

**THE ROLE OF SEROTONIN (5-HT) IN REGULATING THE HYPOXIC
HYPERVENTILATORY RESPONSE OF LARVAL ZEBRAFISH**

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Abstract

Serotonin (5-HT) containing neuroepithelial cells (NECs) are O₂ sensitive chemoreceptors found throughout the skin of larval zebrafish (*Danio rerio*). Zebrafish larvae are sensitive to changes in ambient PO₂ as early as 2 days post fertilisation (dpf) and hyperventilate in response to hypoxia beginning at 3 dpf. Tryptophan hydroxylase (tph) is the rate-limiting enzyme in 5-HT synthesis; three tph paralogs are present in zebrafish (tph1a, tph1b and tph2). Although 5-HT has been implicated as a key neurotransmitter mediating hypoxic hyperventilation, it has not been possible to discern the role of 5-HT specifically contained within the NECs in promoting hypoxic hyperventilation. The purpose of this study was to determine the role of NEC 5-HT in regulating the hypoxic ventilatory response in larval zebrafish. It was hypothesised that 5-HT is a key neurotransmitter released from NECs which contributes to hypoxic hyperventilation.

Immunohistochemistry was used to determine the distribution of tph paralogs and their role in 5-HT production in NECs. Tph1a was present in NECs and nerves innervating NECs. Exposure to the non-selective tph inhibitor, para-chlorophenylalanine (pCPA), or translational gene knockdown of tph1a, diminished 5-HT expression within NECs. Exposure to acute hypoxia (PO₂ = 30 mmHg) revealed a blunted hypoxic ventilatory response (reduced breathing frequency) in fish exhibiting depleted 5-HT in NECs. The hypoxic hyperventilatory response was rescued with application of 5-HT. The results of these experiments demonstrate that tph1a is responsible for 5-HT production in NECs of larval zebrafish, and that 5-HT released from NECs is involved in establishing their hypoxic hyperventilatory response.

Résumé

Les cellules neuro-épithéliales contenant de la sérotonine sont des chémorécepteurs sensibles à l'O₂ et se retrouvent au niveau de la peau chez les larves de poissons-zèbres (*Danio rerio*). Les larves de poissons-zèbres sont sensibles au changement de PO₂ ambiant à partir de 2 jours après la fécondation et hyperventilent en réponse d'hypoxie suivant 3 jours après la fécondation. La tryptophane hydroxylase (thp) est l'enzyme limitante à la synthèse de la sérotonine; trois paralogues de tph sont présents chez le poisson-zèbre (tph1a, tph1b et tph2). Même si la sérotonine est impliquée en tant que neurotransmetteur de l'hyperventilation hypoxique, il n'a pas été possible de discerner spécifiquement le rôle de la sérotonine dans les cellules neuro-épithéliales pour promouvoir l'hyperventilation hypoxique. Le but de cette étude était de déterminer le rôle de la sérotonine dans les cellules neuro-épithéliales en ce qui concerne la régulation de la réponse ventilatoire hypoxique des larves de poissons-zèbres. L'hypothèse est que la sérotonine est un neurotransmetteur clé qui est relâché par les cellules neuro-épithéliales dans le but de contribuer à l'hyperventilation hypoxique. L'immunohistochimie a été utilisée pour déterminer la distribution des paralogues de tph ainsi que leur rôle dans la production de la sérotonine chez les cellules neuro-épithéliales. Tph1a était présent dans les cellules neuro-épithéliales ainsi que dans les nerfs qui innervent les cellules neuro-épithéliales. L'exposition à l'inhibiteur tph non sélectif, para-chlorophenylalanine (pCPA) ou la réduction génique au niveau de la traduction de tph1a diminue l'expression de la sérotonine dans les cellules neuro-épithéliales. L'exposition à l'hypoxie aiguë (PO₂ = 30 mmHg) a révélé une atténuation de la réponse ventilatoire hypoxique (diminution de la fréquence de respiration) chez les poissons exhibant une baisse de sérotonine dans les cellules neuro-épithéliales. La réponse hyperventilatoire hypoxique a été secourue par l'application de la sérotonine. Les résultats de ces expériences démontrent que tph1a est responsable de la production de la sérotonine dans les

cellules neuro-épithéliales chez les larves de poissons-zèbres et que la sérotonine relâchée par les cellules neuro-épithéliales est impliquée dans l'établissement de leur réponse hyperventilatoire hypoxique.

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LIST OF ABBREVIATIONS

5-HT, 5-hydroxytryptamine or serotonin

Aadc, aromatic amino acid decarboxylase

ABO, air-breathing organ

ACh, acetylcholine

AMC, adrenomedullary chromaffin cell

ASR, aquatic surface respiration

ATP, adenosine triphosphate

CB, carotid body

CBS, cystathionine- β -synthase

CSE, cystathionine- γ -lyase

DA, dopamine

dpf, days post fertilisation

Mao, monoamine oxidase

NEB, neuroepithelial bodies

NEC, neuroepithelial cells

NOS, nitric oxide synthase

PaO₂, arterial blood oxygen partial pressure

pCPA, para-chlorophenylalanine

PO₂, oxygen partial pressure

ROS, reactive oxygen species

SERT, serotonin transporter

TH, tyrosine hydroxylase

tph, tryptophan hydroxylase

VAcHT, vesicular acetylcholine transporter

V_f , ventilation frequency

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

The partial pressure of oxygen (PO_2) in aquatic environments is highly variable (Diaz and Breitburg, 2009). The ability to detect and respond to changes in low O_2 (hypoxia) is essential, and many vertebrates are capable of initiating physiological responses to maintain metabolic homeostasis. This is accomplished through the function of specialised chemoreceptor cells that are capable of detecting changes in PO_2 and triggering responses such as hyperventilation (Lopez-Barneo et al., 1988; Jonz et al., 2004). Carotid body (CB) type I (glomus) cells are the primary chemoreceptors in mammals (Gonzalez et al., 1994; Lopez-Barneo, 2008), whereas neuroepithelial cells (NECs) of the skin and gill are the primary chemoreceptors in fish (Jonz et al., 2004). The first gill arch in fish is homologous to the carotid artery in mammals. Therefore, fish NECs are thought to be homologous to mammalian type I cells of the CB (Jonz and Nurse, 2009).

Multiple studies have demonstrated that fish respond to hypoxia by hyperventilating (reviewed by Perry et al., 2009). Fish NECs are trimodal O_2 , CO_2 and NH_3 sensors (Jonz et al., 2004; Qin et al., 2010; Zhang et al., 2011), however relatively little is understood about the downstream signalling mechanisms leading to the hyperventilatory response, including the role of neurotransmitters. Serotonin (5-Hydroxytryptamine; 5-HT) is present in zebrafish (*Danio rerio*) NECs and is a putative neurotransmitter regulating the hypoxic response (Shakarchi et al., 2013).

1.2 The Hypoxic Ventilatory Response

Many aquatic ecosystems face natural episodes of hypoxia, however anthropogenic sources are increasing the number of aquatic habitats facing hypoxia as a result of climate change and nutrient enrichment resulting from agriculture and land use (Diaz and Breitburg,

2009). Fish have been shown to respond to hypoxia by increasing ventilation volume, reducing heart rate (bradycardia), and increasing systemic vascular resistance (Randall and Shelton, 1963; Holeton and Randall, 1967; Hughes, 1973; Perry et al., 2009). The hypoxic ventilatory response arguably is the most physiologically significant of these responses (for review see Perry et al., 2009). The hypoxic hyperventilation is achieved by increases in breathing frequency (V_f) and/or amplitude and varies markedly among different species (reviewed by Gilmour and Perry, 2007). Zebrafish exhibit a significant increase in V_f at a PO_2 of 110 mmHg and below, but breathing amplitude is unchanged (Vulesevic et al., 2006). The increasing water flow over the gills associated with hyperventilation is important in maximising branchial O_2 uptake and minimising the decrease in arterial blood oxygen partial pressure (PaO_2) that accompanies ambient hypoxia (Perry and Gilmour, 2002). Interestingly, larval zebrafish hyperventilate in response to hypoxia prior to the development of gills (Jonz and Nurse, 2005). The functional significance of hyperventilation in larvae lacking gills is unclear. A possible explanation is that hypoxic hyperventilation fulfills a similar function as increased pectoral fin movements at early larval stages (up to 14 days post fertilisation; dpf). Pectoral fin movement increases the flow of water across the body surface, which is where gas exchange occurs prior to gill development (Hale, 2014).

Two additional strategies employed by some fish species for maintaining O_2 uptake are aquatic surface respiration (ASR) and air breathing (Johansen, 1966; Kramer and Mehegan, 1981). Many species utilise ASR, which involves the movement of fish toward the air/water exchange surface where the level of dissolved O_2 is higher. This behavior, which benefits O_2 uptake while minimising increases in V_f , may be initiated by peripheral chemoreceptors (Abdallah et al., 2015). Air breathing involves the uptake of air into an air breathing organ

(ABO). Oxygen is then distributed from the ABO into the bloodstream where it is delivered to the rest of the body (Graham and Wegner, 2010). Air breathing typically is observed in fish inhabiting extreme hypoxic environments and presumably evolved because O₂ uptake from such hypoxic water is not sufficient to meet metabolic demand.

1.3 Peripheral Respiratory Chemoreceptors and Oxygen Sensing

Respiratory chemoreceptors are bimodal O₂ / CO₂ sensors (fish chemoreceptors also detect ammonia) that initiate the aforementioned hypoxic responses necessary for maintaining metabolic homeostasis. Vertebrate chemoreceptors share the following three characteristics; proximity to the environment being detected, innervation, and synaptic vesicles for neurotransmitter storage and release (Gonzalez et al., 1994). The peripheral respiratory chemoreceptors in mammals are type I (glomus) cells of the carotid body (CB) (Lopez-Barneo, 2003; Nurse, 2010), neuroepithelial bodies (NEBs) in pulmonary epithelia, and adrenomedullary chromaffin cells (AMCs), which are active during the perinatal period (Thompson et al., 1997; Cutz et al., 2012; Salman et al., 2013). In fish, the peripheral respiratory chemoreceptors are NECs (Dunel-Erb et al., 1982; Jonz and Nurse, 2004; Burleson et al., 2006; Qin et al., 2010; Zhang et al., 2011).

There are two models for the specific downstream effects that follow O₂ sensing which lead to the hypoxic ventilatory response. The first is known as the mitochondrial hypothesis (Lahiri et al., 2006), which is based on the premise that O₂ is the final electron acceptor in the electron transport chain and that hypoxia results in the production of reactive oxygen species (ROS). According to this model, changes in mitochondrial activity may generate O₂ sensing mechanisms. There is stronger evidence for the second model, known as the membrane hypothesis (Lopez-Barneo et al., 2008) which incorporates the following key components;

- 1) Inhibition of membrane K^+ channels, leading to membrane depolarization.
- 2) An increase in intracellular calcium concentration $[Ca^{2+}]$.
- 3) Release of neurotransmitter from synaptic vesicles onto afferent nerve fibers.

In mammals, the source of Ca^{2+} is extracellular which is transported into the cell by voltage-dependent Ca^{2+} channels, whereas at least in zebrafish, the source of Ca^{2+} is thought to be intracellular stores (Abdallah et al., 2015).

1.3.1 Mammalian Peripheral Respiratory Chemoreceptors

The CB is located at the bifurcation of the carotid artery and consists of a cluster of type I (glomus) cells. These cells are innervated by glossopharyngeal and vagus (cranial nerves IX and X) afferent nerve fibres that send sensory signals to the respiratory center in the brain stem (Ortega-Saenz et al., 2013). Many neurotransmitters and neuromodulators have been implicated in the physiological responses of CB cells to hypoxia, the most prominent being acetylcholine (ACh), adenosine triphosphate (ATP), and dopamine (DA). These transmitters are released onto afferent nerve fibres and act together to produce the net effects observed with exposure to acute hypoxia (Kahlin et al., 2014; Nurse, 2005; Zhang et al., 2000). ATP is a pivotal excitatory neurotransmitter (Kumar and Prabhakar, 2012; Kahlin et al., 2014) and DA is thought to be an inhibitory neuromodulator (Iturriaga and Alcayaga, 2004). Interestingly, species specific effects have been observed for ACh, which is excitatory in rats and cats, but inhibitory in rabbits (Shirahata et al., 2007). The CB also increases in size in response to prolonged hypoxia (McGregor et al., 1984).

Unlike the densely clustered type I glomus cells of the CB, NEBs are a dispersed network of chemoreceptor cells located throughout pulmonary epithelia. These chemoreceptors are innervated by afferent vagus nerves and 5-HT is the key neurotransmitter regulating the response

to hypoxia (Van Lommel, 2009). NEBs contain tryptophan hydroxylase (tph), the rate-limiting enzyme in 5-HT synthesis and 5-HT is released from NEBs in response to hypoxia (Cutz et al., 1993; Fu et al., 2002). The exact physiological function of NEBs is unknown, but these cells are thought to undergo functional transitions throughout development. Because the carotid body is immature during early development, the NEBs may play a key role in the hypoxic response spanning the fetal to postnatal period of development (Cutz et al., 2012). The O₂ sensing mechanism in NEBs involves NADPH oxidase that is linked to O₂ sensitive K⁺ channels (Wang et al., 1996).

AMCs are functional O₂ chemoreceptors during the perinatal period, a period of time during which CB chemoreceptors are immature. These cells release catecholamines (adrenaline, noradrenaline and dopamine) in response to hypoxia to initiate a suite of responses necessary for survival during perinatal development, including lung fluid absorption, surfactant secretion, and cardiac regulation (Thompson et al., 1997; Nurse et al., 2009). The release of catecholamines is a non-neurogenic response until splanchnic innervation of the AMCs occurs around day 3 of postnatal development (Cheung, 1990; Levitsky and Lopez-Barneo, 2009).

1.3.2 Neuroepithelial Cell Development and Morphology

Neurosecretory epithelial cells (NECs) were first identified in the gill epithelium (Dunel-Erb et al., 1982; Jonz and Nurse, 2003). 5-HT positive NECs have been identified in all fish species studied to date (Perry et al., 2009; Jonz et al., 2015). These cells, which are morphologically similar to type I CB cells are trimodal O₂ / CO₂ / NH₃ receptors (Jonz et al., 2004; Qin et al., 2010; Zhang et al., 2011) located on the leading edge of the gill filament in close association with the efferent filament artery, allowing O₂ detection in post-lamellar oxygenated blood as well as from the inspired water (Zachar and Jonz, 2012). NECs are

extensively innervated and contain synaptic vesicles. Extrinsic and intrinsic nerves innervate NECs, but only extrinsic nerve fibers are involved in the hypoxic ventilatory response (Jonz and Nurse, 2005). Additionally, NECs are innervated by afferent and efferent nerve terminals (Bailly et al., 1992; Bailly, 2009).

Several studies have measured the effects of chronic hypoxia or hyperoxia on ventilation and NEC morphology. Exposure to chronic hypoxia had no influence on the V_f of zebrafish exposed subsequently to acute hypoxia, whereas chronic hyperoxia significantly blunted the V_f of zebrafish exposed to acute hypoxia (Vulesevic and Perry, 2006). In adult zebrafish, chronic hypoxia leads to an increase in the size of NECs and the density of non-serotonergic NECs, with no change in the density of serotonergic NECs (Jonz et al., 2004). Exposure to chronic hyperoxia results in a decrease in the density of serotonergic NECs (Vulesevic et al., 2006). In another study, zebrafish raised in hypoxia from embryo to 7 dpf exhibited a decrease in the number of serotonergic NECs (Shakarchi et al., 2013)

Zebrafish are anoxia tolerant until 24 hpf, at which point they become hypoxia sensitive (Padilla and Roth, 2001; Mendelsohn et al., 2008). The demand for O_2 greatly increases at 2 dpf (Barrionuevo and Burggren, 1999). Gill structures begin to develop at 3 dpf (Jonz and Nurse, 2006). Innervated NECs are first present in the gill arch at 3 dpf. At 5 dpf filament NECs appear, but do not become innervated until 7 dpf (Jonz and Nurse, 2006). Cutaneous respiration accounts for nearly all gas exchange in zebrafish up to 14 dpf, and the gills do not become the primary site of respiratory gas exchange until 21 dpf (Rombough, 2002). Acute hypoxia causes an increase in V_f as early as 3 dpf (Jonz and Nurse, 2005). Zebrafish also respond to hypoxia as early as 2 dpf by increasing whole body and pectoral fin movements (Rombough, 1988; Jonz and Nurse, 2005; Coccimiglio and Jonz, 2012). These developmental aspects demonstrate that

extrabranchial chemoreceptors are responsible for sensing and initiating physiological responses to hypoxia in early larval development.

NECs are located throughout the skin of developing zebrafish, with a peak density at 3 dpf (Coccimiglio and Jonz, 2012). There is a gradual developmental reduction in skin NECs as this cell population transitions to the gills. This normal developmental loss of skin NECs is blunted in fish exposed to chronic hypoxia, and accelerated in fish exposed to chronic hyperoxia (Coccimiglio and Jonz, 2012). Skin NECs are innervated and are thought to share the same O₂ sensitive properties as gill NECs. Exposure of larval zebrafish to the neurotoxin 6-OHDA blunted the hyperventilatory response to acute hypoxia (Coccimiglio and Jonz, 2012). The change in skin NEC density with exposure to hypoxia and hyperoxia, and the blunted hyperventilatory effect resulting from the chemical ablation of nerves connected to NECs implicate the skin as the primary site of chemoreceptor prior to the development of functional gills.

1.4 The Role of Respiratory Gasotransmitters in Oxygen Sensing

Gasotransmitters are endogenously produced gases that act as signaling molecules and are thought to contribute to O₂ sensing (Prabhakar, 1998; Perry et al., 2016). Not much is known about the role of these gases in respiratory chemoreception in fish, but recent studies have begun to elucidate their roles in O₂ sensing pathways. The three gasotransmitters that have been studied thus far are carbon monoxide (CO), nitric oxide (NO), and hydrogen sulfide (H₂S).

Carbon Monoxide

The majority of CO is produced from the enzyme heme oxygenase (HO). The two HO isoforms that have been characterised in fish are HO-1 and HO-2 (Perry and Tzaneva, 2016; Perry et al., 2016). HO-1 has been identified in the gills of zebrafish and European sea bass

(*Dicentrarchus labrax*) (Prevot-D'Alvise et al. 2013; Li et al. 2015) and in a sub-population of NECs in larval zebrafish (Tzaneva and Perry, 2016). Inhibition of HO-1 increases the ventilation frequency in goldfish exposed to acute hypoxia and acclimated to 7°C, but has no effect on goldfish exposed to acute hypoxia and acclimated to 25°C (Tzaneva and Perry, 2014). HO-1 inhibition also increased the ventilation frequency in larval zebrafish (4 dpf) exposed to acute hypoxia (Tzaneva and Perry, 2016). CO might function by regulating the level of intracellular Ca^{2+} in NECs, similar to what has been reported in mammalian CB cells (Overholt et al., 1996; Abdallah et al., 2014; Perry and Tzaneva, 2016). The inhibitory effect of CO, as demonstrated in the ventilation measurements mentioned above, could occur as a result of a decrease in intracellular Ca^{2+} and a subsequent decrease in neurotransmitter release.

Nitric Oxide

Nitric Oxide is synthesised from L-arginine by the activity of nitric oxide synthase (NOS). There are two NOS isoforms in fish, neuronal NOS (nNOS) and inducible NOS (iNOS) (reviewed by Donald et al., 2015). Immunolabelling shows that nNOS colocalises with serotonergic NECs in both larval and adult zebrafish (Porteus et al., 2015). Sodium nitroprusside (SNP), a NO donor, significantly blunted the hyperventilatory response to hypoxia in adults. In contrast, SNP did not affect the hyperventilatory response to hypoxia, but significantly increased V_f in normoxic larvae (Porteus et al., 2015). Additionally, adult zebrafish injected with L-NAME, a NO inhibitor, displayed a blunted hypoventilatory response to hyperoxia. In larvae, the hypoxic ventilatory response was inhibited in larvae exposed to L-NAME and those experiencing nNOS gene knockdown (Porteus et al., 2015). Overall, these results demonstrate an inhibitory effect of NO in adult zebrafish, and an excitatory effect in larvae. In mammals, NO plays an inhibitory role in the CB by limiting the release of ACh (Campanucci and Nurse, 2007).

NO may also have an excitatory role in the CNS as it promotes rhythm generation (Pierrefiche et al., 2002).

Hydrogen Sulfide

Two main enzymes, cystathionine- β -synthase (CBS) and cystathionine- γ -lyase (CSE), are known to produce H₂S from L-cysteine. Both enzymes are found in zebrafish and trout gills as well as the skin NECs of larval zebrafish (Olson et al., 2008; Porteus et al., 2014). Trout gills homogenates produce H₂S when exposed to cysteine, and the production of H₂S is prevented with the addition of CBS and CSE inhibitors (Olson et al., 2008). Buccal injection of H₂S elicits hyperventilation and bradycardia in rainbow trout (*Oncorhynchus mykiss*). Removal of the first gill arch pair eliminates the bradycardia response to H₂S (Olson et al., 2008). Exposure to H₂S also elicits depolarisation in isolated zebrafish NECs, which is the same effect observed in NECs exposed to hypoxia (Jonz et al., 2004; Olson et al., 2008). A more recent study demonstrated that CBS and CBE gene knockdown inhibited the hyperventilatory response to hypoxia in larval zebrafish (Porteus et al., 2014). The same study also demonstrated that exposure to Na₂S increased intracellular [Ca²⁺] in zebrafish NECs. The ventilation and intracellular Ca²⁺ data demonstrate an excitatory role of H₂S in promoting hyperventilation in zebrafish. The exact mode of action is unclear, but a number of possibilities have been suggested. The first is that H₂S interacts with membrane K⁺ channels, which leads to membrane depolarisation and neurotransmitter release (Telezhkin et al., 2010). Another model relates to H₂S metabolism; oxidation of H₂S occurs in mitochondria, therefore decreased O₂ slows its oxidation and results in H₂S accumulation. It is therefore suggested that the increase in H₂S resulting from low O₂ levels could ultimately initiate hyperventilation (Olson et al., 2008). Lastly, H₂S increases the level of circulating catecholamines (Perry et al., 2009), which is another possible explanation for

the hyperventilatory response to increased H₂S.

1.5 Putative Neurotransmitters in Fish NEC Chemoreception

Various neurotransmitters have been implicated in the O₂ chemosensory pathways in fish. The most studied thus far include 5-HT, ACh, ATP and catecholamines. Several studies have utilised a combination of immunohistochemistry and pharmacology (agonists and antagonists) to explore the location and general effects of these transmitters. The following section will provide a review of each of these transmitters, including their location and general effects in fish, with a focus on 5-HT and its role as the putative neurotransmitter regulating the hypoxic ventilatory response.

Acetylcholine

The vesicular acetylcholine transporter (VACHT) is used as a marker for ACh. VACHT is not present in the NECs of rainbow trout, goldfish (*Carassius auratus*), or mangrove rivulus (*Kryptolebias marmoratus*) (Regan et al., 2011; Porteus et al., 2013). However, VACHT is present in the extrinsic nerve fibers of the gill in goldfish and trout. The presence of neuronal ACh suggests that these neurons may act as reciprocal synapses that modulate the response to hypoxia by increasing the amount of neurotransmitter release (Porteus et al., 2012). VACHT is present in zebrafish NECs, but not until 14 dpf, suggesting that ACh may play a role in the chemoreceptor response later in development when the gills are fully innervated (Shakarchi et al., 2013). ACh also causes vasoconstriction in the efferent filament artery and arterioles of trout. Vasoconstriction leads to a decrease in blood flow to the gill filaments and a reduction in PaO₂, which may further increase the rate of neurotransmission from the chemoreceptors of the gill (Olson, 2002; Porteus, 2012).

The emersion response of the mangrove rivulus, a behavior that occurs naturally in

response to hypoxia, occurred at a higher O₂ concentration with pre-exposure to ACh (Regan et al., 2011). Administration of ACh also caused an increase in ASR (see section 1.1) in adult zebrafish exposed to hypoxia (Abdallah et al., 2015). Larval zebrafish exposed to nicotine, a cholinergic receptor agonist, responded with an increase in ventilation frequency (Rahbar et al., 2016).

ATP

P2X₃ is an ATP receptor, and P2X₃ positive cells are located in a morphologically distinct subset of serotonergic NECs that are not innervated (Jonz and Nurse, 2003). A more recent study localised P2X₃ in serotonergic neurons and NECs in gill arches (Rahbar et al., 2016). P2X₃ is also present in a subset of non-serotonergic neurons and NECs (Rahbar et al., 2016). Purinergic antagonists attenuate the hyperventilatory response in zebrafish at 14-16 dpf (Rahbar et al., 2016). This suggests that ATP may act on afferent nerve terminals of NECs and may also exhibit paracrine roles in O₂ chemoreception through interaction with P2X₃ receptors.

Catecholamines

Of all the fish species studied to date, tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis has only been found in the NECs of catfish (*Ictalurus punctatus*) (Zaccone et al., 2003; Porteus et al., 2013). Staining for TH was dispersed in the pseudobranch of trout (Porteus et al., 2013). Injection of cyanide (used to induce chemical hypoxia) into the buccal cavity of rainbow trout increased the levels of circulating catecholamines (Reid and Perry, 2003). Exogenous application of dopamine (DA) also inhibited the normoxic ventilation frequency of larval zebrafish (Shakarchi et al., 2013).

Serotonin

A study by Bailly et al. (1992) was the first to demonstrate that NECs contained 5-HT.

Since then, additional studies have described the precise location and morphology of these cells throughout development stages in various fish species, including zebrafish (Jonz et al., 2004; Coccimiglio and Jonz, 2012). Serotonergic NECs have been found in all teleost species studied to date (Perry et al., 2009). In zebrafish, serotonergic NECs are found throughout the skin during early larval stages. At 7 dpf, serotonergic NECs of gill arches and filaments are fully innervated; a population of non-serotonergic NECs is also present in the arches at this stage (Jonz and Nurse, 2006; Shakarchi et al., 2013). In adult zebrafish, gill arches only contain serotonergic NECs, whereas the gill filaments contain a subset of non-serotonergic NECs (Shakarchi et al., 2013). Additionally, chronic hyperoxia was shown to decrease the density of serotonergic filament NECs (Vulesevic et al., 2006).

The rate limiting enzyme in serotonin synthesis is tryptophan hydroxylase (tph), which hydroxylates L-tryptophan to form 5-hydroxytryptophan, which is then converted to 5-HT by aromatic amino acid decarboxylase. Zebrafish express three tph paralogs, tph1a, tph1b, and tph2 (Lillesaar, 2011), which is in contrast to the presence of two tph paralogs in mammals, tph1 and tph2. The additional tph paralog in zebrafish likely resulted from a genome duplication event that took place at the base of teleost evolution (Meyer and Schartl, 1999). In mammals, tph1 is predominately expressed in peripheral tissue, whereas tph2 is centrally located in brain tissue, such as the raphe neurons (Gaspar and Lillesaar, 2012). In zebrafish, tph1a is expressed in the hypothalamus, pineal organ, retina, and spinal cord, tph1b is expressed in the pineal gland and preoptic area, and tph2 is expressed in the mid-hindbrain complex and the pineal organ (Lillesaar et al., 2009; Gaspar and Lillesaar, 2012; Herculano and Miximino, 2014). Interestingly, a recent study demonstrated transient expression of tph1a in intraspinal serotonergic neurons that preceded tph2 expression (Montgomery et al., 2016). Tph1a is present in the intraspinal neurons

at 48 hours post fertilisation (hpf), but at 96 hpf, these neurons express *tph2* instead of *tph1a* (Montgomery et al., 2016). It should also be noted that serotonergic neurons do not have to actively produce 5-HT, because they can absorb 5-HT from the surrounding environment via serotonin transporters (Jafari et al., 2011).

Although 5-HT release from NECs has never been demonstrated directly, there is ample indirect evidence suggesting that 5-HT is a critical initial neurotransmitter mediating the cardiorespiratory responses following hypoxia detection by NECs. As previously discussed, NECs respond to hypoxia by depolarisation resulting from K^+ channel inhibition (Jonz et al., 2004). 5-HT and hypoxia have similar effects on neural activity in isolated trout gill nerve fibers (Burlison and Milsom, 1995). In zebrafish larvae, exogenous 5-HT application initiated hyperventilation and at 10 dpf, the hypoxic hyperventilatory response was blunted by the addition of ketanserin, a 5-HT₂ receptor blocker (Shakarchi et al., 2013). The amphibious fish, mangrove rivulus, which can switch to air breathing in response to hypoxia (see section 1.1) emerged at a higher PO₂ when exposed to 5-HT. This emersion occurred at a lower PO₂ with the addition of ketanserin (Regan et al., 2011). In zebrafish, aquatic surface respiration (see section 1.1) increased in response to 5-HT, a behavior that is also observed during hypoxia (Abdallah et al., 2015).

The majority of serotonergic NECs are extrinsically innervated, although populations of intrinsic neurons also innervate these cells (Jonz and Nurse, 2003). 5-HT is thought to exhibit paracrine regulation, which could suggest a functional role for intrinsic innervation of NECs. Intra-arterial injection of 5-HT in trout caused vasoconstriction (Fritsche et al., 1992). Additionally, 5-HT was shown to have a neuromodulatory role in mammals (Nurse, 2010). The extrinsic innervation of serotonergic NECs and the data summarised in this section point to 5-HT

as a putative neurotransmitter involved in cardiorespiratory reflexes such as hyperventilation. Evidence for its role is largely based on localisation of 5-HT using immunohistochemistry and pharmacological stimulation or inhibition of 5-HT receptors. While these methods demonstrate the potential significance of 5-HT, more direct evidence is needed to clarify the functional significance of 5-HT in regulating cardiorespiratory responses to hypoxia.

1.6 Hypothesis and Research Objectives

The purpose of this research is to examine the role of 5-HT in regulating reflex hyperventilatory responses to hypoxia. The hypothesis being tested is that 5-HT is a key neurotransmitter regulating the hyperventilatory response to hypoxia in larval zebrafish. The research methods used to address this hypothesis are as follows:

1) Localisation of each of the tph paralogs in zebrafish larvae using immunohistochemistry and confocal microscopy. It is predicted that a combination of tph1a and/or tph1b will colocalise with serotonergic NECs.

2) Inhibition of the production of 5-HT in NECs through pharmacological treatment or gene knockdown. It is predicted that exposing larval zebrafish to the non-selective tph inhibitor, p-chlorophenylalanine (pCPA) (Koe and Weissman, 1966; Airhart et al., 2012) will reduce 5-HT within NECs. Additionally, gene knockdown using antisense morpholino oligonucleotides targeting tph will prevent 5-HT production in NECs.

3) Measurement of ventilation frequency of larval zebrafish to compare the hypoxic response of fish with normal and diminished levels of 5-HT in NECs. It is predicted that zebrafish with reduced expression of 5-HT within NECs will have an attenuated ventilatory response when exposed to acute hypoxia.

CHAPTER 2

THE ROLE OF SEROTONIN (5-HT) IN REGULATING THE HYPOXIC HYPERVENTILATORY RESPONSE OF LARVAL ZEBRAFISH

2.1 Introduction

An ability to respond to changes in the ambient partial pressure of oxygen (PO_2) is necessary for metabolic homeostasis in vertebrates, but of particular importance in fish because fluctuations in dissolved O_2 levels are more common in aquatic environments (Diaz and Breitburg, 2009). The sensing and responses to changes in environmental and/or internal PO_2 are mediated by peripheral chemoreceptors. In mammals, Type I (glomus) cells of the carotid body (CB), neuroepithelial bodies (NEBs), and adrenomedullary chromaffin cells (AMCs) are examples of O_2 / CO_2 sensitive respiratory chemoreceptors (Thompson et al., 1997; Lopez-Barneo, 2003; Cutz et al., 2010; Nurse, 2010; Salman et al., 2013). Although relatively few species have been examined (zebrafish, *Danio rerio*; channel catfish, *Ictalurus punctatus*; goldfish, *Carassius auratus* and rainbow trout, *Oncorhynchus mykiss*), it is generally assumed that in fish, neurosecretory epithelial (neuroepithelial) cells (NECs) function as O_2 sensitive chemoreceptors (Jonz et al., 2004; Burlison et al., 2006; Zachar and Jonz, 2012) and are thought to be homologous to Type I cells of the CB (Jonz and Nurse, 2009). In zebrafish, the NECs are trimodal sensors of O_2 , CO_2 and NH_3 (Qin et al., 2010; Abdallah et al., 2015; S.F. Perry, unpublished data). In rainbow trout, the NECs also were implicated in NH_3 sensing (Zhang et al., 2011). NECs have been found in all teleost species studied to date (Saltys et al., 2006; Coolidge et al., 2008; Perry et al., 2009; Porteus et al., 2012; Zachar and Jonz, 2012). Fish respond to low levels of O_2 (hypoxia) by increasing ventilation volume, reducing heart rate (bradycardia), and increasing systemic vascular resistance (Randall and Shelton, 1963; Holeton and Randall, 1967; Hughes, 1973; reviewed by Perry et al., 2009). The hypoxic ventilatory response arguably is the most physiologically significant of these responses (Perry et al., 2009).

In adult zebrafish, NECs are primarily located on the leading edge of the gill filament in

close association with the efferent filament artery, allowing for O₂ detection from post-lamellar oxygenated blood as well as from the inspired water (Jonz and Nurse, 2003; Zachar and Jonz, 2012). Gill NECs, however, are not innervated until 7 days post fertilisation (dpf) (Jonz and Nurse, 2005) yet the hyperventilatory response to hypoxia is present as early as 3 dpf (Jonz and Nurse, 2005) suggesting the presence of extrabranchial respiratory chemoreceptors in larval zebrafish. Indeed, the NECs of the skin are presumed to be the putative peripheral respiratory chemoreceptors (Coccimiglio and Jonz, 2012) prior to maturation of the gill. Innervated NECs are present throughout the skin of larval zebrafish beginning at 24 hours post fertilisation (hpf) and reach a peak density at 3 dpf (Coccimiglio and Jonz, 2012). After 3 dpf there is a gradual reduction in skin NECs as this cell population transitions to the gills. The normal developmental loss of skin NECs was blunted in zebrafish exposed to chronic hypoxia, and accelerated in zebrafish exposed to chronic hyperoxia (Coccimiglio and Jonz, 2012; Shakarchi et al., 2013).

Relatively little is known about the downstream effects leading to the hypoxic ventilatory response in fish. In zebrafish, there is strong evidence for the membrane hypothesis, which suggests that hypoxia causes background potassium channel inhibition, leading to membrane depolarisation (Jonz et al., 2004), an increase in intracellular calcium (Ca²⁺) (Abdallah et al., 2015), and neurotransmitter release (for review see Jonz et al., 2015). Various neurotransmitters have been implicated in O₂ sensing pathways in fish, although their precise roles remain unclear. The neurotransmitters studied thus far include serotonin (5-hydroxytryptamine; 5-HT; Regan et al., 2011; Shakarchi et al., 2013; Abdallah et al., 2015), acetylcholine (ACh; Regan et al., 2011; Shakarchi et al., 2013; Abdallah et al., 2015), adenosine triphosphate (ATP; Jonz and Nurse, 2003; Rahbar et al., 2016) and dopamine (DA; Porteus et al., 2013; Rahbar et al., 2016) (reviewed by Jonz et al., 2015), as well as the gasotransmitters hydrogen sulfide (H₂S; Porteus et

al., 2014), nitric oxide (NO; Porteus et al., 2015), and carbon monoxide (CO; Tzaneva and Perry, 2014; 2016) (reviewed by Perry et al., 2016; Perry and Tzaneva, 2016).

There is indirect evidence that NEC 5-HT is a critical neurotransmitter mediating the cardiorespiratory responses following hypoxia detection. Innervated serotonergic NECs are present as early as 24 hpf (Coccimiglio and Jonz, 2012) and multiple studies have found that exogenous 5-HT caused behaviours resembling those observed in response to hypoxia, such as hyperventilation and aquatic surface respiration (ASR) (Regan et al., 2011; Shakarchi et al., 2013; Abdallah et al., 2015; Rahbar et al., 2016). In zebrafish, application of ketanserin, a 5-HT₂ receptor blocker, blunted the hypoxic hyperventilatory response (Shakarchi et al., 2013) and increased ASR (Abdallah et al., 2015).

Evidence for a role of 5-HT in the respiratory chemoreceptor pathway is largely based on its localisation using immunohistochemistry and pharmacological stimulation or inhibition of 5-HT receptors. While these methods demonstrate the potential overall significance of 5-HT, they cannot distinguish the effects of 5-HT potentially released from the peripheral NECs versus 5-HT acting at one or more downstream sites including the central nervous system and motor neurons. Thus, direct evidence is needed to clarify the functional significance of 5-HT within the NECs in initiating the cardiorespiratory responses to hypoxia. Tryptophan hydroxylase (tph) is the rate-limiting enzyme in 5-HT synthesis. Zebrafish express three tph paralogs; tph1a, tph1b and tph2 (Lillesaar, 2011). The purpose of this study was to determine which tph paralog is responsible for 5-HT production in NECs to allow a reverse genetics approach to prevent the expression of 5-HT in NECs during early development. We hypothesised that 5-HT is the key neurotransmitter released from NECs and which initiates the reflex hyperventilatory responses to hypoxia. It is predicted that larval zebrafish with diminished 5-HT in NECs will exhibit a blunted

hyperventilatory response to acute hypoxia.

2.2 Materials and Methods

2.2.1 Zebrafish Care

Wild-type adult zebrafish (*Danio rerio*) were raised at the University of Ottawa aquatic care facility where they were kept in 10 l acrylic tanks in closed, re-circulating flow-through aquatic housing systems (Aquatic Habitats, Apopka, FL, USA). The tanks were supplied with aerated dechloraminated City of Ottawa tap water and maintained at a 12h:12h light:dark cycle at 28°C. For zebrafish breeding, adult zebrafish were placed in a 2 l tank and male and female fish were separated by a divider using a ratio of 1 male: 2 females (Westerfield, 2000). Zebrafish were acclimated in the tanks overnight, and the following day the water was changed and the divider removed to allow for spawning. Embryos were collected using a fine mesh net and placed in a petri dish containing E3 embryo medium (0.33 mM CaCl₂·2H₂O, 0.17 mM KCl, 0.33 mM MgSO₄·6H₂O, 5 mM NaCl, 0.0001% methylene blue). Embryos were stored in an incubator maintained at 28°C, and embryo medium was changed daily until the appropriate developmental age was reached for experiments (4 dpf). Care and handling of zebrafish was performed according to the University of Ottawa Veterinary Service and Canadian Council on Animal Care (CCAC) guidelines.

2.2.2 Immunohistochemistry

Zebrafish larvae were euthanised at 4 dpf using MS-222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma Aldrich Inc.) and subsequently fixed overnight at 4°C in phosphate buffered saline (PBS) solution containing 4% paraformaldehyde (PFA). Larvae were then rinsed a total of 5 X in 1X PBS solution containing 0.1% tween-20 (PBST), and then placed in a 1:1 mixture of PBST:100% methanol for 5 min at room temperature. Larvae were then placed in

100% methanol for 5 min, followed by another 1:1 mixture of PBST:100% methanol for 5 min. Larvae were then rinsed 5 X with PBST. Antigen retrieval was performed by placing larvae in a 150 mM tris-HCl solution (pH 9.0) for 10 min at room temperature, followed by 15 min at 65°C. Larvae were placed in a blocking solution containing 0.8% Triton-X 100 and 2% bovine serum albumin (BSA) dissolved in PBS for 1 h at room temperature. Primary antibodies (Table 1) were added and larvae were placed on a shaker overnight at 4°C. Larvae were then rinsed 5 X 5 min in PBST. Secondary antibodies (Table 1) were added to a solution containing 0.8% Triton-X 100 in PBST and left in the dark at room temperature for 1 h. Larvae were rinsed 5 X 5 min in PBST and placed in a concave depression on a glass slide for imaging. Images were taken using a confocal microscope with the appropriate lasers for each fluorophore (Nikon Instruments Inc., USA) and analysed using Nikon Elements software (Nikon Instruments Inc., USA).

Table 1. Primary and Secondary Antibodies used for Immunohistochemistry

Antibody	Dilution	Antigen	Host	Source	Secondary antibody
Primary					
5-HT	1:250	serotonin	rabbit	Sigma	Alexa 488
5-HT	1:250	serotonin	goat	ImmunoStar	Alexa 594
tph1a	1:250	tph1a	rabbit	GenScript ¹	Alexa 488
tph1b	1:250	tph1b	rabbit	GenScript ¹	Alexa 488
tph2	1:250	tph2	rabbit	GenScript ¹	Alexa 488
zn-12	1:100	neuronal cell surface	mouse	DSHB ²	Alexa 546 / CF633
Secondary					
Alexa 488	1:500	rabbit	donkey	Thermo Fisher	
Alexa 546	1:200	mouse	goat	Thermo Fisher	
Alexa 594	1:500	goat	donkey	Thermo Fisher	
CF 633	1:200	mouse	donkey	Sigma	

¹Custom antibody produced by GenScript

²University of Iowa, Developmental Studies Hybridoma Bank

2.2.3 Western Blots / Protein Quantification

Zebrafish larvae (4 dpf) were homogenised (10 μ L per larva) in RIPA buffer (150 mM NaCl, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride; PMSF) with the addition of protease inhibitor (1 tablet per 10 mL RIPA buffer; Roche, USA). A bicinchoninic acid (BCA) assay was used to determine protein content. A dilution series of protein was prepared, whereby it was determined that 12.5 μ g was the appropriate loading amount for gel electrophoresis based on the linear dynamic range (Taylor and Posch, 2014). Protein samples were separated using a 12% TGX Stain-Free FastCast Acrylamide gel (Bio-Rad, USA). The gel was activated for 1 min under UV light to visualise total protein prior to membrane transfer. The separated samples were then transferred to a PVDF membrane (Bio-Rad, USA) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, USA) and the total protein transferred on the membrane was visualised. The membrane was blocked in a 5% milk solution for 2 h at room temperature. The membrane was then added to a 2% milk solution containing a 1:1,000 dilution of the antibody of interest (tph1a, tph1b, or tph2; Table 1) and placed at 4°C overnight. The membrane was rinsed 3 X 10 min in a solution containing 0.1% tween-20 in 1X TBS (TBST) and probed with a goat anti-rabbit secondary antibody (1:5000; Pierce, USA) for 1 h at room temperature. Additional 3 X 10 min TBST rinses were performed and the membrane was imaged after 2 min exposure to Luminata Classico Western HRP Substrate (EMD Millipore, Germany). The abundance of the protein of interest and the total amount of protein in each lane were measured and normalised to a pooled sample to obtain the relative density (ImageLab software; Bio-Rad, USA).

2.2.4 Antisense Morpholino Injection

Zebrafish embryos were injected at the border of the cell and yolk sac at the one cell

stage with an antisense oligonucleotide (Gene Tools, LLC) designed to bind to the translation start site of *tph1a* (5'-TCGAGTACATGCTGATGTTTGCTGA-3'). Morpholino stock was diluted in 1X Danieau buffer [5.0 mmol l⁻¹ Hepes (pH 7.6), 58 mmol l⁻¹ NaCl, 0.4 mmol l⁻¹ Ca(NO₃)₂, 0.7 mmol l⁻¹ KCl], and 0.05% phenol red. The working stock of morpholino was 8ng nl⁻¹; 1 nl was injected into each embryo. A dose of 8 ng was chosen because it was the lowest injection dose that significantly reduced *tph1a* protein expression. Injection needles were calibrated prior to use to ensure accuracy and precision of injections. On each day of injections, a separate group of sham embryos was injected with 1 nl of a control morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3') prepared to a final concentration of 8 ng nl⁻¹. This morpholino has no known target sequence in zebrafish. The purpose of the sham treatment was to control for the effects of microinjection and nucleotide loading. After injection, embryos were placed in a petri dish containing E3 medium and maintained in an incubator at 28°C.

2.2.5 Ventilation Frequency (Vf) Measurements

At 4 dpf, larvae were placed in a perfusion chamber and mildly anesthetised using 0.06 mg ml⁻¹ MS-222 adjusted to pH 7.4. Larvae were provided continuously with temperature controlled water (28°C) flowing at a rate of 125 ml h⁻¹. Levels of dissolved O₂ in the water were controlled by continuous gassing of the water with gas mixers provided by digital mass flow controllers. Hypoxia (partial pressure of O₂ (PO₂) 30 mmHg) was achieved by bubbling a mixture of 19% air and 81% nitrogen into the water. For all experiments which monitored breathing, larvae were first exposed to normoxic (154 mmHg) water and given 10 min to recover after being placed into the perfusion chamber prior to recording ventilation. They were then exposed to 5 min of continuing normoxia followed by 30 min of hypoxia. Larval breathing movements were recorded for the duration of the experiment using a video camera (3CCD

camera, Dage-MTI, USA) mounted to a dissecting microscope (CMZ1500, Nikon, USA) and connected to a computer. Videos were captured using CyberLink Power Director Software (Santa Clara, CA, USA). V_f was measured by counting the number of opercular movements. Normoxic values were recorded as the average V_f during the entire 5 min exposure. During hypoxia, opercular movements were counted for 60 sec every 5 min (total of six data points during hypoxia; 6 total counts), and the peak V_f was used for data analysis.

A separate group of embryos was exposed to hypoxia and 5-HT. These experiments followed the standard protocol except that fish were exposed to an additional 30 min of hypoxic water (30 mm Hg) containing 50 μ M 5-HT. This concentration was based on a previous study that measured the effects of exogenous 5-HT on V_f in zebrafish larvae (Shakarchi et al., 2013).

2.2.6 Exposure to pCPA

Fish were treated with the general tph inhibitor p-chlorophenylalanine (pCPA; CAS number 14173-40-1, Sigma Inc.) to deplete NECs of 5-HT. The protocol for pCPA exposure was based on methods outlined in Airhart et al. (2012). Zebrafish were raised in petri dishes containing embryo medium and dechorionated at 23 hpf. Larvae were then exposed to 100 μ M pCPA dissolved in embryo medium from 24 to 48 hpf. The pCPA concentration of 100 μ M was selected on the basis of preliminary experiments using a range of pCPA levels because it was the lowest concentration to consistently reduce the number of 5-HT positive cells on the skin. A separate group of fish was dechorionated but not treated with pCPA to control for the effects of dechoriation. At 48 hpf, fish were placed back in standard embryo medium until they were used for ventilation experiments and immunohistochemistry at 4 dpf.

2.2.7 Cell Counts

Immunohistochemistry was used to label serotonergic NECs (see immunohistochemistry

section 2.2) of *tph1a* morpholino injected, sham injected, pCPA treated and untreated dechorionated larvae. Rabbit 5-HT primary antibody and Alexa 488 secondary antibody were used for immunolabelling (Table 1). For each fish, the eye closest to the viewing surface, the ventral region of the yolk sac and the posterior region of the tail were imaged. The surface area and total number of serotonergic NECs of each image were counted using Nikon Elements software (Nikon Instruments Inc., USA).

2.2.8 Statistical Analysis

Sigma-Plot (version 12.2, SPSS Inc.) was used for all statistical analyses. Data are represented as means \pm SEM. All ventilation frequency measurements were analysed using two way repeated measures analysis of variance (ANOVA) followed by a Holm-Sidak post-hoc test when significant differences were detected. Protein quantification and cell count data were analysed using a t-test. The limit of significance for all analyses was 0.05.

2.3 Results

2.3.1 Antibody Specificity

A western blot was performed to test the specificity of the tph1a morpholino and to ensure it was not reducing the levels of tph1b or tph2 protein. Western blots using protein extracted from sham-injected and tph1a MO larvae and probed separately with each of the three tph antibodies (Fig. 1A), revealed a distinct band at 55 kDa in all lanes except for the tph1a MO protein lane that was probed with tph1a antibody (Fig. 1D). These results confirm that the tph1a antibody was indeed specifically detecting tph1a and that tph1a morpholino was specific for the single tph1a paralog.

2.3.2 Localisation of Tryptophan Hydroxylase (tph) Paralogs in Larval Zebrafish

The distribution of each tryptophan hydroxylase (tph) paralog was determined by immunohistochemistry using primary antibodies designed for each paralog (Fig. 1A). Larvae were also immunolabelled using serotonin (5-HT) and zn-12 primary antibodies. Co-labelling of tph1a and zn-12 revealed a string-like tph1a distribution within many zn-12 positive neurons (Fig. 2). Triple staining for tph1a, zn-12 and 5-HT revealed a similar string-like distribution of tph1a positive nerves innervating neuroepithelial cells (NECs) (Fig. 3G-I, Fig. 4). Additionally, tph1a was expressed within a sub-population of NECs (Fig. 3G, I; Fig. 4). A distinct fibre-like pattern of tph1a was also observed, which did not colocalise with zn-12 or 5-HT (Fig. 3H).

Immunolabelling for tph1b was not observed in the tail region (Fig. 5A-C), however, labelling was present in one or more distinct cell types in the yolk sac which were 5-HT negative and which resembled ionocytes (Appendix 1). All 5-HT positive NECs were tph1b negative. Immunolabelling for tph2 showed staining in neuromast cells that colocalised with 5-HT, however, tph2 was not detectable in 5-HT positive NECs (Fig. 5D-F).

2.3.3 Neuroepithelial Cells in para-chlorophenylalanine (pCPA) Treated Larvae

A 5-HT primary antibody was used to identify 5-HT positive NECs in pCPA treated and control larvae at 4 days post fertilisation (dpf). Representative images of the eye, yolk sac and tail revealed diminished 5-HT positive NEC labelling in pCPA treated larvae (Fig. 6A-B). Quantitative analysis revealed that the density of 5-HT positive NECs was significantly reduced in pCPA treated larvae (25.7 ± 6.2 cells mm^{-2}) compared to control larvae (189.2 ± 17.6 cells mm^{-2} ; Fig. 6C).

2.3.4 Ventilation Frequency (V_f) in pCPA Treated Larvae

Exposure to acute hypoxia significantly increased the V_f of control larvae from 25.9 ± 6.4 to 102.3 ± 22.1 breaths min^{-1} . The hypoxic hyperventilatory response was abolished in pCPA treated larvae (normoxic $V_f = 34.1 \pm 5.8$ breaths min^{-1} ; hypoxic $V_f = 40.1 \pm 8.1$ breaths min^{-1} ; Fig. 7A). The addition of 5-HT to hypoxic fish partially recovered the hypoxic ventilatory response in pCPA treated larvae (Fig. 7B). The combined exposure to acute hypoxia and exogenous 5-HT caused a significant increase in V_f (from 40.9 ± 4.5 to 105.7 ± 9.6 breaths min^{-1} ; Fig. 7B). Addition of 5-HT did not affect the hypoxic hyperventilatory response of control fish (hypoxic V_f was 146 ± 7.4 and 138.3 ± 15.6 breaths min^{-1} in hypoxic fish without and with 5-HT, respectively). Despite the increase in V_f associated with the addition of 5-HT to pCPA treated hypoxic fish, the hyperventilatory response remained partially blunted when compared to control fish (138.3 ± 15.6 and 105.7 ± 9.6 breaths min^{-1} in control and pCPA treated fish, respectively; Fig. 7B).

2.3.5 Confirmation of *tph1a* Knockdown

Protein extracted from 4 dpf larvae revealed a distinct band at approximately 55 kDa corresponding to the expected size of *tph1a* (Fig. 1B). The relative expression of this protein was

diminished approximately 10 fold in *tph1a* MO larvae compared to shams (Fig. 1B-C). This band was absent in both preadsorption (using the synthetic peptide antigen) and preimmune serum controls (Fig. 1B). A band was also detected at about 15 kDa in both *tph1a* MO and sham-injected larvae (Fig. 1B). This band was still present in the pre-adsorption controls, but disappeared in the pre-immune serum controls (Fig. 1B).

Knockdown of *tph1a* was also confirmed by performing immunohistochemistry using the *tph1a* primary antibody. Immunoreactivity for *tph1a* was present in shams, whereas no immunoreactivity for *tph1a* was observed in *tph1a* MO larvae (Fig. 1E-F).

2.3.6 NECs in *tph1a* Morphant (MO) Larvae

A primary antibody for 5-HT was used to label 5-HT positive NECs in *tph1a* MO and sham-injected 4 dpf larval zebrafish. Representative images of the eye, yolk sac and tail revealed diminished 5-HT positive NEC labelling in *tph1a* MO larvae compared to shams (Fig. 8A-B). Quantitative analysis demonstrated that the density of 5-HT positive NECs was significantly reduced in *tph1a* MO larvae (47.8 ± 13.1 cells mm^{-2}) compared to shams (121.9 ± 24.0 cells mm^{-2} ; Fig. 8C).

Exposure to acute hypoxia significantly increased the V_f of sham-injected larvae from 14.1 ± 5.5 to 97.6 ± 17.2 breaths min^{-1} (Fig. 9A). The hypoxic hyperventilatory response was blunted in *tph1a* MO larvae (normoxic $V_f = 44.6 \pm 6.8$ breaths min^{-1} ; hypoxic $V_f = 68.6 \pm 8.5$ breaths min^{-1} ; Fig. 9A). No significant difference was observed between the hypoxic V_f of *tph1a* MO and sham-injected larvae; however, a significant difference was detected between normoxic treatments for the two groups. Additionally, the magnitude of the hyperventilatory response (the change in V_f (ΔV_f) between normoxic and hypoxic fish) was reduced significantly in the *tph1a* MO fish. Sham-injected larvae exhibited an approximate 3.5 fold increase in ΔV_f compared to

tph1a MO larvae (Fig. 9B). In a separate experiment, the hyperventilatory response to hypoxia was blunted in tph1a MO larvae (normoxic $V_f = 32.2 \pm 10.4$ breaths min^{-1} ; hypoxic $V_f = 43.6 \pm 11.1$ breaths min^{-1} ; Fig. 10), and no significant difference was observed between normoxic treatments. The combined exposure to acute hypoxia and exogenous 5-HT caused a significant increase in V_f (from 32.2 ± 10.4 to 75.7 ± 11.0 breaths min^{-1} ; Fig. 10). Addition of 5-HT did not affect the hypoxic hyperventilatory response of control fish (hypoxic V_f was 139.9 ± 2.8 and 153.0 ± 3.7 breaths min^{-1} in hypoxic fish without and with 5-HT, respectively; Fig. 10).

Figure 1. Tryptophan hydroxylase (tph) antibody specificity. Peptide sequences and alignments for tph1a, tph1b, and tph2 (**A**). The peptide sequence of each antibody is listed first, followed by the peptide sequences of tph1a, tph1b, and tph2. Amino acids that align with the peptide antigen sequence are highlighted in red. Panel **B** shows representative western blot bands for tph1a MO and sham larvae, and pre-adsorption and pre-immune controls. Expression of tph1a was reduced in tph1a MO larvae compared to shams (**C**). An asterisk indicates a significant difference between groups; t-test, $P < 0.05$. $N = 6$. The tph1a morpholino was specific to the tph1a paralog and did not reduce tph1b and tph2 protein expression as shown by western blotting in tph1a MO and sham larvae (**D**). Immunoreactivity for tph1a was detected in the tail of sham larvae (**E**), but not in tph1a MO larvae (**F**). The inserts in E and F show magnified regions of the panels indicated by the dashed box. Arrows indicate tph1a. Scale bar = 50 μm in E-F.

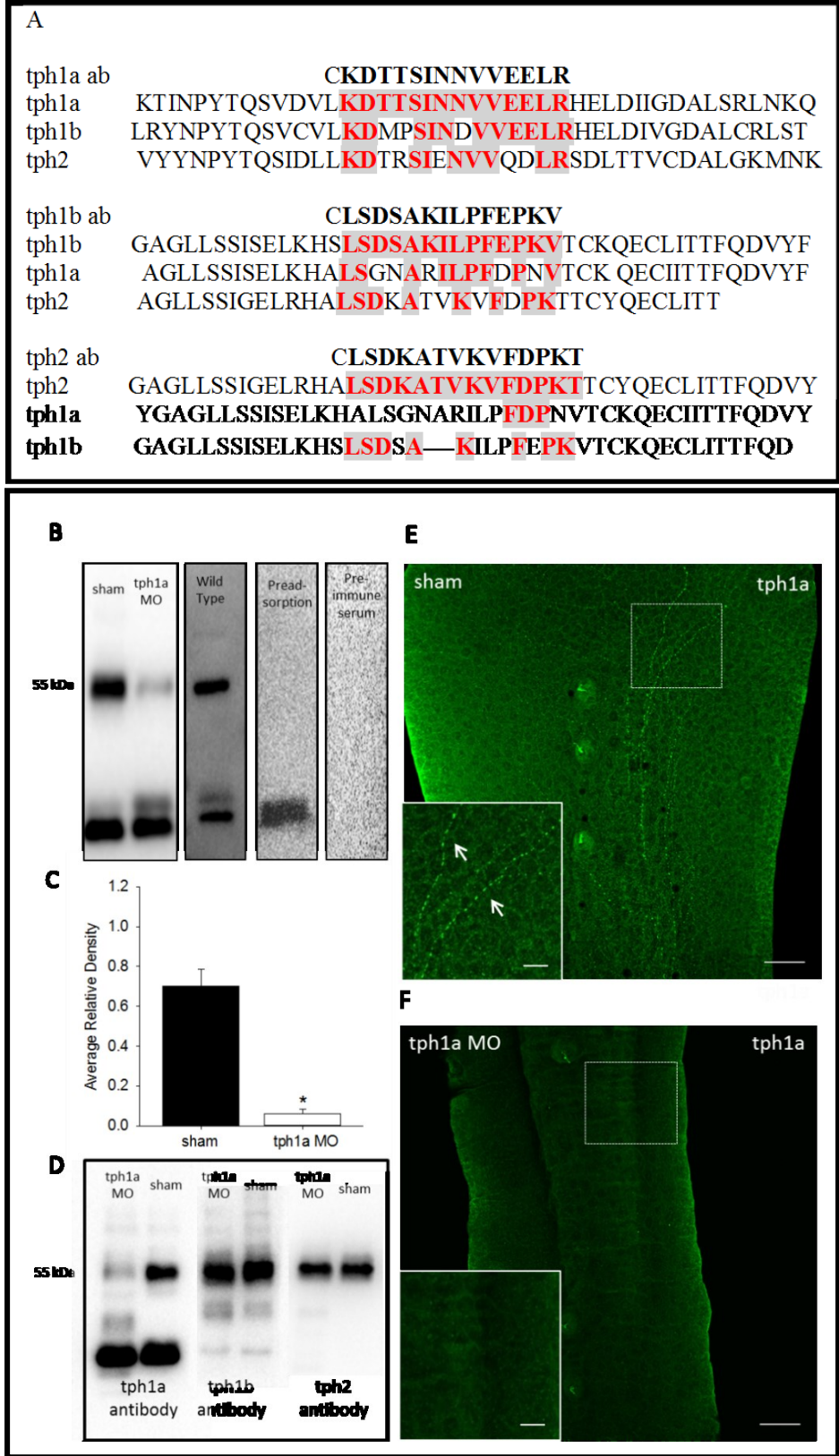


Figure 1

Figure 2. Distribution of tryptophan hydroxylase 1a (tph1a) (**A**) and zn-12 positive nerve fibres (**B**) in the tail of 4 day post fertilisation (dpf) zebrafish larvae. The merged image of tph1a and zn-12 shows the localisation of tph1a in nerve tissue (**C**). Insert **D** is a magnified view of tph1a and zn-12 colocalisation. Panel **E** represents the location from which the images were captured. Arrows indicate tph1a and arrow heads indicate colocalisation of tph1a and zn-12. Scale bars = 50 μm in A-C; 25 μm in D.

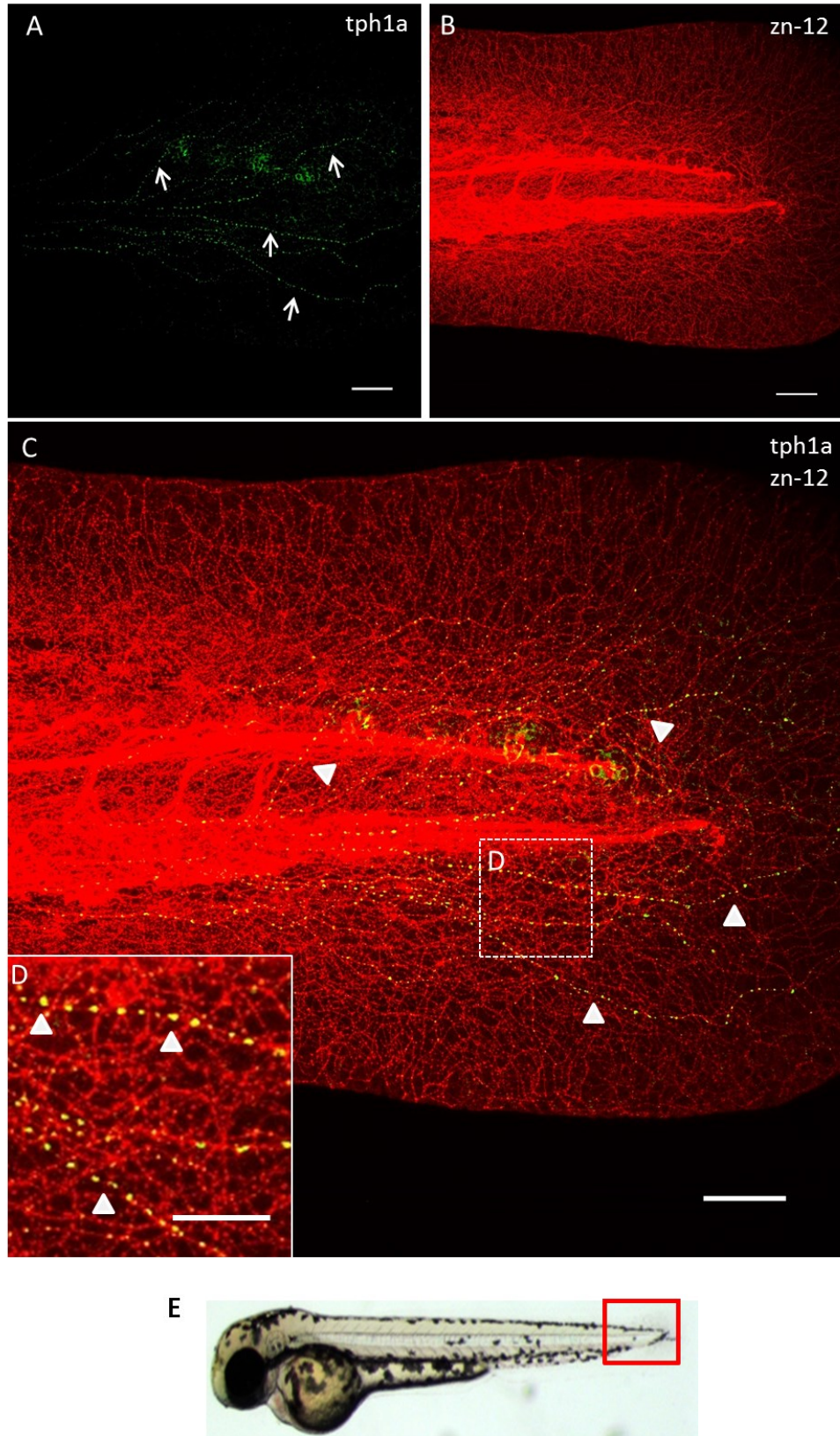


Figure 2

Figure 3. Distribution of tph1a (**A**), serotonin (5-HT) positive neuroepithelial cells (NECs) (**C**), and zn-12 positive nerve fibres (**E**) in the tail of 4 day post fertilisation (dpf) zebrafish larvae. The merged image of tph1a, 5-HT and zn-12 shows the colocalisation of tph1a in nerves innervating NECs and expression of tph1a within a NEC (**G**). Insert **H** is a magnified view of tph1a and zn-12 colocalisation in nerves innervating a NEC. Tph1a also exhibited a distinct fibre-like pattern that did not colocalise with zn-12 or 5-HT. Insert **I** is a magnified view showing the presence of intracellular tph1a within a NEC. Panels **B**, **D**, and **F** are representative negative control images in which the primary antibodies were omitted. Panel **J** represents the location from which the images were captured. Arrows indicate tph1a and 5-HT in panels A and C, and colocalisation of tph1a and 5-HT in panel I. Arrow heads indicate colocalisation of tph1a and zn-12. Hollow double arrows indicate tph1a staining in panel H. Scale bars = 20 μm in A, C, E, G and H; 50 μm in B, D and F; 10 μm in I.

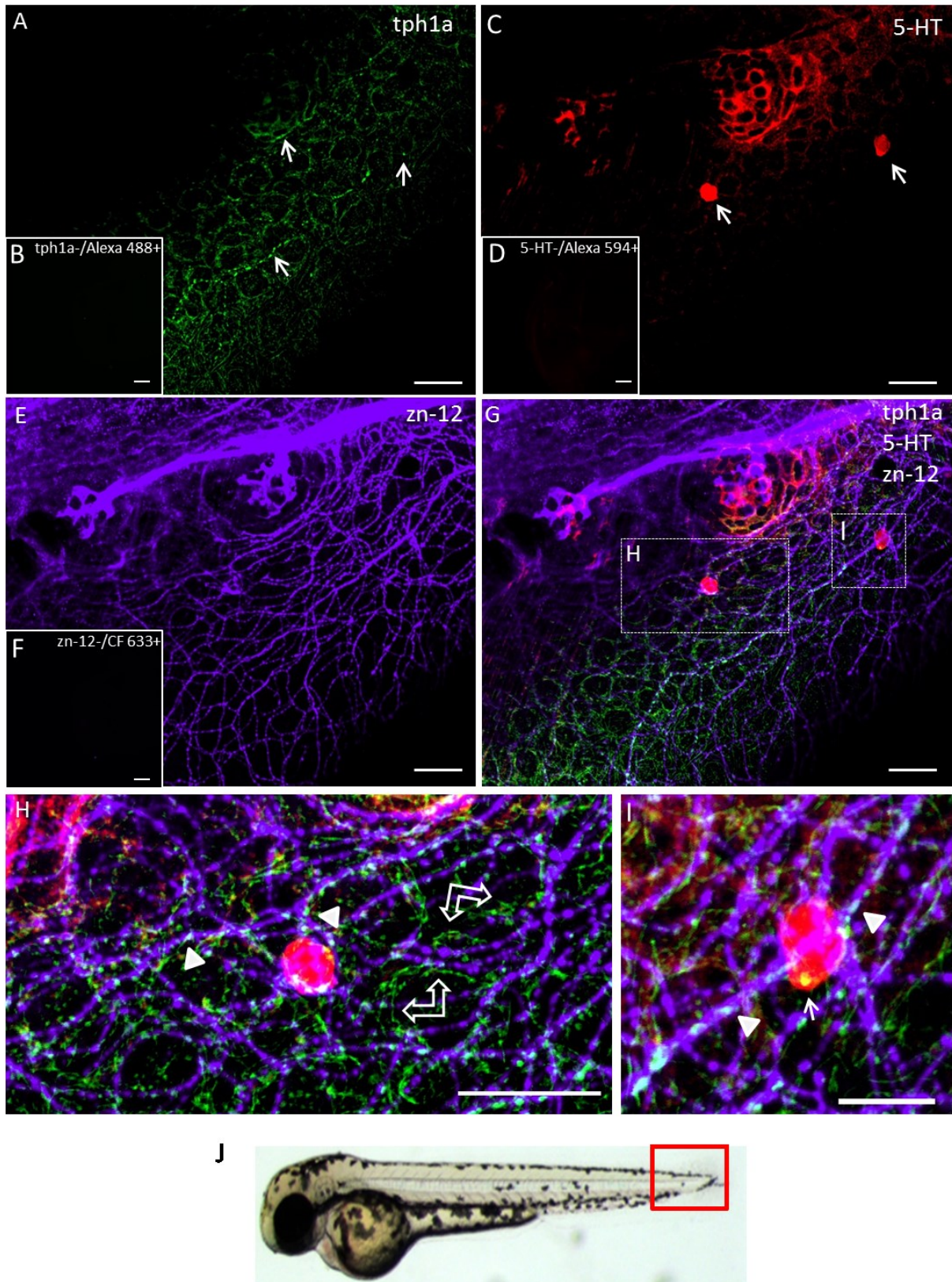


Figure 3

Figure 4. Representative orthogonal and volume views of z-stack series confocal micrograph images taken from panel I in Fig. 3. Panel **A** shows XZ, XY and YZ orthogonal views that illustrate tph1a staining within a 5-HT positive neuroepithelial cell (NEC). Panel **B** shows orthogonal views that illustrate zn-12 positive nerve fibres contacting the 5-HT positive NEC. Panels **C** and **D** are 3D volume views that show tph1a positive nerve fibres contacting a 5-HT positive NEC. Arrows indicate intracellular tph1a. Arrow heads indicate tph1a positive nerve fibres innervating a 5-HT positive NEC. Scale bars = 5 μm in A-D.

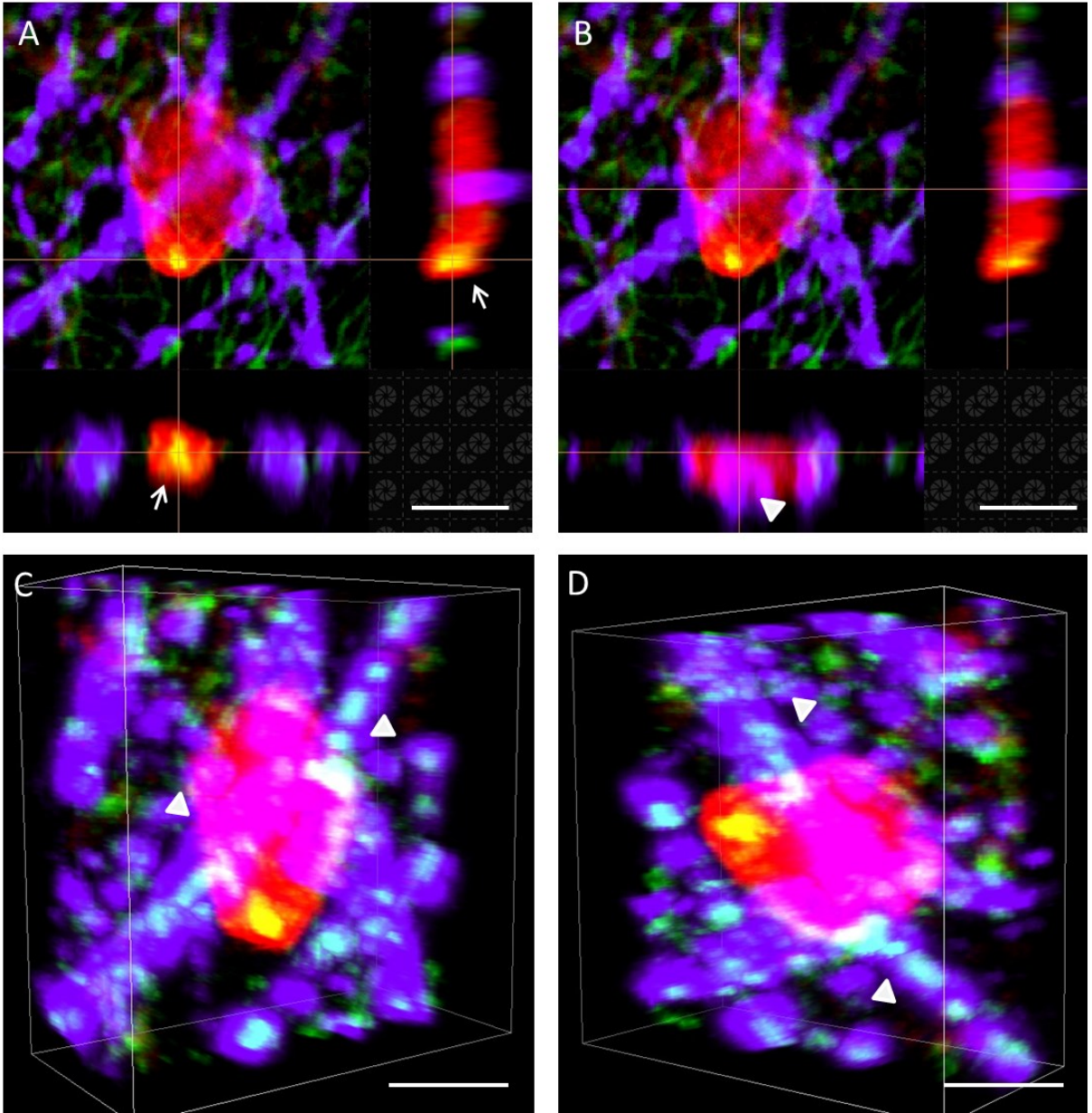


Figure 4

Figure 5. Distribution of tryptophan hydroxylase 1b (tph1b) (**A**), tph2 (**D**) and serotonin (5-HT) (**B** and **E**) in the tail of 4 days post fertilisation (dpf) zebrafish larvae. Panel **A** and the merged image of tph1b and 5-HT (**C**) demonstrate the absence of detectable tph1b expression in the tail region including within 5-HT positive neuroepithelial cells (NECs). The merged image of tph2 and 5-HT shows colocalisation of tph2 and 5-HT in neuromast cells, and no co-expression of tph2 in 5-HT positive NECs (**F**). Panel **G** represents the location from which the images were captured. Arrows indicate tph2 and 5-HT. Arrow heads indicate colocalisation of tph2 and 5-HT. Scale bars = 50µm in A-F

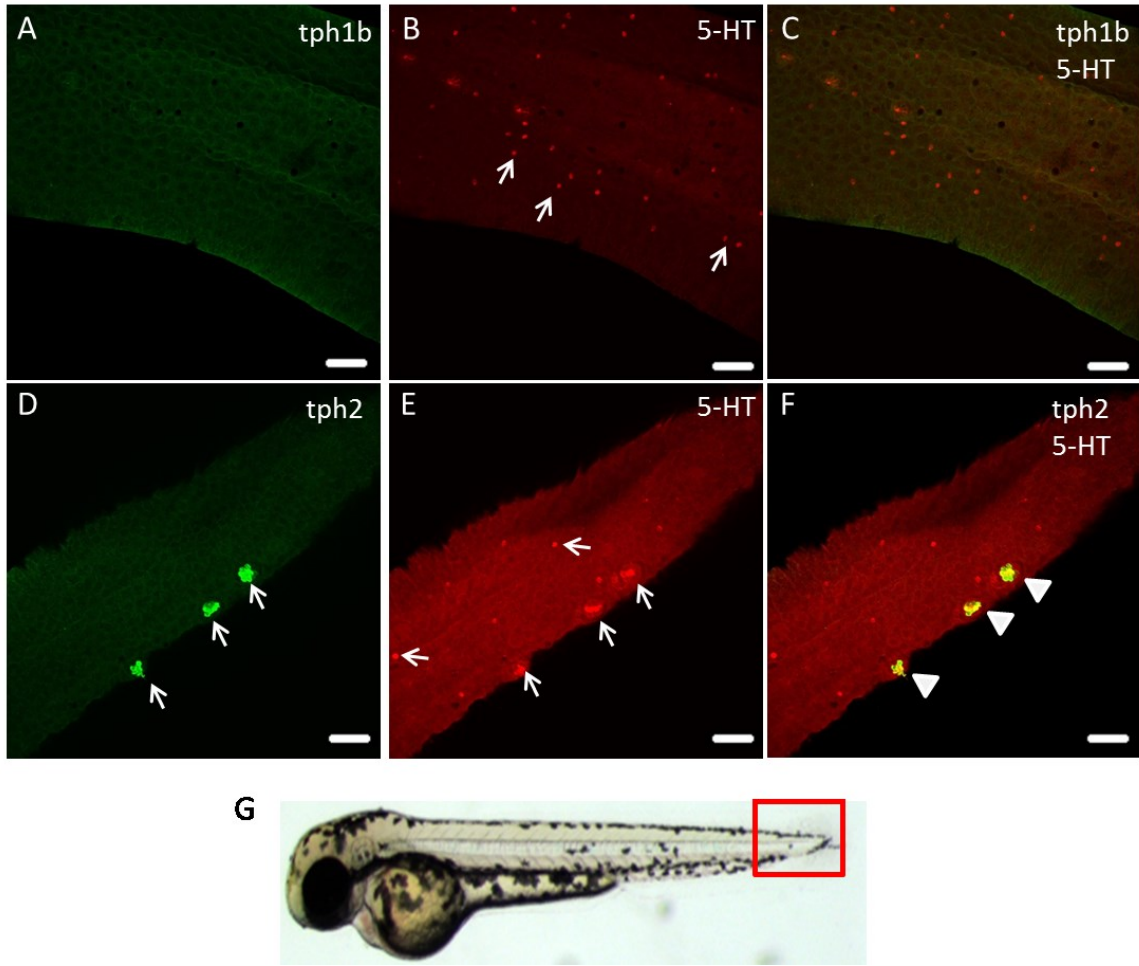
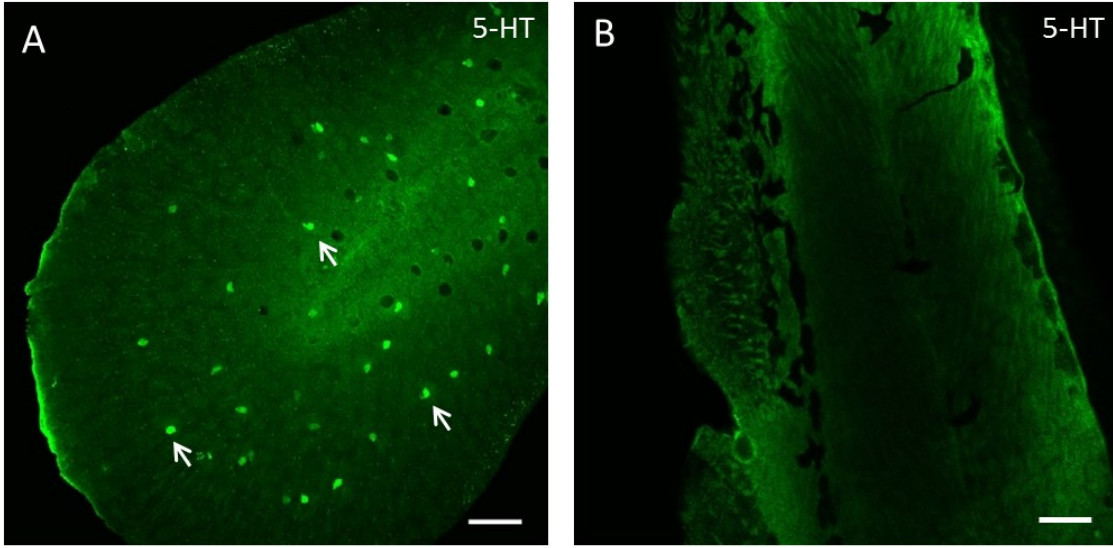


Figure 5

Figure 6. Exposure to pCPA reduces the number of 5-HT positive neuroepithelial cells (NECs) in 4 days post fertilisation (dpf) zebrafish larvae. Representative images of control fish (**A**) and pCPA treated fish (**B**) immunolabelled with a 5-HT primary antibody. There was a decrease in the number of 5-HT positive NECs in larvae treated with pCPA compared to control larvae (**C**). Larvae exposed to pCPA exhibited a reduction in the density of 5-HT positive NECs. An asterisk indicates a significant difference between treatments; t-test, $P < 0.05$. $N = 5$ for pCPA treated, 7 for controls. Arrows indicate 5-HT positive NECs. Scale bars = $50 \mu\text{m}$ in A-B.



C

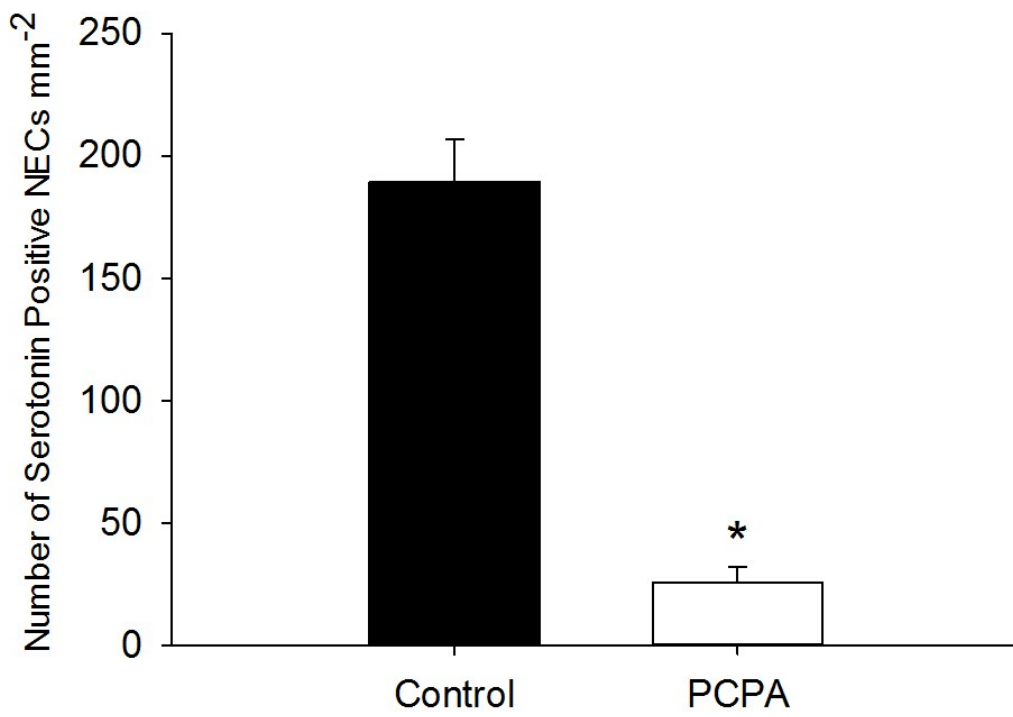
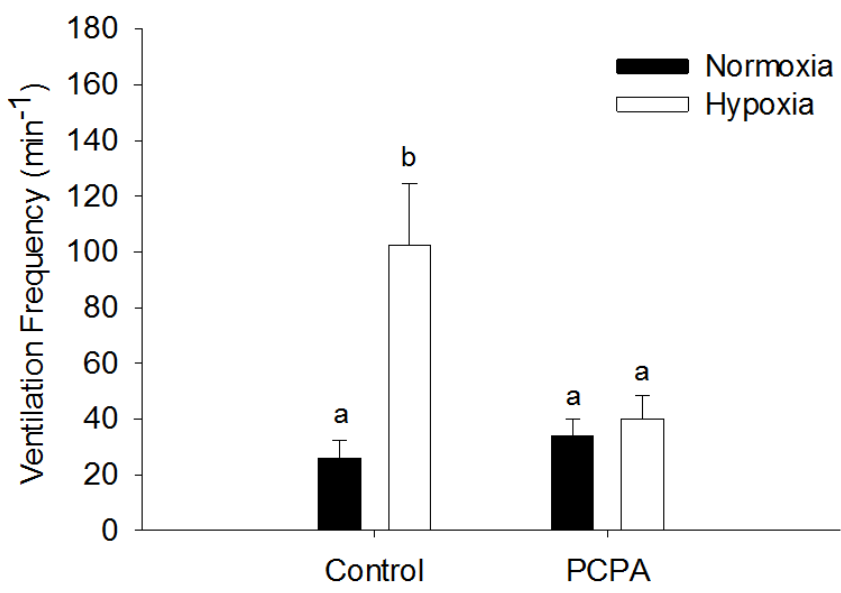


Figure 6

Figure 7. The hypoxic hyperventilatory response was blunted in para-chlorophenylalanine (pCPA) treated fish and partially restored with exposure to exogenous serotonin (5-HT). Ventilation frequency (V_f) in breaths min^{-1} of control and pCPA treated 4 days post fertilisation (dpf) larval zebrafish exposed to normoxia (154 mmHg) followed by hypoxia (30 mmHg) (**A**). Two-way ANOVA, $p < 0.05$. $N = 6$ control, 7 pCPA. Ventilation frequency in breaths min^{-1} of control and pCPA treated 4 dpf larval zebrafish exposed to normoxia (154 mmHg), hypoxia (30 mmHg), and hypoxia (30 mmHg) followed by 50 μM 5-HT (**B**). Different letters indicate significant difference between groups. Two-way repeated measures ANOVA, $P < 0.05$. $N = 6$ control, 7 pCPA.

A



B

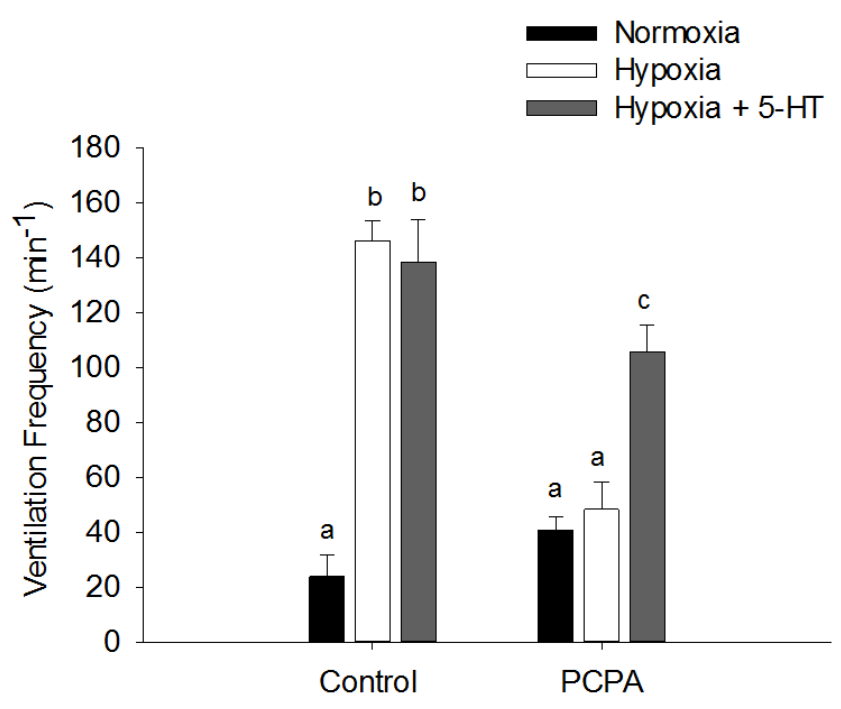


Figure 7

Figure 8. Tryptophan hydroxylase 1a (*tph1a*) gene knockdown reduced the number of serotonin (5-HT) positive neuroepithelial cells (NECs) in 4 day post fertilisation (dpf) zebrafish larvae. Representative images of sham fish (**A**) and *tph1a* morphant (MO) fish (**B**) immunolabelled with a 5-HT primary antibody. There was a decrease in the number of 5-HT positive NECs in *tph1a* MO larvae (N=7) compared to shams (N=6) larvae (**C**). An asterisk indicates a significant difference between groups; t-test, $P < 0.005$. Arrows indicate 5-HT positive NECs. Scale bar = 50 μm in A-B.

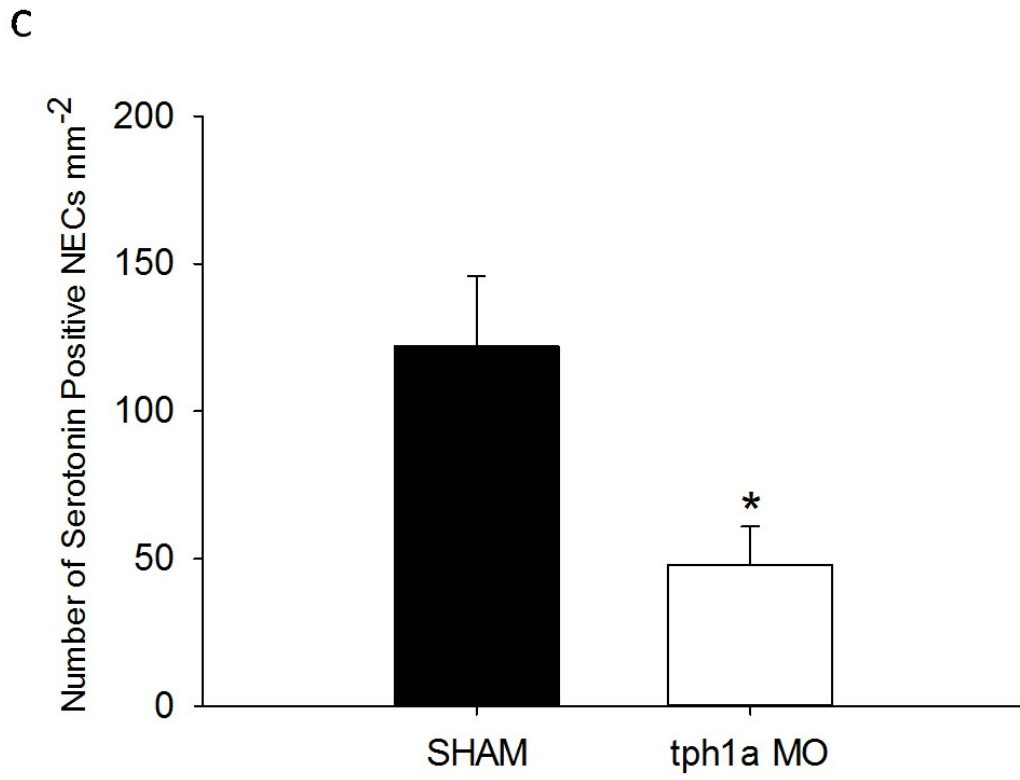
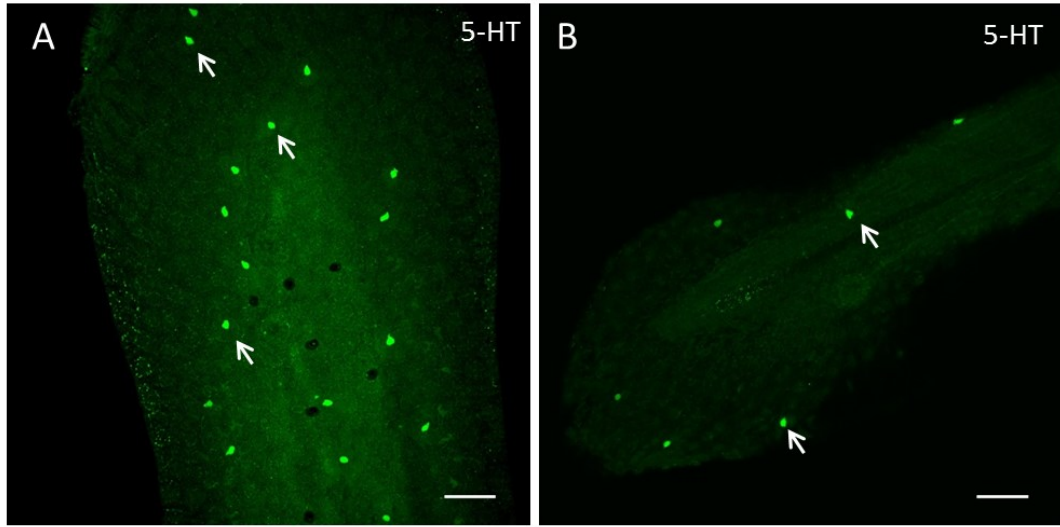


Figure 8

Figure 9. The hypoxic hyperventilatory response was blunted in tryptophan hydroxylase 1a (tph1a) morphant (MO) fish. Ventilation frequency in breaths min^{-1} of sham and tph1a MO larval zebrafish at 4 days post fertilisation (dpf) exposed to normoxia (154 mmHg) followed by hypoxia (30 mmHg) (**A**). Two-way ANOVA, $p < 0.05$; $N = 8$. Change in ventilation frequency as calculated by the difference between normoxic and hypoxic breaths min^{-1} of sham and tph1a MO larvae (**B**). Different letters indicate a significant difference within or between groups (panel A); An asterisk indicates a significant difference between groups in (panel B); t-test, $P < 0.05$.

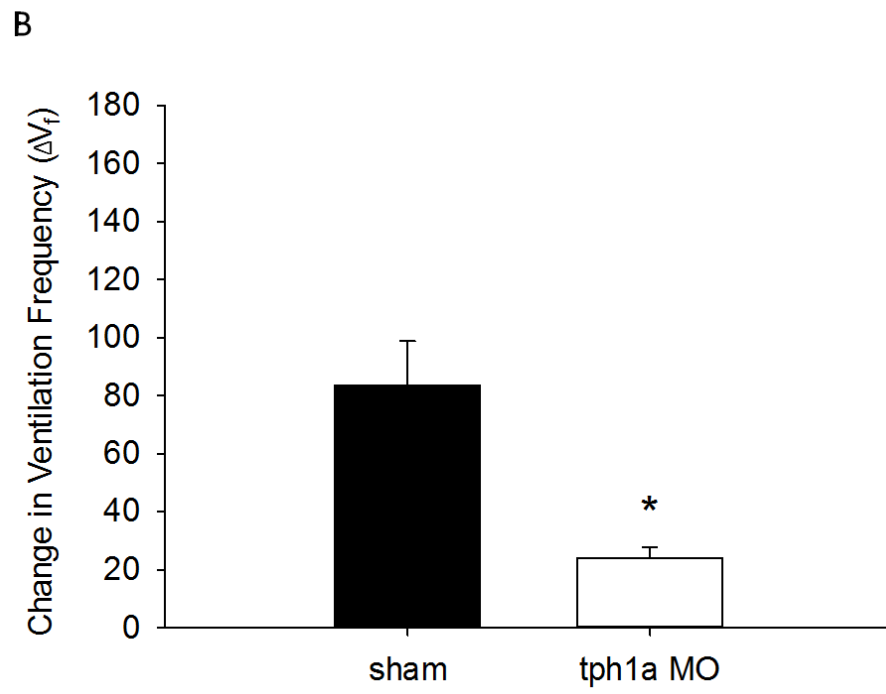
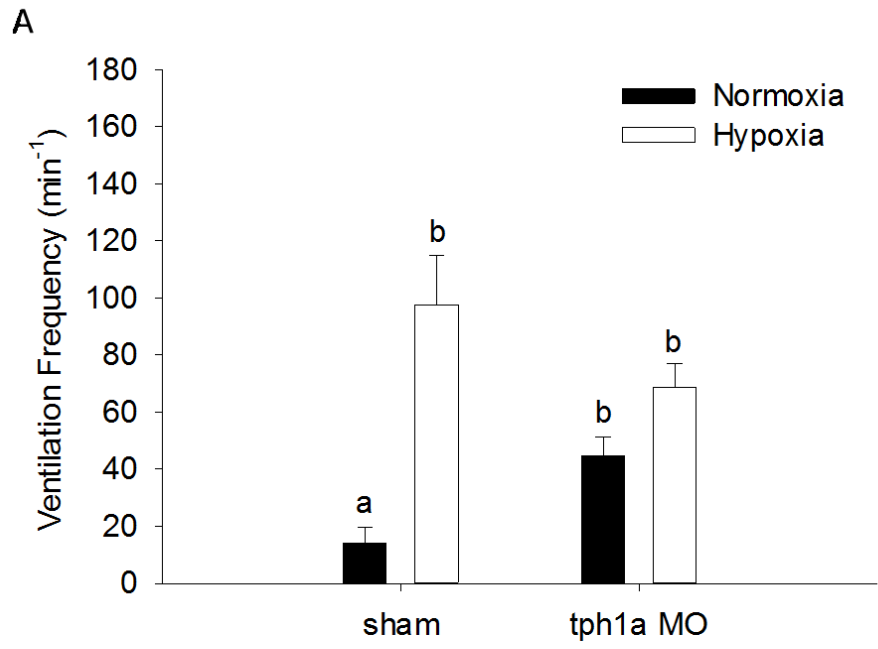


Figure 9

Figure 10. The hypoxic hyperventilatory response was blunted in tryptophan hydroxylase 1a (tph1a) morphant (MO) fish and partially restored with exposure to exogenous serotonin (5-HT). Ventilation frequency in breaths min^{-1} of sham and tph1a MO larval zebrafish at 4 days post fertilisation (dpf) exposed to normoxia (154 mmHg), hypoxia (30 mmHg), and hypoxia (30 mmHg) followed by 50 μM 5-HT. Different letters indicate significant difference between groups. Two-way repeated measures ANOVA, $P < 0.05$. $N = 7$ sham, 7 tph1a MO.

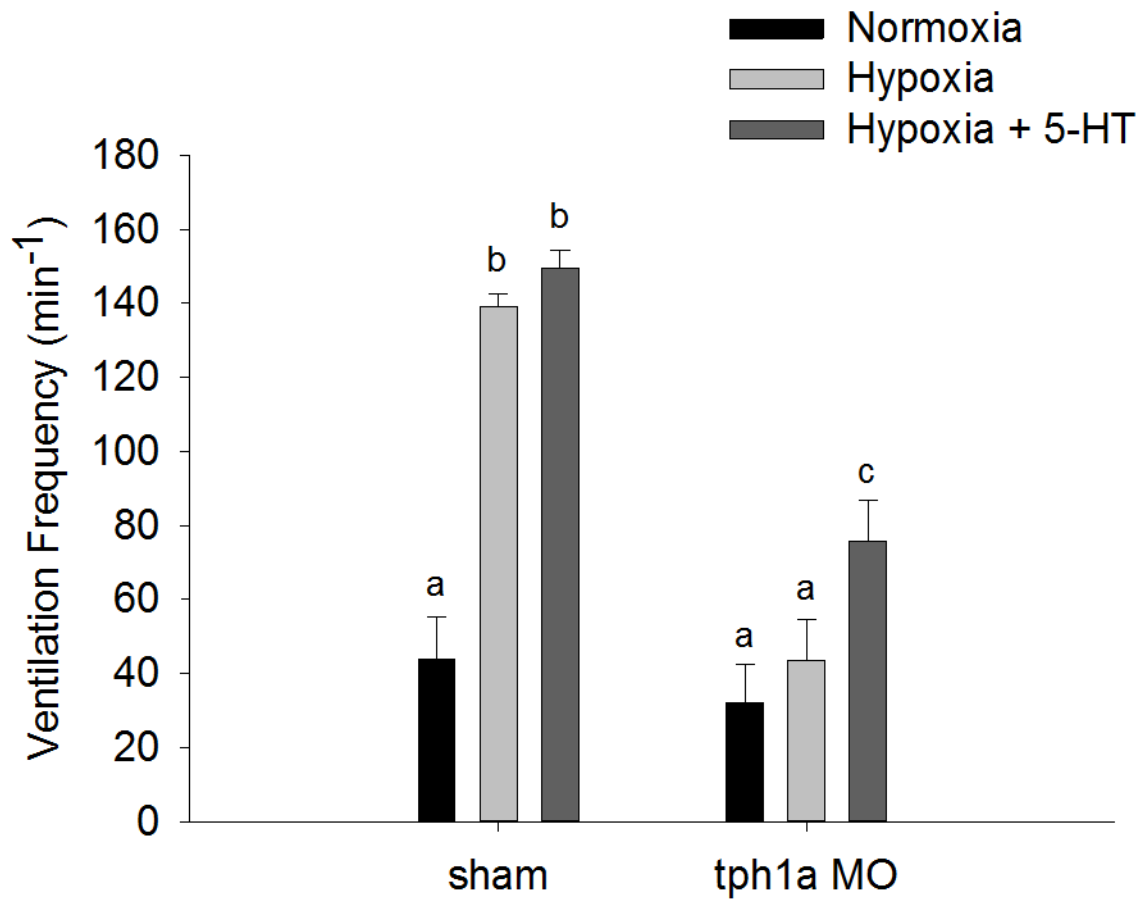


Figure 10

2.4 Discussion

The purpose of this study was to determine the enzymatic source of 5-HT contained within NECs and the role of this NEC 5-HT in the hypoxic hyperventilatory response of larval zebrafish. 5-HT is the predominant neurotransmitter stored within zebrafish NECs (Jonz and Nurse, 2003) and although previous studies have implicated 5-HT in the O₂ chemoreception pathway (Shakarchi et al., 2013; Abdallah et al., 2015), its specific role within this pathway remains largely unknown. Experiments in which exogenous 5-HT was administered *in vivo* revealed the potential for stimulating breathing in larvae after 7 dpf (Shakarchi et al., 2013) or aquatic surface respiration in adults (Abdallah et al., 2015). Moreover, the hyperventilatory response to hypoxia was abolished by the 5-HT₂ receptor antagonist ketanserin in larvae older than 7 dpf (Shakarchi et al., 2013). However, to date, there is no direct evidence that 5-HT specifically stored and released from NECs is the key neurotransmitter promoting hyperventilation. Indeed, previous experiments employing 5-HT receptor agonists or antagonists were unable to distinguish between the potential central effects of 5-HT from those specifically mediated by 5-HT stored within NECs. The main findings of the current study are that i) *tph1a* is responsible for 5-HT production in NECs of larval zebrafish and ii) the hypoxic hyperventilatory response is inhibited in larvae with depleted levels of 5-HT in NECs. Thus, this study is the first to provide direct evidence that 5-HT is a key neurotransmitter released from O₂-sensing NECs and which promotes the reflex hyperventilatory responses to hypoxia.

2.4.1 Tryptophan hydroxylase and 5-HT Synthesis in NECs

Previous studies describing the location of the three zebrafish *tph* paralogs (*tph1a*, *tph1b* and *tph2*) in larval zebrafish focused on their presence in the brain and spinal cord (Bellipanni et al., 2002; Lillesaar, 2011). The focus of this study was to assess the distribution of *tph* paralogs

in the skin NECs of larval zebrafish to determine which paralogs are responsible for 5-HT production in these O₂ chemosensory cells. 5-HT is used as a marker for NECs in both larval and adult zebrafish, though the source and mode of delivery of 5-HT is unknown. 5-HT positive cells can produce 5-HT directly, or take up 5-HT through serotonin transporters (Jafari et al., 2011). In this study, we demonstrate that *tph1a* is the specific paralog responsible for 5-HT production within larval NECs. Immunolabelling of zebrafish larvae with antibodies against 5-HT and *tph1a* revealed co-expression of *tph1a* and 5-HT in a subset of NECs (Fig. 3) where *tph1a* appeared to be densely and specifically localised to specific regions of the cell. Interestingly, the presence of 5-HT in discrete cellular regions was in marked contrast to the broad distribution of 5-HT throughout the NEC. A possible explanation is that *tph1a* is part of a multi-enzyme complex within NECs. The synthetic pathway for L-tryptophan, the substrate that *tph* interacts with to form 5-hydroxytryptophan, is part of a multi-enzyme complex in bacteria, yeasts and plants (reviewed by Dunn et al., 2008). Biosynthesis of L-tryptophan is absent in fish, therefore dietary L-tryptophan is required for 5-HT synthesis. It has been demonstrated that 5-HT production in fish is influenced by the level of circulating L-tryptophan (Johnston et al., 1990), which emphasises the importance of this essential amino acid in the serotonergic system. Multi-enzyme complexes have been shown to decrease reaction rates as a result of the close proximity of enzymes and substrates within these complexes (Welch and Gaertner, 1975). Therefore, it is possible that L-tryptophan and *tph1a* form part of a multi-enzyme complex within NECs that produce 5-HT; once produced, 5-HT can diffuse throughout the cytoplasm and be stored within synaptic vesicles.

In larval zebrafish, skin NECs are extensively innervated (Jonz and Nurse, 2005; Coccimiglio and Jonz, 2012), an essential feature of chemosensory cells. There are multiple

markers for 5-HT producing neurons, such as monoamine oxidase (Mao), L-aromatic amino acid decarboxylase (Aadc), and tph; however, tph is the only marker specific to serotonin, because Mao and Aadc are involved in the synthesis of additional monoamines. Immunolabelling using antibodies against tph1a and zn-12 revealed multiple serotonergic neurons in the tail region of larval zebrafish indicated by the co-expression of tph1a and zn-12 (Fig. 2). Tph1a was dispersed throughout neurons and the greatest staining was found in varicosities, which exhibited a pattern reminiscent of “beads on a string”. Triple immunolabelling using antibodies against tph1a, zn-12 and 5-HT showed that serotonergic (based on tph1a staining; see below) neurons innervate NECs (Fig. 3, 4). Interestingly, tph1a positive / zn12 positive nerve fibres did not co-express 5-HT. The apparent absence of serotonin in tph1a-containing nerve fibres is puzzling because tph is the rate-limiting enzyme for 5-HT synthesis and is the standard marker used to identify serotonergic neurons (Lillesaar, 2011). One possible explanation is that neuronal 5-HT is below the level of detection using standard immunohistochemical techniques. It is unclear whether nerve fibres innervating NECs in zebrafish larvae originate from spinal or cranial nerves. In adult zebrafish, NECs in the gill are innervated by cranial nerves IX and X (glossopharyngeal and vagus) (Jonz and Nurse, 2009).

Immunohistochemistry experiments using antibodies against tph1b and tph2 did not implicate either of these paralogs in the production of 5-HT in NECs. Neither tph1b nor tph2 was localised within 5-HT positive NECs or associated with peripheral zn-12 positive nerve fibres. Because the tph1b and tph2 antibodies detected appropriate sized proteins on western blots and immunoreactivity for tph1b and tph2 was observed in presumptive ionocytes on the yolk sac and in tail neuromasts, respectively, it is unlikely that the absence of tph1b and tph2 staining in NECs can be explained on the basis of non-functional antibodies. Moreover, while in situ hybridisation

revealed the presence of tph1b in the preoptic area and tph2 in the mid-hindbrain (unpublished data; see also Bellipanni et al., 2002; Lillesaar et al., 2008), there was no evidence of staining epithelial cells of the skin. Thus, it highly likely that the 5-HT within zebrafish larvae NECs is derived exclusively from tph1a.

Further evidence for the role of tph1a in 5-HT production in NECs was demonstrated by knockdown of tph1a. Knockdown of tph1a protein expression caused a reduction in the number of 5-HT positive NECs (Fig. 8). Specificity of the tph1a morpholino was also confirmed using western blot analysis to ensure that the reduction in NECs did not result from knockdown of tph1b or tph2. Taken collectively, the findings from immunohistochemical studies and tph1a gene knockdown demonstrate that tph1a is the rate-limiting enzyme responsible for serotonin production in NECs. The presence of intracellular and neuronal tph1a suggest that 5-HT accumulates in NECs either by its synthesis in the NEC itself, or the delivery and uptake (via serotonin transporters) of 5-HT released from the serotonergic fibres innervating the NECs.

2.4.2 Serotonin and the Hypoxic Hyperventilatory Response

Previous studies have shown that exogenous 5-HT can elicit hyperventilatory responses in zebrafish larvae which resemble those observed in response to hypoxia (Shakarchi et al., 2013, Rahbar et al., 2016). The neurotransmitter 5-HT has been observed in NECs of all teleost species and is used as a marker for zebrafish NECs (reviewed by Perry et al., 2009). In this study, we explored for the first time the functional role of 5-HT by comparing the reflex ventilatory responses to hypoxia in control (or sham) larvae with those experiencing specific depletion of intracellular 5-HT in NECs (achieved via translational gene knockdown of tph1a) or widespread (including central) non-specific depletion of 5-HT (achieved via pharmacological inhibition of all tph paralogs). The results clearly demonstrated that the hypoxic hyperventilatory response

was blunted in fish experiencing selective 5-HT depletion in NECs as well as those experiencing widespread depletion.

Larvae exposed to the drug para-chlorophenylalanine (pCPA) from 24 - 48 hpf exhibited a significantly reduced number of 5-HT positive NECs at 4 dpf (Fig. 6) and the hyperventilatory response to hypoxia was abolished in these larvae. The elimination of hyperventilation in fish unable to produce 5-HT implicates 5-HT as the key neurotransmitter regulating the hypoxic hyperventilatory response. To test this idea further, an additional experimental group of pCPA-treated larvae was exposed to acute hypoxia in the presence of exogenous 5-HT to determine whether the hyperventilatory response could be rescued. Indeed, a significant increase in V_f was observed in this group of larvae, further demonstrating the importance of 5-HT in the hyperventilatory response to hypoxia.

Because pCPA is a non-selective tph inhibitor and it was shown that tph1a is exclusively responsible for 5-HT production in NECs, targeted tph1a knockdown was used to selectively deplete 5-HT in NECs. Knockdown of tph1a was clearly effective at 4 dpf, as demonstrated by western blot analysis (Fig. 1). Immunolabelling of tph1a MO larvae showed a significant reduction in 5-HT positive NECs compared to sham-injected larvae (Fig. 8), the same effect that was observed in pCPA-treated fish. While there was a trend toward a small increase in V_f during hypoxia in tph1a MO larvae, the data were not statistically significant ($P = 0.13$; Fig. 9). Additionally, when calculating the change in V_f (ΔV_f) between normoxia and hypoxia, the tph1a MO larvae exhibited an approximate 3.5 fold decrease in ΔV_f compared to sham-injected larvae. Although there was a significant increase in V_f of tph1a MO larvae during normoxia, it is unlikely that the higher resting V_f constrained the ability of these fish to raise V_f further because the average values for hypoxic V_f in fish that mounted a significant hyperventilatory response

were much higher (68.6 breaths min⁻¹ in tph1a MO larvae compared to 97.6, 105.7, 138.3 and 146.0 breaths min⁻¹ in controls and shams). The hyperventilatory response to hypoxia was abolished in tph1a MO larvae, and partially rescued in the presence of exogenous 5-HT. These results, which were similar to those observed in pCPA-treated fish, further demonstrate that 5-HT produced by the tph1a paralog is responsible for eliciting hypoxic hyperventilation. Although the results of the tph1a knockdown experiments indicate that that 5-HT expressed specifically within NECs is the predominant factor regulating hypoxic hyperventilation, it is premature to exclude a role for central 5-HT until future experiments are conducted which target tph1b and tph2 for knockdown.

Mammalian NEBs are known to release 5-HT in response to hypoxia and may possibly play a role during fetal development prior to maturation of the CB, the primary mammalian respiratory chemoreceptor (see section 1.2.1). AMCs are also important respiratory chemoreceptors active during the perinatal period in mammals. Skin NECs of developing zebrafish are similar to NEBs and AMCs in that they are functional chemoreceptors that initiate cardiorespiratory responses in early development prior to maturation of functional gill chemoreception. We have shown that 5-HT is a key neurotransmitter regulating respiratory chemoreception in skin NECs. A large population of gill NECs are also 5-HT positive, so it is possible that our findings may be extended to O₂ chemoreception in adults. However, elucidating the specific role of 5-HT in the adult hypoxic hyperventilatory response will require further study.

2.4.3 The Role of Additional Neurotransmitters and Gasotransmitters

The vesicular acetylcholine transporter (VAChT) was localised to non-serotonergic cells in the gill filament of adult zebrafish and in gill filaments of larvae older than 14 dpf (Shakarchi

et al., 2013). In addition, treatment with ACh induced hyperventilation in late stage larvae (14 – 21 dpf; Shakarchi et al., 2013) and the cholinergic receptor agonist nicotine stimulated V_f in adults (Rahbar et al., 2016). The results of these experiments reveal a potential stimulatory role of ACh in the adult response to hypoxia. While 5-HT acts as the primary excitatory neurotransmitter in larval NECs, other transmitters may impact the ventilatory response to hypoxia through excitatory or inhibitory mechanisms. For example, exposure of larvae to dopamine resulted in an inhibition of V_f (Shakarchi et al., 2013). Additionally, the family of gasotransmitters, H_2S , NO, and CO have all been implicated in the hypoxic ventilatory response through both excitatory and inhibitory mechanisms (reviewed by Perry et al., 2016). The chemoreception pathway in NECs likely involves the interplay of multiple transmitters and signalling molecules, allowing fish to mount appropriate physiological responses to changes in respiratory gases to maintain metabolic homeostasis.

2.4.4 The Significance of Hyperventilation in Larvae and the Mechanism of Action of 5-HT

Skin NECs in developing zebrafish share many of the chemosensory properties of mammalian and gill respiratory chemoreceptors, namely the presence of synaptic vesicles, innervation, and proximity to the sampling environment (Jonz and Nurse, 2005; Coccimiglio and Jonz, 2012). Zebrafish NECs are innervated by 2 dpf, and are abundantly located throughout the skin until the cell population begins to transition to the gills. Bulk diffusion across the skin accounts for nearly all respiratory gas exchange in early larval development (Rombough, 2002) and thus the functional significance of hyperventilation might be to minimise boundary layers by increasing water flow over the cutaneous surface to allow for increased rates of respiratory gas diffusion.

5-HT can act as a typical neurotransmitter or act in a paracrine fashion through release

outside of the synapse (Fuxe et al., 2010). The collective evidence from this study and others examining NEC morphology and the role of 5-HT receptors suggests that the 5-HT expressed in larval NECs acts as a typical neurotransmitter. Ketanserin a 5-HT₂ receptor blocker, inhibited the hypoxic hyperventilatory response in larvae (Shakarchi et al., 2013). 5-HT positive NECs also contain synaptic vesicles, demonstrating a site of neurotransmitter storage in these cells. The findings from this study demonstrate the presence of tph1a positive serotonergic neurons innervating NECs. Collectively, the results of these studies demonstrate the likelihood that 5-HT acts as a typical neurotransmitter, however, further studies are needed to identify the location of specific 5-HT receptors associated with the afferent pathway of the hypoxic ventilatory response. Additionally, NECs have been shown to depolarise in response to hypoxia (Jonz et al., 2004), but no studies have yet demonstrated the release of 5-HT from NECs. Such experiments are required to definitively conclude that 5-HT stored within NECs functions as a typical neurotransmitter.

2.5 Perspectives and Future Directions

This study demonstrates the importance of 5-HT in initiating hyperventilation in response to hypoxia in zebrafish larvae however further investigation is required to elucidate the downstream mechanisms of 5-HT. According to the membrane model of chemoreception (Lopez-Barneo et al., 2008), neurotransmitters are released onto afferent nerve fibres in response to hypoxia. Currently, it is unclear which 5-HT receptor sub-types are found on the afferent nerve terminals. Elucidation of the 5-HT pathway requires histological and molecular analysis of 5-HT receptors. Once identified, these receptors can be targeted for knockdown (or knockout) in early development. Characterisation and localisation of serotonin transporters might also help determine whether 5-HT accumulates in NECs through uptake via the serotonin re-uptake transporter (SERT).

The hypoxic ventilatory response was blunted in fish targeted for *tph1a* gene knockdown. Morpholino knockdown is transient and at 4 dpf it is likely (based on western blots) that some *tph1a* activity persisted. Thus, it is possible that the trend for increased V_f during hypoxia in the *tph1a* MO fish resulted from incomplete knockdown of *tph1a*. Future studies could explore the effects of *tph1a* knockout on hypoxic ventilation frequency by establishing a *tph1a* CRISPR-Cas9 knockout model. A major advantage of this approach is the ability to take measurements in both larvae and adult fish. It would also be useful to measure the V_f of larval fish targeted for *tph1b* and *tph2* knockdown or knockout. Assuming no role for central 5-HT, it is predicted that the hypoxic V_f would not be inhibited in these fish because they should express normal 5-HT levels in NECs. Although it is tempting to extrapolate the results of the current study to adult zebrafish, additional studies exploring the role of 5-HT in adults are necessary to provide a uniform model of O_2 chemoreception across all developmental stages.

The presence of serotonergic innervation of NECs suggests that 5-HT is delivered to NECs, released from NECs, or both. While we do not have direct evidence for the mode of 5-HT transmission and storage in NECs, Fig. 11 outlines a possible pathway based on the location of *tph1a* observed in this study.

Figure 11. Proposed model for the storage and release of serotonin (5-HT) in neuroepithelial cells. **(1)** Tryptophan hydroxylase 1a (tph1a) produces 5-HT in nerves and in NECs **(2)** 5-HT accumulates in NECs **(3)** 5-HT is released onto afferent neurons in response to hypoxia

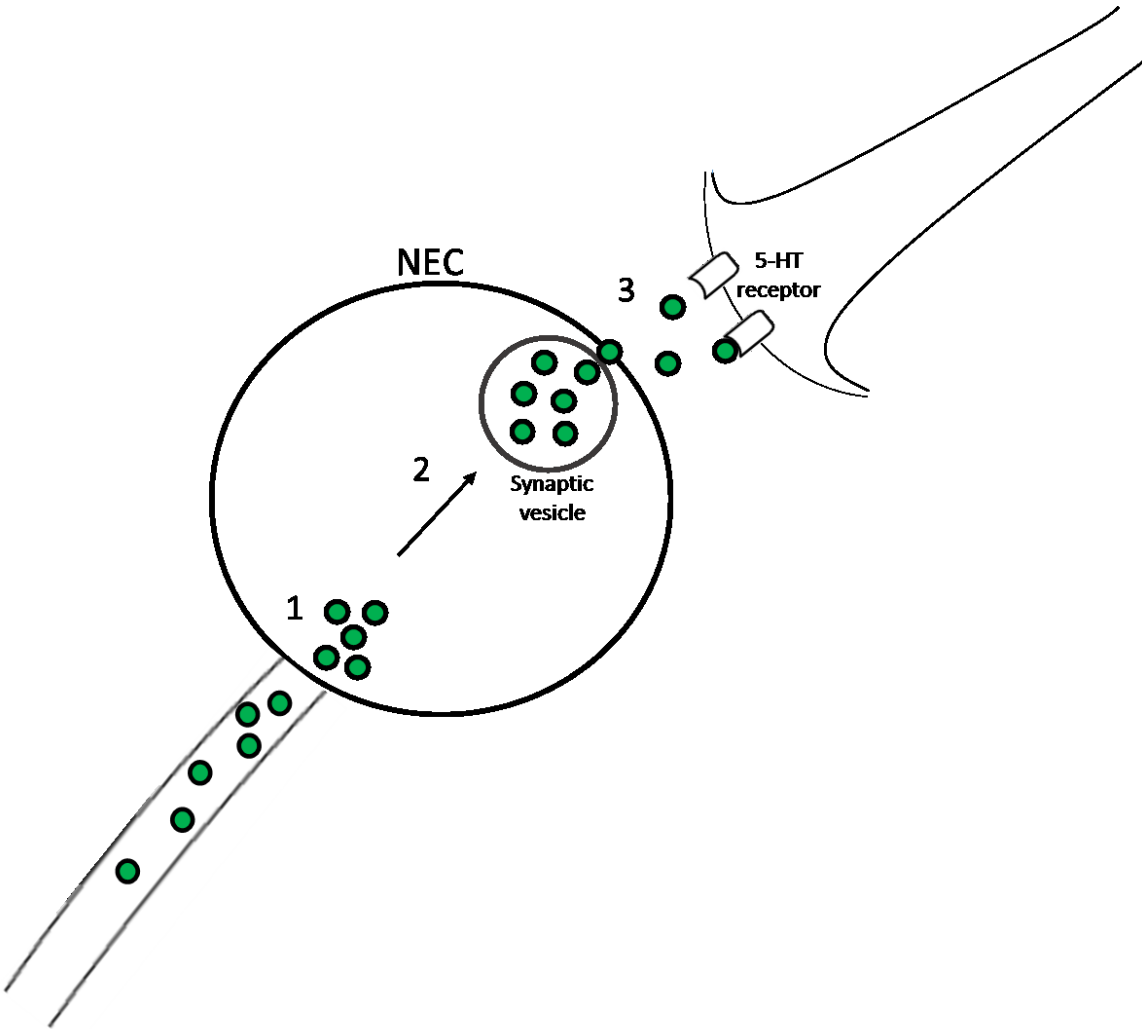


Figure 11

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Appendix

Appendix 1. Distribution of tryptophan hydroxylase 1b (tph1b) (**A**) and serotonin (5-HT) (**B**) in the tail of 4 dpf zebrafish larvae. Panel A and the merged image of tph1b and 5-HT (**C**) demonstrate tph1b labelling in one or more distinct cell types in the yolk sac which were 5-HT negative and which resembled ionocytes. Additionally 5-HT positive NECs did not localise with tph1b. Panel E represents the location from which the images were captured. Arrows indicate tph1b. Arrow heads indicate 5-HT. Scale bars = 50 μm in A-C; 20 μm in D

