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

by

Kamila Shakarchi, Peter C. Zachar and Michael G. Jonz*

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

Running title: 5-HT and ACh in gill O₂ sensing

Editor: Dr. Ken Lukowiak

Key words: 5-HT, ACh, hypoxia, zebrafish, chemoreceptor, NEC.

*Author for correspondence (email: mjonz@uottawa.ca)

Michael G. Jonz, PhD

Department of Biology

University of Ottawa

30 Marie Curie Pvt.

Ottawa, ON, Canada, K1N 6N5

phone: +1 613 562 5800 x6051

fax: +1 613 562 5486
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 O₂-sensitive gill NECs, acetylcholine (ACh) plays a more prominent role in O₂ 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 (PO₂=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 (Vf) in early stage (7-10 d.p.f.) and late stage larvae (14-21 d.p.f.), while ACh increased Vf only in late stage larvae. In time-course experiments, application of ketanserin inhibited the hyperventilatory response to acute hypoxia (PO₂=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.
INTRODUCTION

Respiratory chemoreceptors are specialized cells that detect changes in the partial pressure of environmental or arterial O₂ and CO₂ (PO₂ and PCO₂) 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 O₂, or hypoxia, is important for the development and survival of many organisms, so as to maintain internal O₂ levels within a normal physiological range to protect cells and tissues. In teleost fish, O₂/CO₂-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 PCO₂) with ion channel inhibition and membrane depolarisation (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 O₂-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, b). 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., 2012). Thus, it appears that the hypoxic response initiated by O₂ chemoreceptors may include both serotonergic and
cholinergic mechanisms. What is lacking in the literature, however, is information about which neurotransmitter(s) are 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 O2-chemosensory mechanisms and their development in vertebrates. Zebrafish embryos are initially anoxia-tolerant but become hypoxia-sensitive larvae within the first 2 to 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 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 O2 chemosensitivity from an extrabranchial site to the gills (Jonz and Nurse, 2006; Coccimiglio and Jonz, in press), 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 O2 sensing in zebrafish, but ACh is not required during early development.
MATERIALS AND METHODS

Animals

Wild type adult zebrafish, *Danio rerio*, 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 14-10 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 and placed in an incubator at 28.5°C. Embryo medium contained the following: 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄ at pH 7.8. After hatching, larvae were placed in 1 litre 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 PO₂ was reduced to 75 mmHg by injection with 95% N₂ and measured with a thermal conductivity O₂ sensor and feedback system. This level of hypoxia was selected for acclimation as it resulted in a low level of mortality. Critical PO₂ for zebrafish under 10 d.p.f. is about 70-75 mmHg (Barrioneuvo 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 N₂ to achieve a PO₂ of 75 mmHg using a Pegas 4000 gas mixer (Columbus Instruments, Columbus, OH, USA). Water PO₂ was measured daily with an O₂ meter (Model 55, YSI Inc., Yellow Springs, OH). 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

Neuroepithelial cells (NECs) and other cell types of the gills in zebrafish were identified and studied using previously established procedures (Jonz and Nurse, 2003, 2005). Zebrafish used for imaging were euthenised with 1 mg ml⁻¹ MS 222 (tricaine methanesulfonate; Syndel Laboratories, Vancouver, BC, Canada) or rapidly stunned and decapitated. Whole larvae and
gill complexes (i.e. containing 4 bilateral gill pairs) from adults were then fixed by immersion in a phosphate buffered solution (PBS) containing 4% paraformaldehyde at 4°C overnight. PBS contained the following: 137 mM NaCl, 15.2 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄ at pH 7.8 (Bradford et al., 1994; Jonz and Nurse, 2003). After rinsing whole larvae, or gill complexes from adults, were permeabilized for 24 to 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-serotonin (5-HT) and anti-SV2 for 24 h. The tissue was then rinsed, treated with secondary antibodies (as described above) and the gill arches were isolated.

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 sulfate complex conjugated with bovine serum albumin (manufacturer specifications). In addition, antibodies against the synaptic vesicle protein, 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 characterised (Trevarrow et al., 1990; Jonz and Nurse, 2003, 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 vs. NECs of the filaments. zn-12 was raised in mouse against membrane fractions from adult zebrafish CNS 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 60-248 kDa (see Metcalfe et al., 1990).

Antibodies against the vesicular acetylcholine transporter (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 recognises 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 (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) 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. Sample sizes (i.e. number of larvae) are indicated in the legend of Figure 2. 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 vs.
Hypoxia and 7 vs. 10 d.p.f.) and Bonferroni post test with GraphPad Prism v.5.03 software (GraphPad Software Inc., San Diego, CA).

**Ventilation frequency measurements**

Ventilation frequency ($V_f$) experiments were performed following 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 that was fitted with a Sylgard (Dow Corning Corporation, Midland, MI, USA) mould that created a well approximately 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 stereo microscope (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 serotonin (5-HT, 50 µmol l$^{-1}$), acetylcholine (ACh, 50 µmol l$^{-1}$) and dopamine (DA, 50 µmol l$^{-1}$) were quantified by recording $V_f$, as observed by the number of buccal and opercular movements min$^{-1}$. Exogenous application of these neurotransmitters of similar concentrations was 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 $V_f$ was recorded to establish a reliable baseline measurement. Hypoxia, 5-HT, ACh or DA was then administered for an additional 3 min and $V_f$ 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-test (p<0.05). These
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 analyzed 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<sub>2</sub> receptor antagonist, 100 µmol l<sup>-1</sup>) or
hexamethonium (a nicotinic ACh receptor antagonist, 100 µmol l<sup>-1</sup>) 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. These drugs were previously used to modify the
emersion response to hypoxia in *K. marmoratus*, when applied exogenously at the same
concentration (Regan et al., 2011). Data are presented as means ± S.E.M. and were analyzed by
repeated measures ANOVA (p<0.05). All drugs used in V<sub>f</sub> experiments were purchased from
Sigma-Aldrich (Oakville, ON, Canada) and were dissolved in system water containing 0.05 mg
ml<sup>-1</sup> MS 222 adjusted to a pH of 7.8 at room temperature. Ketanserin is poorly soluble in water
and was first dissolved in dimethyl sulfoxide (DMSO, 0.1% final concentration). This
concentration of DMSO alone did not affect V<sub>f</sub> in our experiments or in a previous study (Jonz
and Nurse, 2005).
RESULTS

Serotonergic and non-serotonergic neuroepithelial cells 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, 2005; Zachar and Jonz, 2012): the gill arch NECs that 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 taste buds that are located along the oral aspect of the gill arch epithelium and the gill rakers (Figs. 1 and 2A-D). In Figure 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 compared to the gill arches (Figs. 1 and 2).

Acclimating developing zebrafish to hypoxia reduced the number of serotonergic gill neuroepithelial cells 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 to controls raised in normoxia (150 mmHg), at 7 d.p.f. and at 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 to 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 of the gill arch (2.9 ± 0.4 to 3.6 ± 0.4) or gill filaments (0.9 ± 0.1 to 1.0 ± 0.1) compared to controls (Fig. 2C, D, F). We quantified the ratio of the mean number of NECs following hypoxic acclimation to controls (NECs$_{Hyp}$/NECs$_{Cont}$; 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 developed 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 dopamine (DA) in behavioural assays and changes in ventilation frequency (Vf) were recorded. Dopamine is an inhibitory neurotransmitter in the carotid body (Nurse, 2010) and fish gill (Burleson and Milsom, 1995a, b) and was used in the present study to demonstrate inhibition of Vf 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 Figure 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 Vf, 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 and DA upon Vf. Early larvae produced hyperventilatory responses when exposed to 5-HT (Fig. 3B), but reduced Vf when confronted with dopamine (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 dopamine in the same manner as younger larvae (i.e. with increased and decreased Vf, respectively; Fig. 3B and D) with the additional effect of a significant rise in Vf in response to exogenous application of ACh (Fig. 3C). Thus, at the concentration tested, the stimulatory effects of 5-HT and ACh on Vf mimicked those of hypoxia, with ACh having effects only on late stage larvae, while dopamine inhibited Vf in both early and late stage larvae.

To further demonstrate that a cholinergic mechanism of 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, Vf was significantly increased by hypoxia within 3 min (Fig. 4A-F). Subsequent addition of the 5-HT₂ receptor antagonist, ketanserin (100 µmol l⁻¹), to the superfusate reversibly reduced Vf 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 Figure 3, addition of the nicotinic ACh receptor antagonist, hexamethonium (100 µmol l$^{-1}$), in similar time-course experiments had no effect upon $V_f$ at 10 d.p.f. (Fig. 4D). At 12 and 21 d.p.f., however, hexamethonium significantly reduced the hypoxia-stimulated rise in $V_f$ (Fig. 4E, F).

**Immunolocalization of the vesicular acetylcholine transporter (VACHT) in the developing gill corresponded 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 $V_f$ 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.

**Characterisation of VACHT-positive cells in the gill**

We used gills from adult zebrafish to map the distribution of VACHT cells in the filaments and 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. 6A, B). 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) organised 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 neuroepithelial cells (NECs) that are present in the gills during early larval stages, and a population of cells expressing the vesicular acetylcholine transporter (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 O2 sensing are still forming between 14 and 21 days post-fertilization (d.p.f.). The results of our study are summarized chronologically in Figure 7 and show the developmental stage at which these observations occurred.

Development of serotonergic and SV2-positive neuroepithelial cells

Neuroepithelial cells 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 than observed in larvae: both serotonergic and non-serotonergic NECs will occupy the gill filaments in adults (and respiratory lamellae), while only serotonergic NECs have been observed in the gill arches (Jonz and Nurse, 2003, 2005). Presumably, this change in distribution of non-serotonergic NECs from the gill arches to the filaments reflects a developmental program. It has been proposed that NECs of the gill filaments that do not express serotonin (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 larave 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: only these cells have been shown to possess chemoreceptor properties in the gill (Jonz et al., 2004; Qin et al., 2010); NECs appear to be functional as chemoreceptors in vivo beginning at 7 d.p.f. (Jonz and Nurse, 2005); and this time frame represents an important developmental period when the systems of gas exchange (Rombough, 2002) and O₂ 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, in press), 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 ventilatory frequency (V̇₀) and an altered ventilatory response to hypoxia. Results of this and the present study suggest that hypoxic acclimation during development may postpone the transition of O₂ 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, since by 10 d.p.f. the number of NECs in the gill are not reduced (or have 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. corresponded 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 had a stimulatory effect on \( V_f \) in early stage larvae (7-10 d.p.f.). Moreover, by using hypoxia to first increase \( V_f \) in time-course experiments, we also demonstrated that ketanserin (a 5-HT\(_2\) receptor antagonist) abolished the hyperventilatory response to hypoxia as early as 10 d.p.f. This suggests that hypoxia stimulated 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 \( V_f \) (Burleson and Milsom, 1995a, b). In addition, in the amphibious fish, \textit{K. marmoratus}, pre-exposure to 5-HT increased 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 (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 \( V_f \), 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, ionotrophic 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 demonstrate that during these early larval stages zebrafish were sensitive to exogenous application of dopamine (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-localised \textit{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., 2012).

**VACHT-positive cells are a separate population of neurosecretory cell 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 likely retain synaptic vesicles that actively store acetylcholine (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., 2012). In the latter study, VACHT-positive neurons contained serotonin
and were similar in morphology to the serotonergic filament neurons of zebrafish (Jonz and
Nurse, 2003). In the present study, however, 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 if VACHT
cells are innervated, as are NECs, and if they may participate in a neural or paracrine role in the
gill. Closer examination of these cells may also determine if they should be considered
“paraneurons”, which share structural and functional properties of neurons (Fujita, 1989;
Zaccone et al., 1997), as do O₂-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 nor SV2 were localised
to the terminal branches of the gills, where neighbouring NECs were found (Saltys et al., 2006).
In addition, since 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 O2-chemosensory system in the zebrafish gill appears to develop at a later stage. Unlike 5-HT, ACh did not affect Vf 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 Vf 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 Vf (Burleson and Milsom, 1995a, b).

Accordingly, nicotine had similar effects in these studies (Burleson and Milsom, 1995a, b), 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 was 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 localised 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.

Acetylcholine is the favoured candidate for mediating fast excitatory neurotransmission (along with ATP) in the carotid body, and nicotonic ACh receptors have been localised to post-synaptic nerve terminals of petrosal neurons as well as 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 O₂ 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 O₂ 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 O₂ chemosensory response in the gills.

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 et al., 2012), that the effects of the
drugs on Vf 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, b; Regan et al., 2011), and
that our data from ventilation experiments correspond well with our immunohistochemical
results, changes in Vf 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 et al., 2012).

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 Vf during early
developmental stages. An interesting question then arises: What are the relative roles of serotonergic and cholinergic control of $V_f$ and of NECs vs. 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, in press), 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 $V_f$. Both the carotid body and $O_2$-sensitive pulmonary neuroepithelial bodies (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

<table>
<thead>
<tr>
<th>#</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>2</td>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>3</td>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>4</td>
<td>d.p.f.</td>
<td>days post-fertilisation</td>
</tr>
<tr>
<td>5</td>
<td>NEB</td>
<td>neuroepithelial body</td>
</tr>
<tr>
<td>6</td>
<td>NEC</td>
<td>neuroepithelial cell</td>
</tr>
<tr>
<td>7</td>
<td>PO\textsubscript{2}</td>
<td>partial pressure of oxygen</td>
</tr>
<tr>
<td>8</td>
<td>PCO\textsubscript{2}</td>
<td>partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>9</td>
<td>SV2</td>
<td>synaptic vesicle protein</td>
</tr>
<tr>
<td>10</td>
<td>VACHT</td>
<td>vesicular acetylcholine transporter</td>
</tr>
<tr>
<td>11</td>
<td>V\textsubscript{f}</td>
<td>ventilation frequency</td>
</tr>
<tr>
<td>12</td>
<td>zn-12</td>
<td>zebrafish neuron-specific antigen</td>
</tr>
</tbody>
</table>

# ACKNOWLEDGEMENTS

zn-12 and SV2 antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, 52242.

# FUNDING

This research was supported by the Natural Sciences and Engineering Research Council of Canada [grant number 342303-2007], the Canadian Foundation for Innovation and the Ontario Research Fund [grant number 16589].
References


Qin, Z., Lewis, J.E. and Perry, S.F. (2010) Zebrafish (Danio rerio) gill neuroepithelial cells are sensitive chemoreceptors for environmental CO\textsubscript{2}. J. Physiol. 588, 861-872.


Rombough, P. (2002). Gills are needed for ionoregulation before they are needed for O\textsubscript{2} uptake in developing zebrafish, Danio rerio. J. Exp. Biol. 205, 1787-1794.


Figure legends

Figure 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 5-HT (green) and SV2 (red). Panels labelled 1, 2 and 3 in A through C denote 5-HT, SV2 and 5-HT with SV2 labelling, respectively. Zebrafish larvae were raised until 7 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 panels of A-C. Scale bar in D1 is 10 µm and applies to all panels in D.

Figure 2. Acclimation to hypoxia reduced the number of serotonergic neuroepithelial cells (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 serotonin (5-HT, green) and zn-12 (red) in isolated gills. Panels labelled 1 and 2 in A through D denote 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 ± 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 (Hyp) compared to the mean number of NECs in controls (Cont). The data are replotted from panels E and F and show an increase in NEC number between 7 and 10 d.p.f.

Figure 3. Effects of hypoxia and application of neurotransmitters on ventilation frequency in zebrafish larvae in vivo. Ventilation frequency (Vf, min⁻¹) is indicated for control (solid bars) and treated (open bars) 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 serotonin (5-HT), (C) 50 μmol l⁻¹ acetylcholine, or (D) 50 μmol l⁻¹ dopamine. Means ± S.E.M. are indicated. *, significantly different from control values (two-way ANOVA, Bonferroni, p<0.05).

Figure 4. *In vivo* inhibition of the hyperventilatory response to hypoxia by ketanserin and hexamethonium in zebrafish larvae. In time-course experiments, larvae of 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) were subsequently applied for 2 min (solid horizontal line). 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.

Figure 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 through D denote 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.

Figure 6. Cells containing the vesicular acetylcholine transporter (VACHT) do not contain serotonin (5-HT) or the synaptic vesicle protein 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 away from the midline of the filament. B: Panels labelled 1, 2, 3 and 4 denote 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. A 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 B2-4.

Figure 7. Chronology summarizing the developmental timing of morphological and ventilatory events from the present study. The horizontal line represents the development of zebrafish from egg to adult in days post-fertilization (d.p.f.). Details above the line are taken from immunohistochemical experiments (Figs. 1, 2, 5, 6), and details below the line are taken from behavioural experiments (Table 1, Figs. 3 and 4). Abbreviations: ↑, increase; ↓, decrease; +, positive immunoreaction; - , negative immunoreaction; 5-HT, 5-hydroxytryptamine or serotonin; ACh, acetylcholine; DA, dopamine; Hex, hexamethonium; HV, hyperventilatory; Ket, ketanserin; NEC, neuroepithelial cell; VACHT, vesicular acetylcholine transporter. ¹Jonz and Nurse (2003).
Table 1. Details of primary and secondary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Antigen</th>
<th>Host</th>
<th>Source</th>
<th>Cat. No.</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT(^1)</td>
<td>1:250</td>
<td>serotonin</td>
<td>rabbit</td>
<td>Sigma (polyclonal)</td>
<td>S5545</td>
<td>FITC(a)(^3) or Alexa 405</td>
</tr>
<tr>
<td>SV2(^2)</td>
<td>1:200</td>
<td>SV2</td>
<td>mouse</td>
<td>DSHB(^4) (monoclonal)</td>
<td>SV2</td>
<td>Alexa 594</td>
</tr>
<tr>
<td>VChA(^5)</td>
<td>1:200</td>
<td>vesicular transporter</td>
<td>guinea pig</td>
<td>Millipore (polyclonal)</td>
<td>AB1588</td>
<td>FITC(b)</td>
</tr>
<tr>
<td>zn-12(^6)</td>
<td>1:100</td>
<td>neuron surface</td>
<td>mouse</td>
<td>DSHB (monoclonal)</td>
<td>zn-12</td>
<td>Alexa 594</td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alexa 405</td>
<td>1:100</td>
<td>rabbit</td>
<td>goat</td>
<td>Invitrogen</td>
<td>A31556</td>
<td>–</td>
</tr>
<tr>
<td>Alexa 594</td>
<td>1:100</td>
<td>mouse</td>
<td>goat</td>
<td>Invitrogen</td>
<td>A11005</td>
<td>–</td>
</tr>
<tr>
<td>FITC(a)</td>
<td>1:50</td>
<td>rabbit</td>
<td>goat</td>
<td>Cedar Lane</td>
<td>111-095-003</td>
<td>–</td>
</tr>
<tr>
<td>FITC(b)</td>
<td>1:50</td>
<td>guinea pig</td>
<td>goat</td>
<td>Millipore</td>
<td>AQ108F</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^1\)5-hydroxytryptamine; \(^2\)Fluorescein isothiocyanate; \(^3\)Synaptic vesicle protein; \(^4\)Developmental Studies Hybridoma Bank, University of Iowa; \(^5\)Vesicular acetylcholine transporter; \(^6\)Zebrafish-derived neuronal antibody.
Table 2. Effects of *in vivo* application of acute hypoxia (PO$_2$ = 25 mmHg) and neurochemicals on ventilation frequency (V$_f$) in zebrafish larvae. Developmental stages are indicated in days post-fertilization (dpf). Mean ± S.E.M. frequencies (min$^{-1}$) are indicated for each group with sample size (n) in parentheses. Values in italics indicate a significant difference from control at that stage (two-way ANOVA, P<0.05). The concentration of each chemical was 50 µM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>7 dpf</th>
<th>10 dpf</th>
<th>12 dpf</th>
<th>14 dpf</th>
<th>21 dpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>74.7 ± 10.8 (20)</td>
<td>75.5 ± 11.0 (20)</td>
<td>99.0 ± 8.6 (20)</td>
<td>105.0 ± 8.9 (20)</td>
<td>102.7 ± 12.1 (18)</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>144.3 ± 8.2 (20)</td>
<td>136.5 ± 16.0 (20)</td>
<td>161.6 ± 12.0 (20)</td>
<td>168.3 ± 8.5 (20)</td>
<td>156.7 ± 16.8 (18)</td>
</tr>
<tr>
<td>Control</td>
<td>46.9 ± 7.5 (10)</td>
<td>27.6 ± 6.5 (10)</td>
<td>32.0 ± 11.9 (9)</td>
<td>50.8 ± 10.0 (10)</td>
<td>62.9 ± 7.5 (10)</td>
</tr>
<tr>
<td>Serotonin$^1$</td>
<td>106.9 ± 23.8 (10)</td>
<td>63.6 ± 10.7 (10)</td>
<td>84.7 ± 15.1 (9)</td>
<td>113.4 ± 8.6 (10)</td>
<td>109.1 ± 18.7 (10)</td>
</tr>
<tr>
<td>Control</td>
<td>110.0 ± 12.2 (20)</td>
<td>60.5 ± 10.1 (20)</td>
<td>103.7 ± 12.4 (20)</td>
<td>69.6 ± 8.5 (20)</td>
<td>104.9 ± 10.6 (20)</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>115.1 ± 14.0 (20)</td>
<td>70.0 ± 11.9 (20)</td>
<td>145.4 ± 16.5 (20)</td>
<td>103.9 ± 12.1 (20)</td>
<td>156.1 ± 12.5 (20)</td>
</tr>
<tr>
<td>Control</td>
<td>75.2 ± 7.3 (10)</td>
<td>61.6 ± 14.9 (10)</td>
<td>78.3 ± 12.4 (8)</td>
<td>83.8 ± 8.9 (10)</td>
<td>123.6 ± 19.4 (10)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>22.0 ± 9.1 (10)</td>
<td>13.1 ± 7.5 (10)</td>
<td>25.5 ± 12.9 (8)</td>
<td>32.6 ± 11.2 (10)</td>
<td>76.8 ± 14.5 (10)</td>
</tr>
</tbody>
</table>

$^1$5-hydroxytryptamine, 5-HT.