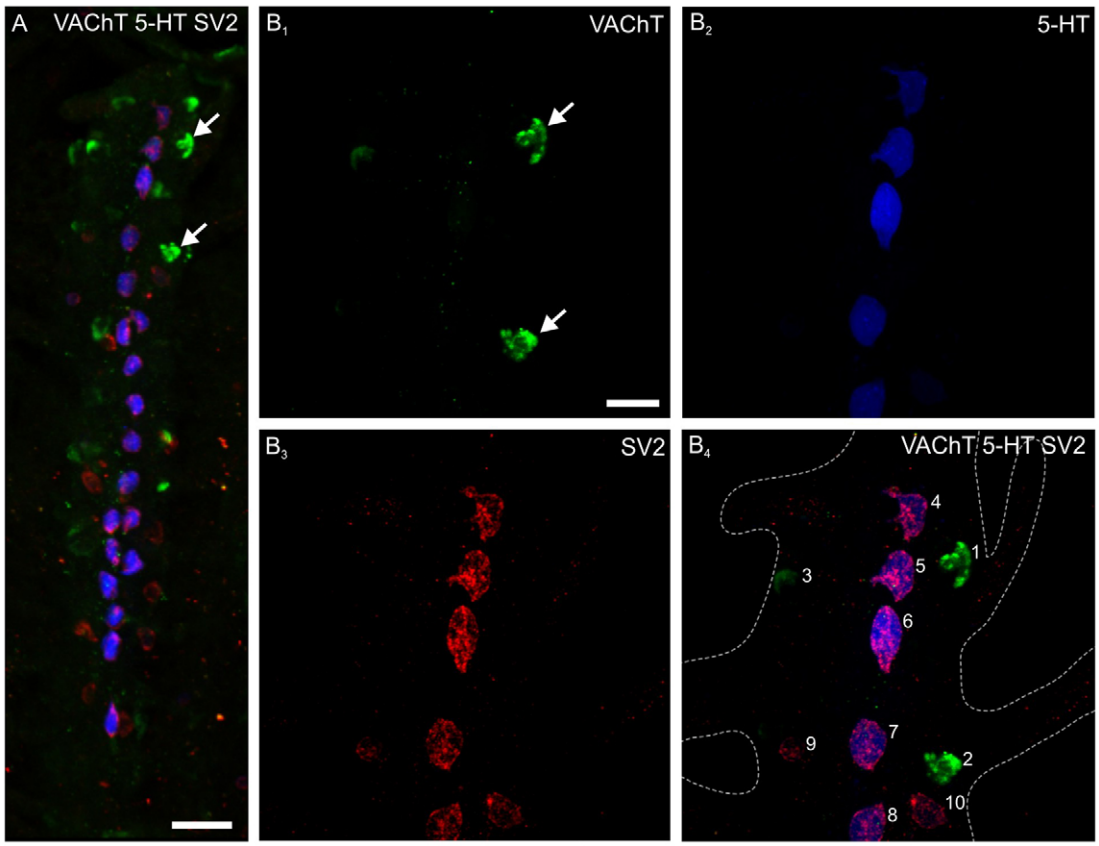


Fig. 6. Cells containing VAChT do not contain 5-HT or SV2. Confocal micrographs from an adult gill filament demonstrate triple immunohistochemical labelling with antibodies against VAChT (green), 5-HT (blue) and SV2 (red). (A) Imaging of the distal half of a gill filament showing all three cell types. The distal tip is located near the top of the image. Cells immunoreactive for VAChT (arrows) were observed 10–20 μm from the midline of the filament. (B) Panels labelled 1, 2, 3 and 4 show labelling by VAChT, 5-HT, SV2 and VAChT with 5-HT and SV2, respectively. In B4, 10 cells are indicated: cells 1–3 are VAChT positive, cells 4–8 are 5-HT and SV2 positive, and cells 9 and 10 are only SV2 positive. The dashed outline indicates the position of the lamellae. Scale bar in A is 20 μm. Scale bar in B1 is 10 μm and applies to all panels in B.

HT, ACh did not affect $f_V$ during early larval stages. In correspondence with these observations, time course experiments demonstrated that hexamethonium (a nicotinic ACh receptor antagonist) failed to inhibit hypoxia-induced hyperventilation at 10 d.p.f. In later stage larvae, ACh had a stimulatory effect on $f_V$ and, at 12 and 21 d.p.f., hexamethonium inhibited the hyperventilatory response to hypoxia. This suggests that hypoxia stimulated endogenous release of ACh only in later stage larvae, and that ACh may be an important mediator of the hypoxic response *via* nicotinic receptors. The findings are consistent with those of previous studies in trout, in which ACh stimulated receptors within the gill and increased glossopharyngeal nerve discharge and $f_V$ (Burleson and Milsom, 1995a; Burleson and Milsom, 1995b). Accordingly, nicotine had similar effects in these studies (Burleson and Milsom, 1995a; Burleson and Milsom, 1995b), and ACh and hexamethonium increased or reduced, respectively, sensitivity of the emersion response to hypoxia in *K. marmoratus* (Regan et al., 2011).

We further showed by immunohistochemistry that weak detection of the VAChT protein in the gill filaments can be observed as early as 14 d.p.f., and distinct cells immunoreactive for VAChT were present at 21 d.p.f. VAChT is a marker of cholinergic activity and has been localized to rat carotid body type I cells *in situ* (Zhang and Nurse, 2004). These results indicate the later development (between 14 and 21 d.p.f.) of a population of cells that presumably stores ACh.

ACh is the favoured candidate for mediating fast excitatory neurotransmission (along with ATP) in the carotid body, and nicotonic ACh receptors have been localized to post-synaptic nerve terminals of petrosal neurons as well as to pre-synaptic type I cells (Shirahata et al., 2007; Nurse, 2010). The cholinergic mechanism in the carotid body is tightly linked to postnatal development of chemoreceptor function, with increases in ACh synthesis and nicotinic receptor expression increasing with age (Bairam et al., 2007; Shirahata et al., 2007). Our results suggest that, as in the carotid body, a cholinergic component of $O_2$ sensing in the gills develops relatively late.

### Conclusions and significance

The present study used *in vivo* drug application and immunohistochemistry to explore potential neurochemical mechanisms of $O_2$ sensing, and their development, in the gills of zebrafish. We examined the sensitivity of ventilatory changes in zebrafish larvae to exogenous application of neurochemicals, and identified serotonergic and cholinergic pathways in the gill that potentially contribute to the control of the hypoxic ventilatory response at different developmental stages. These studies may help guide future pharmacological investigations in zebrafish that link endogenous neurotransmitters to mechanisms of synaptic transmission or modulation of the $O_2$ chemosensory response in the gills.

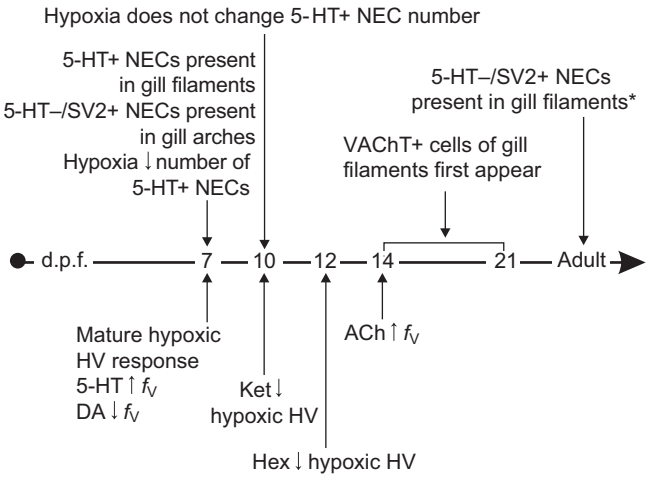


Fig. 7. Chronology of the development of morphological and ventilatory events from the present study. The horizontal arrow represents the development of zebrafish from egg to adult (d.p.f.). Details above the arrow are taken from immunohistochemical experiments (Figs 1, 2, 5, 6), and details below are taken from behavioural experiments (Table 1, Figs 3, 4). ↑, increase; ↓, decrease; +, positive immunoreaction; –, negative immunoreaction; HV, hyperventilatory. *Previously published information (Jonz and Nurse, 2003).

Similar preparations, in which drugs were applied exogenously to the water, have been used to study ventilatory responses in zebrafish and other species (Jonz and Nurse, 2005; Turesson et al., 2006; Regan et al., 2011). A caveat of this approach is the potentially non-specific actions of these drugs on the central nervous system. However, given that the blood–brain barrier begins to develop in zebrafish at 3 d.p.f. (Rihel and Schier, 2012), that the effects of the drugs on $f_V$ in zebrafish in our study are consistent with those of previous isolated gill and *in vivo* perfusion studies in other species (Burleson and Milsom, 1995a; Burleson and Milsom, 1995b; Regan et al., 2011), and that our data from ventilation experiments correspond well with our immunohistochemical results, changes in $f_V$ reported in the present study would seem to have arisen from the direct effects of these drugs on chemosensory mechanisms in the gills. In our experiments, however, we could not differentiate the specific sites of action in the gills of the drugs tested, and so cannot yet deduce specific functional roles for 5-HT, ACh and DA. Our study also demonstrates that this preparation may be useful for screening the effects of a variety of drugs, or classes of drugs, on hypoxic ventilatory control and their potential biomedical importance. Similar large-scale screens using zebrafish have already been employed (Rihel and Schier, 2012).

The results from this study suggest that a cholinergic mechanism is not required for production of the hyperventilatory response to hypoxia in developing zebrafish. A serotonergic system therefore appears to sufficiently regulate hypoxia-induced changes in $f_V$ during early developmental stages. An interesting question then arises: what are the relative roles of serotonergic and cholinergic control of $f_V$, and of NECs *versus* VAChT-positive cells? Perhaps serotonergic NECs in zebrafish mediate initial responses to hypoxia during early development, such as increased frequency of body movement and buccal pumping (Jonz and Nurse, 2005; Coccimiglio and Jonz, 2012), and direct the transition of $O_2$ sensing from an extrabranchial site to the developing gills. Accordingly, serotonergic and cholinergic systems may both be important during later larval stages and adulthood when the gills and the circulatory system are fully developed and required for gas exchange (Rombough, 2007; Schwerte, 2009). It is also tempting to speculate on the evolutionary significance of this pattern of development, in which a serotonergic system in the gill precedes cholinergic control of $f_V$. Both the carotid body and $O_2$-sensitive pulmonary NEBs of mammals are found in tissues that are derivatives of embryonic arches III and IV, respectively, and correspond to the same sites as gill NECs in fish (Burleson and Milsom, 2007; Jonz and Nurse, 2009). In this manner, gill NECs are evolutionary precursors of mammalian $O_2$ chemoreceptors. Perhaps the chronological sequence of the development of a serotonergic system followed by a cholinergic system in the fish gill reflects a primitive condition that was antecedent to the differentiated roles of these respective systems in mammals, in which early development of serotonergic NEBs of the pulmonary epithelium became important in facilitating the transition to extrauterine life during the perinatal period (much like serotonergic NECs may have a similar role in the developing gills), and later maturation of the cholinergic phenotype of carotid body type I cells led to their dominance as $O_2$ chemoreceptors in adults. Although this may explain how ACh and 5-HT each became dominant neurotransmitters in type I cells and NEBs, respectively, it would not explain how both ACh and 5-HT came to be expressed in type I cells while they occupy different cell types in the fish gill.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-HT | 5-hydroxytryptamine (serotonin) |
| ACh | acetylcholine |
| DA | dopamine |
| d.p.f. | days post-fertilization |
| $f_V$ | ventilation frequency |
| NEB | neuroepithelial body |
| NEC | neuroepithelial cell |
| $P_{CO_2}$ | partial pressure of carbon dioxide |
| $P_{O_2}$ | partial pressure of oxygen |
| SV2 | synaptic vesicle protein |
| TH | tyrosine hydroxylase |
| VAChT | vesicular acetylcholine transporter |
| zn-12 | zebrafish neuron-specific antigen |

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